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TGF- β and BMP2 Regulation of MicroRNA-200 Family in Chronic Pancreatitis

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Abstract

Objectives—To investigate regulation of microRNA (miR)-200 family (a, b, c, 141, and 429) in chronic pancreatitis (CP). This was accomplished by examining miR-200 family levels in a mouse model in vivo and their regulation in pancreatic cells in vitro.

Methods—CP was induced by cerulein for 4 weeks (50 μ g/kg, 5 hourly intraperitoneal injections/day, and 3 days/week). Control mice received normal saline. The pancreata were harvested for fibrosis assessment by Sirius red staining and for miR, collagen, and fibronectin levels by quantitative PCR. In vitro, human primary pancreatic stellate cells (hPSCs) and fibroblasts (hPFBs), and rat pancreatic epithelial AR42J cells were treated with vehicle, TGF- β (1 ng/ml), or BMP2 (50 ng/ml) for 24 hours and then harvested for miR analysis.

Results—In CP, miR-200s were decreased by 56–70% and inversely correlated with pancreatic fibrosis, miR-21, and miR-31 ($P < 0.05$). In vitro, TGF- β inhibited miR-200b in AR42J cells by 62%, while BMP2 increased miR-200b in all three cell types in a range of 1.5–3.4 folds and inhibited miR-21 in hPFBs by 21% ($P < 0.05$).

Conclusions—Both in vivo and in vitro studies suggest an anti-fibrogenic function of miR-200s in chronic pancreatitis. TGF- β and BMP2 may function through inverse regulation of miR-200b levels.

Keywords

chronic pancreatitis; cerulein; microRNA-200 family; pancreatic cells; TGF- β ; BMP2

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INTRODUCTION

Chronic pancreatitis (CP) is a progressive inflammatory disease of the pancreas, characterized by pancreatic inflammation, fibrosis, and loss of exocrine and endocrine functions, resulting in clinical manifestations of unremitting severe pain, malnutrition, and diabetes.¹ Therapeutic options for CP are only supportive cares; none are effective in stopping the ultimate progression to extensive fibrosis and tissue loss.^{2,3} This urgent and unmet need for CP therapy is largely due to fundamental gaps in knowledge regarding the complex mechanisms that regulate CP progression. Therefore, a better understanding of the mechanisms driving CP progression is crucial for development of effective and specific therapy.

Multiple signaling pathways are involved during the disease progression, among which transforming growth factor (TGF)- β signaling pathway plays a key pro-fibrogenic role.⁴⁻⁶ In contrast, bone morphogenetic proteins (BMPs), a major subgroup of the TGF- β superfamily, are anti-fibrogenic in the lungs, kidneys and liver.⁷⁻¹⁰ We have reported that BMP2 attenuates TGF- β 's pro-fibrogenic effects in vitro in pancreatic stellate cells (PSCs), the major effector cells in CP.¹¹ Knockout of BMP receptor type 2 in mice enhances pancreatic fibrosis in experimental CP.¹² However, the intracellular mechanisms by which these signaling pathways conduct their pro-fibrogenic or anti-fibrogenic responses have not been clearly defined.

microRNAs (miRs) are small non-coding RNA molecules (~22 bp) that modulate gene expression post-transcriptionally. They bind to 3'-untranslated region of mRNAs, leading to degradation of target mRNAs or suppression of protein translation, and thus, play a critical role in regulation of physiological functions and diseases.¹³ miR-200 family consists of five members, a, b, c, 141, and 429, and has anti-fibrogenic functions in the lung¹⁴ and kidney.¹⁵ They inhibit epithelial to mesenchymal transition, while promoting mesenchymal to epithelial transition, thus suppressing production of extracellular matrix (ECM) proteins.^{16,17} In renal and pulmonary fibrosis, miR-200 family levels decrease compared to normal control. TGF- β inhibits miR-200 family levels in the epithelial cells of these organs.^{14,15} However, the role of miR-200 family members and their regulation in CP are not clear.

In this study, we evaluated whether the members of miR-200 family are altered in CP and whether TGF- β and BMP2 regulate miR-200s in pancreatic cells in vitro. We found that miR-200 family levels were suppressed and inversely correlated with pancreatic fibrosis and pro-fibrogenic miRs in vivo, and that TGF- β and BMP2 had opposing effects on the member miR-200b in pancreatic cells in vitro. Thus, our studies suggest that miR-200b is anti-fibrogenic in CP and inversely regulated by the pro-fibrogenic factor TGF- β and the anti-fibrogenic factor BMP2 in pancreatic cells.

MATERIALS AND METHODS

Materials

Cerulein, the decapeptide analog of the potent pancreatic secretagogue cholecystokinin, was purchased from Bachem Americas, Inc. (Torrance, Calif); recombinant human TGF- β (=TGF- β 1) and BMP2 were purchased from R&D Systems, Inc. (Minneapolis, Minn).

Animals and In Vivo CP model

All animal experiments were performed according to the protocols approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston. Adult C57BL/6 mice (Charles River, Wilmington, Mass) were housed in a climate-controlled room with an ambient temperature of 23°C and a 12:12-hour light-dark cycle. Animals were fed standard laboratory chow, given water ad libitum, and randomly assigned to control or experimental CP groups (n = 4 mice/group).

For CP induction, the mice were given 5 hourly intraperitoneal injections of cerulein (50 μ g/kg) for alternative 3 days/week for 4 weeks. The control mice received equal volume and frequency of normal saline injections. Four days after the last cerulein injection (completion of CP induction), the mice were euthanized, and the pancreata were harvested.^{11,12} The pancreas samples were fixed in 10% formalin for morphological studies, or frozen in liquid nitrogen and stored at -80°C for RNA extraction.

Sirius Red Staining

Paraffin-embedded pancreas samples were sectioned (5 μ m), stained with Sirius red as described previously.^{11,12}

Cells

Human primary pancreatic stellate cells (hPSCs) were purchased from ScienCell Research Laboratories (Carlsbad, Calif), human primary pancreatic fibroblasts (hPFBs) were obtained from Vitro Biopharma (Golden, Colo). AR42J cells were obtained from ATCC (Manassas, Va). The cells were seeded and cultured in tissue culture dishes following specific instructions from the companies.

Quantitative PCR

Total RNAs were extracted from pancreatic tissue samples using TRIzol (Thermo Fisher Scientific, Pittsburgh, Pa) and from the cells using miRNeasy Mini Kit (Qiagen, Valencia, Calif). For analysis of collagen and fibronectin mRNA levels, total RNA samples were reversely transcribed to cDNA using RETROscript kit (Life Technology Co., Grand Island, N.Y.). Quantitative PCR (qPCR) was performed using TaqMan gene expression master mix and specific gene probe sets as previously described.¹⁸ The probe sets of mouse collagen 1a1 (Coll1a1, Mm00801666_g1), Coll1a2 (Mm00483888_m1), fibronectin (Mm01256744_m1), and 18S (Hs99999901_s1) (Life Technology Co.) were used in the study. The specific signals acquired were normalized to the signals acquired from 18S. For miR analysis, total RNA samples were reversely transcribed using TaqMan MicroRNA Reverse Transcription Kit. qPCR was conducted using respective mouse miR assay kits

(Life Technology Co.). The specific signals acquired were normalized to the signals acquired from U6.

Statistical Analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). Differences between two groups were analyzed using Student's *t*-test. Differences among multiple groups were analyzed using one-way ANOVA test. Pearson correlation was performed to detect correlation between miRs and pancreatic fibrosis. *P* value < 0.05 is considered significant.

RESULTS

miR-21 and miR-31 Levels are Elevated, While miR-200b and miR-27b Levels are Suppressed in Mouse CP Pancreas

To begin to investigate the role of miRs in CP, we selected and surveyed a panel of miRs, miR-21, -31, -27b, 29b, and -200b, from a large body of miR array data generated in the past decade on fibrotic diseases. miR-21, miR-27b, miR-29b, and miR-200b are regulated by TGF- β in multiple organ fibrosis,^{19–21} and miR-31 is dramatically increased in activated PSCs.²²

First, we confirmed the presence of significant pancreatic fibrosis after CP induction via repeated cerulein injections for 4 weeks. Consistent with our previous reports, dramatic collagen deposition is detected by Sirius red staining in CP pancreas compared to the saline control group. mRNA levels of Col1a1, Col1a2, and fibronectin in CP pancreas are elevated by 6.5–11.0 folds compared to the control ($P < 0.05$, Figure 1).

Next, we examined the levels of the selected miR panel. Compared to the control, levels of miR-21 and miR-31 significantly increase in 4 weeks' CP pancreas by 2.5 and 4.1 folds respectively, while levels of miR-27b and miR-200b decrease in CP pancreas by 51% and 68%, respectively ($P < 0.05$, Figure 2). miR-29b is undetectable (data not shown). These data demonstrate that the pro-fibrogenic miR-21 and miR-31 are upregulated and the anti-fibrogenic miR-27b and miR-200b are downregulated in CP.

miR-200 Family Levels are Correlated With miR-27b Levels, but Inversely Correlated With Pancreatic Fibrosis, miR-21, and miR-31 Levels

We focused on miR-200 family members since their role in CP is not clear. We found that levels of all five members of miR-200 family, a, b, c, 141, and 429 in 4 weeks' CP pancreas show significant decreases by 56–70% ($P < 0.05$) (Figure 3). To begin to describe potential role of the suppressed miR-200 family in relate to pancreatic fibrosis in CP, we performed Pearson correlation analysis. We found a significantly negative correlation between miR-200a/b and Col1a1/Col1a2/fibronectin, between miR-200c and fibronectin. In addition, a significantly positive correlation is observed between miR-31 and Col1a1/fibronectin (Fig. 4A). Furthermore, we found a significantly positive correlation between all five miR-200 members, and between miR-200 members and miR-27b. Whereas a significantly negative correlation is observed between miR-200a/b and miR-21/-31. In addition, a significantly positive correlation is observed between miR-21 and miR-31 ($P < 0.05$) (Fig.

4B). Taken together, these findings suggest that members of miR-200 family may be anti-fibrogenic since they are suppressed in CP and negatively correlated with pancreatic fibrosis and the pro-fibrogenic miR-21 and -31.

TGF- β and BMP2 Have Opposing Effects on miR-200b in Pancreatic Cells In Vitro

We reported that BMP2 opposes the pro-fibrogenic effects of TGF- β and plays an anti-fibrogenic role in CP.^{11,12} To determine whether TGF- β and BMP2 contribute to the changes of the above selected miR panels in CP, we utilized 3 types of pancreatic cells, hPSCs, hPFBs, and AR42J cells and examined direct effects of TGF- β and BMP2 on the miRs in vitro. hPSCs, the key effector cells in CP, were treated with vehicle, TGF- β , or BMP2 for 24 hours. The cells were then harvested for analysis of miRs. We found that compared to the vehicle control, BMP2 treatment increases miR-200b by 1.5 folds ($P < 0.05$), and marginally increases the other miR-200 members ($P > 0.05$). TGF- β treatment does not alter miR-200 levels (Fig. 5A). A similar pattern is observed in hPFBs, except that BMP2 inhibits miR-21 levels in hPFBs (Fig. 5B). Interestingly, in AR42J cell, BMP2 treatment increases miR-200b levels while TGF- β inhibits miR-200b levels (Fig. 5C). Taken together, these findings demonstrate the opposing effects of BMP2 and TGF- β on miR-200b in pancreatic cells.

DISCUSSION

The purpose of this study was to evaluate the regulation of miR-200 family members in CP. We found that levels of all five members of miR-200 family are suppressed in cerulein-induced mouse CP pancreas, which is associated with the suppressed level of the anti-fibrogenic miR-27b and the increased levels of pancreatic fibrosis and the pro-fibrogenic miR-21 and miR-31. In vitro in pancreatic cells, TGF- β and BMP2 have different effects on miR-200b. Our findings suggest that miR-200 family may represent anti-fibrogenic miRs in CP, and the balance of TGF- β and BMP2 signaling may contribute to the suppressed miR-200b levels in CP.

Aberrant expressions of miRs are observed during development of organ fibrosis. Our in vivo data are consistent with literature that miR-200s and -27b are suppressed in organ fibrosis, while -21 and -31 are elevated, and support their roles in the pancreas as anti-fibrogenic and pro-fibrogenic respectively. TGF- β -regulated miR-21, miR-200s, and miR-29 have been shown to modulate renal fibrosis.^{19,20,23} In pulmonary cells, TGF- β suppresses miR-27b for extracellular matrix production.²¹ Though our in vitro results of TGF- β 's effects on miRs may differ from these studies, we used different cells with our studies. We found that BMP2 induces miR-200b levels in all three types of pancreatic cells, stellate cells, fibroblasts, and epithelial cells, and suppresses miR-21 levels in fibroblasts, while TGF- β only inhibits miR-200b in the epithelial cells. Nevertheless, the regulation patterns on the miRs support the anti-fibrogenic property of BMP2 and the pro-fibrogenic characteristic of TGF- β .

Of note, we observe more dramatically altered miR levels in the in vivo studies than the in vitro studies. This may be due to the fact that the in vitro data are obtained from an individual cell type treated with a single factor, while interaction of multiple cell types and factors in the pancreas may contribute to the miR changes during CP induction in vivo.

Therefore, whether and how TGF- β and BMP2 regulate miRs in CP in vivo warrants further investigation. Furthermore, the limitation of using an individual cell type to mimic in vivo pathological changes once again leads us to appreciate the complexity of the whole pancreatic organ in physiological and pathological states, to effectively utilize the in vivo animal models, and to design better in vitro cell models to faithfully mimic in vivo cellular and molecular distribution, thus delineating cellular and molecular mechanisms.

In summary, miR-200 family is suppressed in mouse CP pancreas and is inversely correlated with pancreatic fibrosis and the pro-fibrogenic miR-21 and -31, suggesting that miR-200s may be anti-fibrogenic in the pancreas. TGF- β and BMP2 have opposing effects on miR-200b in pancreatic cells. Whether TGF- β and BMP2 regulate miR-200s in CP to execute their respective pro-fibrogenic or anti-fibrogenic functions deserves further investigation. Understanding cellular and molecular mechanisms for the opposing effects of TGF- β and BMP2 on pancreatic fibrosis will provide novel insights and facilitate future therapeutic development for CP.

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FIGURE 1. Pancreatic fibrosis induced in cerulein CP mouse model

CP was induced by repeated cerulein injections for 4 weeks (4w). A, Representative images of Sirius red staining for collagen deposition in the mouse pancreas. B, mRNA levels of Col1a1, Col1a2, and fibronectin. The mRNA levels were normalized against 18s and quantified as fold of control (CON). * $P < 0.05$ compared with CON.

FIGURE 2. Pancreatic miR-21/-31 and miR-27b/-200b levels inversely altered in the mouse CP Levels of miR-21, -31, -27b, and -200b in mouse pancreas under cerulein-induced CP for 4w. miR levels were measured by qPCR, normalized against U6, and quantified as fold of CON. * $P < 0.05$ compared with CON.

FIGURE 3. miR-200 family levels reduced in the mouse CP

Levels of miR-200a, -200b, 200c, -141, and -429 in mouse pancreas under cerulein-induced CP for 4w. miR levels were measured by qPCR, normalized against U6, and quantified as fold of CON. * $P < 0.05$ compared with CON.

FIGURE 4. miR-200 family levels correlated with miR-27b, inversely correlated with pancreatic fibrosis and miR-21/-31 in the mouse CP

A, Pearson coefficients of miR-200s with Col1a1, Col1a2, and FN. B, Pearson coefficients of miR-200s with miR-27b, -21, and -31. Positive value indicates a correlation relationship, and negative value indicates an inverse relationship. * $P < 0.05$.

FIGURE 5. miR-200b levels inversely regulated by TGF- β and BMP2 in pancreatic cells
 Levels of miRs in A, hPSCs, B, hPFBs, and C, AR42J. Cells were seeded in triplicate wells and cultured for 16–24 hours. The cells were starved in the media supplemented with 0.1% FBS for 4 hours and then treated with vehicle (Veh, 0.1 % BSA, 4 mM HCl, used for dilution of TGF- β and BMP2), TGF- β (=TGF- β 1, 1 ng/ml), or BMP2 (50 ng/ml) for 24 hours. The cells were harvested and total RNAs were prepared. The miR levels were measured by qPCR, normalized against U6, and quantified as fold of Veh. * $P < 0.05$ compared with Veh.