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Human amniotic fluid stem cells attenuate cholangiocyte apoptosis in a bile duct injury model of liver ductal organoids

Sinobol Chusilp [a,b], Carol Lee [a], Bo Li [a], Dorothy Lee [a], Masaya Yamoto [a], Niloofar Ganji [a], Paisarn Vejchapipat [c], Agostino Pierro [a,*]

[a] Division of General and Thoracic Surgery, Translational Medicine Program, The Hospital for Sick Children, Toronto, ON M5G 1X8, Canada
[b] Division of Pediatric Surgery, Department of Surgery, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
[c] Division of Pediatric Surgery, Department of Surgery, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

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ABSTRACT

Purpose: Biliary atresia (BA) is a fibro-obliterative cholangiopathy that involves both extrahepatic and intrahepatic bile ducts in infants. Cholangiocyte apoptosis has an influence on the fibrogenesis process of bile ducts and the progression of liver fibrosis in BA. Human amniotic fluid stem cells (hAFSCs) are multipotent cells that have ability to inhibit cell apoptosis. We aimed to investigate whether hAFSCs have the potential to attenuate cholangiocyte apoptosis and injury induced fibrogenic response in our *ex vivo* bile duct injury model of liver ductal organoids.

Methods: The anti-apoptotic effect of hAFSCs was tested in the acetaminophen-induced injury model of neonatal mouse liver ductal organoids (AUP #42681) by using direct and indirect co-culture systems. Cell apoptosis and proliferation were evaluated by immunofluorescent staining. Expression of fibrogenic cytokines was analyzed by RT-qPCR. Data were compared using one-way ANOVA with *post hoc* test.

Results: In our injury model, liver ductal organoids that were treated with hAFSCs in both direct and indirect co-culture systems had a significantly smaller number of apoptotic cholangiocytes and decreased expression of fibrogenic cytokines, transforming growth factor beta-1 (TGF-β1) and platelet-derived growth factor-BB (PDGF-BB). Moreover, hAFSCs increased cholangiocyte proliferation in injured organoids.

Conclusion: hAFSCs have the ability to protect the organoids from injury by decreasing cholangiocyte apoptosis and promoting cholangiocyte proliferation. This protective ability of hAFSCs leads to inhibition of the fibrogenic response in the injured organoids. hAFSCs have high therapeutic potential to attenuate liver fibrogenesis in cholangiopathic diseases such as BA.

Biliary atresia (BA) is an idiopathic cholangiopathy that involves both extrahepatic and intrahepatic bile ducts in infants. The fibro-obliteration of bile ducts in BA leads to bile flow obstruction and liver fibrosis, which progresses rapidly into liver cirrhosis. The progression of liver fibrosis in BA is accelerated by complicated etiology, including the progression of disease in intrahepatic bile ducts, persistent inflammation and immune response, and biliary epithelial-mesenchymal transition [1–4]. Moreover, the fibrogenic cascades in the liver of biliary atresia are still ongoing even after the surgical treatment, Kasai portoenterostomy, is performed. The development of a novel therapy to prevent or reverse the progression of liver fibrosis is essential to improve clinical outcomes in biliary atresia patients.

Cholangiocyte apoptosis has been observed in both extrahepatic and intrahepatic bile ducts in BA patients [5,6] and is involved in pathogenesis of BA [7,8]. The rhesus rotavirus-induced BA mouse model has indicated that cholangiocyte apoptosis happens in the early stages of disease, first involving extrahepatic bile ducts, followed by intrahepatic bile ducts. Moreover, the apoptosis of intrahepatic cholangiocytes occurs earlier before liver fibrosis can be observed [9,10]. These findings suggest that cholangiocyte apoptosis plays an important role in the initiation of liver fibrogenesis in BA.

Amniotic fluid stem cells (AFSCs) are multipotent cells present in amniotic fluid [11]. These stem cells has been described as a novel cell source for stem cell therapy due to their high differentiation and proliferation potential, lack of tumorigenicity and the possibility to be collected and expanded for subsequent application in the perinatal period [12,13]. Various studies have demonstrated that AFSCs reduce tissue injury, stimulate tissue regeneration, and inhibit cell apoptosis through their paracrine effects [14–18]. We hypothesized that the ability of AFSCs in minimizing tissue injury and inhibiting cell apoptosis can attenuate the initiation of liver fibrogenesis in BA.

Liver ductal organoids are mini-organ structures composed of cholangiocytes that harbor liver progenitor cells [19,20], which

* Corresponding author at: University of Toronto, Division of General and Thoracic Surgery, The Hospital for Sick Children, 1526-555 University Ave, Toronto, ON M5G 1X8, Canada. Tel.: +1 416 813 7340; fax: +1 416 813 7477.
E-mail addresses: agostino.pierro@sickkids.ca anne.apong@sickkids.ca (A. Pierro).

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recapitulate the functionality of *in vivo* bile ducts and are suitable for disease modeling. In this study, we utilized our *ex vivo* bile duct injury model of liver ductal organoids combined with the co-culture systems to investigate an anti-apoptotic potential of human amniotic fluid stem cells (hAFSCs) in the injured organoids.

1. Material and methods

1.1. Liver ductal organoid culture

We derived liver ductal organoids from intrahepatic bile ducts of neonatal mouse pups (AUP #42681) and cultured according to a method recently developed in our laboratory [21]. Briefly, intrahepatic bile ducts were isolated from the liver by digestion using tissue dissociation cocktail (StemCell Technologies, Cambridge, MA) and cultured in Matrigel dome (CORNING, Corning, NY). Mouse HepatiCult organoid growth medium (StemCell Technologies, Cambridge, MA) supplemented with penicillin–streptomycin (100 U/ml) was used as the culture medium.

1.2. Injury model in liver ductal organoids

We used our recently developed a a novel model of injured liver ductal organoids to investigate cholangiocyte apoptosis with relevance to biliary atresia [21]. Briefly, injury was induced in liver ductal organoids on day 4 of culture by administrating acetaminophen (3 mg/ml) (Sigma-Aldrich, St. Louis, MO), a hepatotoxicity agent that induces cell apoptosis [22,23], in the culture medium for 24 h. In the direct co-culture system of hAFSCs, the organoids and hAFSCs were co-cultured in Matrigel dome for 4 days before injury induction, whereas in indirect co-culture system, the transwell membrane insert containing hAFSCs was placed above the organoids in Matrigel dome without direct contact on day 4 of culture during the injury induction (Fig. 1).

1.3. Direct and indirect co-culture of hAFSCs and liver ductal organoids

To assess the effect of hAFSCs in the injured organoids, we established the direct and indirect co-culture systems of hAFSCs and the organoids (Fig. 1). The direct co-culture system, in which the organoids were co-cultured together with hAFSCs in the Matrigel dome, was developed to mimic *in vivo* stem cell transplantation. The indirect co-culture system using transwell membrane insert, in which hAFSCs do not have direct contact with the organoids, was developed to evaluate the paracrine effect of hAFSCs. Cholangiocyte apoptosis in the injured liver ductal organoids was evaluated by immunofluorescent staining of cleaved caspase-3, the marker of cell apoptosis (Fig. 2). In addition, we assessed cholangiocyte proliferation by immunofluorescent staining of the organoids using cell proliferation marker Ki67 (Fig. 2).

hAFSCs were purchased from Celprogen (Celprogen, Torrance, CA) and were cultured in human amniotic fluid expansion media with serum (Celprogen, Torrance, CA) in an incubator at 37 °C and 5% CO_2. After reaching 80–100% cell confluency, hASFCs were collected and suspended in Matrigel at a density of 500 cells per well together with liver ductal organoid of 200 fragments per well. hAFSCs were co-cultured with liver ductal organoids in Matrigel dome and maintained in an incubator at 37 °C and 5% CO_2.

Indirect co-culture system of hAFSCs with liver ductal organoids was established using a transwell membrane insert (6.5 mm diameter, 0.4 μm pore size, COSTAR, CORNING, Corning, NY). hAFSCs were seeded at a density of 10,000 cells on transwell membrane insert and placed above the Matrigel dome containing liver ductal organoids in each well without direct contact.

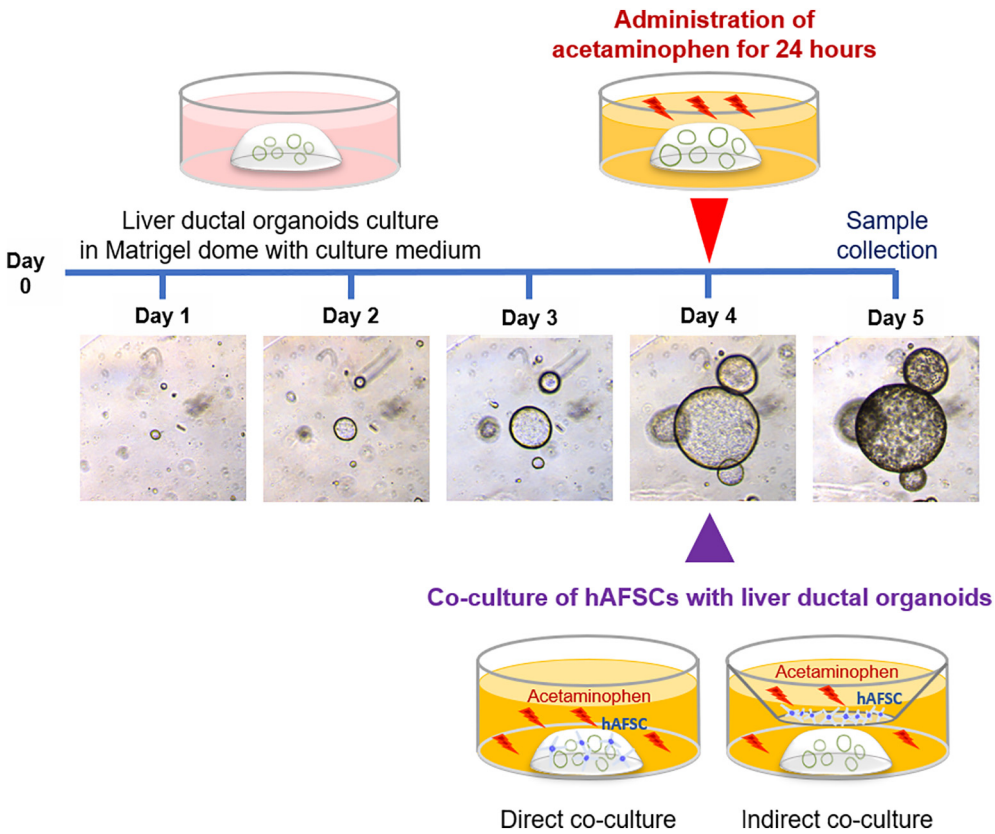

Fig. 1. Injury model of liver ductal organoids. (A) Liver ductal organoids were cultured in Matrigel dome for 4 days before induction of injury by administration of acetaminophen (3 mg/ml) in culture medium for 24 h. For direct co-culture system, hAFSC were co-cultured with the organoids in Matrigel dome for 4 days before inducing injury. For indirect co-culture system, hAFSC were cultured on transwell membrane insert placed above the Matrigel dome containing the organoids on day 4 of culture during the injury induction.

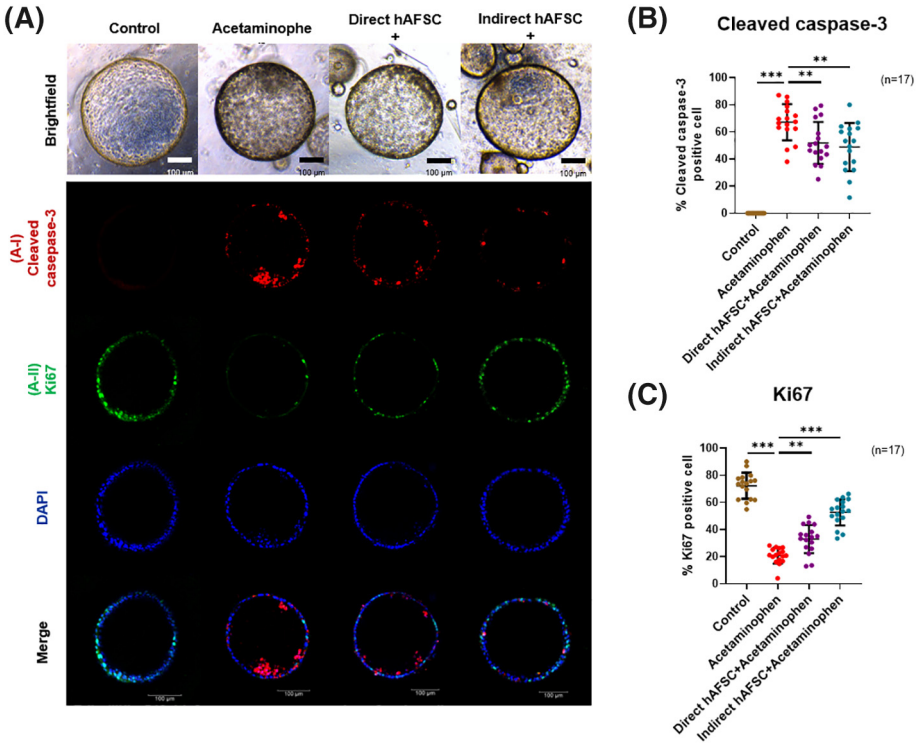

Fig. 2. Cholangiocyte apoptosis and proliferation in liver ductal organoids. (A) Representative images of liver ductal organoids in brightfield and immunofluorescent double staining of (A-I) cell apoptosis marker cleaved caspase-3 (red), and (A-II) cell proliferation marker Ki67 (green). Nuclei were stained with DAPI (blue). (B) Percentage of cleaved caspase-3 positive cell in each organoid. (C) Percentage of Ki67 positive cell in each organoid. Data are presented as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

1.4. RT-qPCR

Total RNA was extracted from the organoids using Trizol reagent (Invitrogen, Carlsbad, CA) followed by complementary DNA (cDNA) synthesis using qScript cDNA SuperMix (Quantabio, Beverly, MA) and S1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was performed using advanced qPCR Master Mix and CFX384 Real-Time System (Bio-Rad Laboratories, Hercules, CA). The housekeeping gene *Gapdh* was used to normalize the expression of each gene. The sequence of the primers using in this study were listed below:

Tgf-β1: sense 5′-ATTCCTGGCGTTACCTTGG-3′ and antisense 5′-AGCCCTGTATTCCGTCTCCT-3′; *P d g f-bb*: sense 5′- GGGGCTTCCAGGAGTGATACCA-3′ and antisense 5′-GCCCGAGCAGGTCAGAACAAA-3′; *Gapdh*: sense 5′-TGAAGCAGGCATCTGAGGG-3′ and antisense 5′-CGAAGGTGGAAGAGTGGGAG-3′.

1.5. Immunofluorescent staining

Liver ductal organoids fixed with 4% paraformaldehyde and permeabilized with 1% Triton X-100 were used for immunofluorescent staining. After blocking with 5% bovine serum albumin, the organoids were double immunostained with primary antibodies for cleaved caspase-3 (1:200) (Cell Signaling Technology, Danvers, MA) and Ki67 (1:200) (Invitrogen, Carlsbad, CA), TGF-β1 (1:200) (Invitrogen, Carlsbad, CA) and PDGF-BB (1:200) (Invitrogen, Carlsbad, CA). Then the organoids were incubated with Alexa Fluor-conjugated secondary antibody (Invitrogen, Carlsbad, CA) (1:400) and DAPI (Vector Laboratories) (1:400). Leica SP8 lightning confocal microscopy (Leica Microsystems, Wetzlar, Germany) was used for imaging.

1.6. Statistics

GraphPad Prism 8 (GraphPad Software, San Diego, CA) was used for statistical analyses. Results are reported as means ± SD and compared using one-way ANOVA with Bonferroni correction. Differences were considered statistically significant when $P < 0.05$.

2. Results

2.1. hAFSCs reduced cholangiocyte apoptosis in the injured liver ductal organoids

We found that co-culture of hAFSCs in both direct and indirect systems significantly reduced apoptotic cholangiocytes as shown by significantly decreased number of cleaved caspase-3 positive cells in the injured organoids compared to injured organoids without hAFSC treatment (Fig. 2A, B). These findings indicate that hAFSCs can protect liver ductal organoids against the injury through a paracrine effect.

2.2. hAFSCs increased cholangiocyte proliferation in the injured liver ductal organoids

hAFSCs administration in both direct and indirect co-culture systems significantly enhanced the proliferation of cholangiocytes in injured organoids as shown by an increased number of Ki67 positive cells compared to injured organoids without hAFSC treatment (Fig. 2A, C).

2.3. Fibrogenic response in liver ductal organoids is attenuated by hAFSC

Direct and indirect co-culture of hAFSCs with liver ductal organoids significantly decreased the mRNA expressions of *Tgf-β1* and *Pdgf-bb* (Fig. 3A), the potent cytokines involved in the stimulation of fibrogenesis. Protein expressions of these cytokines were confirmed by immunofluorescent double staining of TGF-β1 and PDGF-BB (Fig. 3B), which also showed consistent results with decreased cytokines expression in injured organoids and increased with hAFSCs direct/ indirect co-culture. These findings indicate that hAFSCs have the ability to decrease fibrogenic response in these injured organoids.

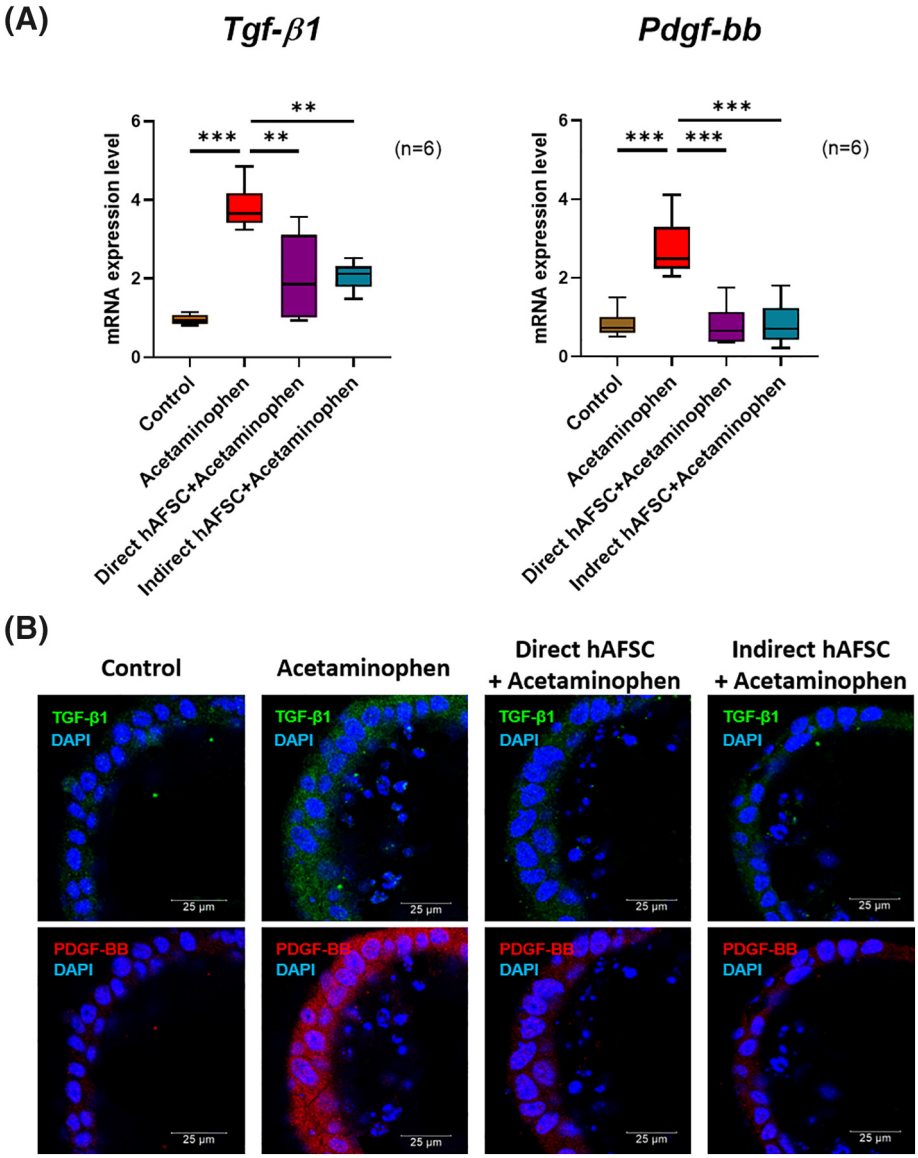

Fig. 3. Fibrogenic response in liver ductal organoids. (A) mRNA expressions of fibrogenic cytokines, *Tgf-β1* and *Pdgf-bb*. Data are presented as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$ (B) Representative images of immunofluorescent double staining of TGF-β1 (green) and PDGF-BB (red). Nuclei were stained with DAPI (blue).

3. Discussion

In this study, we tested the anti-apoptotic effect of hAFSCs in our injury model of liver ductal organoids by using the co-culture system, which mimics *in vivo* stem cell transplantation. Our results demonstrated that co-culture of hAFSCs with the organoids in both direct and indirect co-culture system could attenuate cholangiocyte injury by reducing cell apoptosis and promoting cell proliferation, which led to the decreased expression of fibrogenic cytokines in injured cholangiocytes (Fig. 4).

Various experimental models of tissue injury have demonstrated that the protective effects of AFSCs against injury are mediated through their anti-inflammatory and anti-apoptotic properties [14–18]. For example, in a rat model of neonatal intestinal injury, Zani et al. revealed that AFSCs reduce intestinal inflammation by stimulating the cyclooxygenase-2 (COX-2) pathway, thereby decreasing enterocyte apoptosis and promoting epithelial regeneration by upregulation of Wnt signaling [14]. A recent study by Koike et al. has also illustrated that AFSCs reduce intestinal inflammation and damage induced by ischemia and reperfusion through tumor necrosis factor-induced protein 6 (TSG-6) [24]. In a renal ischemia and reperfusion injury model, Monteiro Carvalho Mori da Cunha et al. demonstrated that AFSCs protect renal damage, increase cell proliferation and reduce renal interstitial fibrosis in the long term [25].

Our results in the injury model of liver ductal organoids are consistent with those of Zani et al. and Monteiro Carvalho Mori da Cunha et al., indicating that AFSCs have the ability to minimize cell injury and apoptosis and increase cell proliferation. Furthermore, the protection against injury by AFSCs leads to inhibition of the fibrogenic response in injured cholangiocytes as shown by the decreased expression of fibrogenic cytokines TGF-β1 and PDGF-BB. Previous studies have demonstrated that these cytokines play an important role in liver fibrogenesis by activating hepatic stellate cells to transdifferentiate into myofibroblasts, which produce collagen and extracellular matrix components in the liver [26–28]. Moreover, the increased expression of TGF-β1 in intrahepatic bile ducts correlates with the increased severity of liver fibrosis in BA patients [29]. Thus, the results from our study suggest that hAFSCs have the potential to intervene the fibrogenic cascade at an earlier stage of liver fibrosis in BA. However, the mechanism of hAFSCs in reducing cholangiocyte injury and apoptosis needs to be further explored.

In a mouse model of carbon tetrachloride-induced liver fibrosis, Peng et al. demonstrated the benefit of AFSCs in amelioration of liver fibrosis

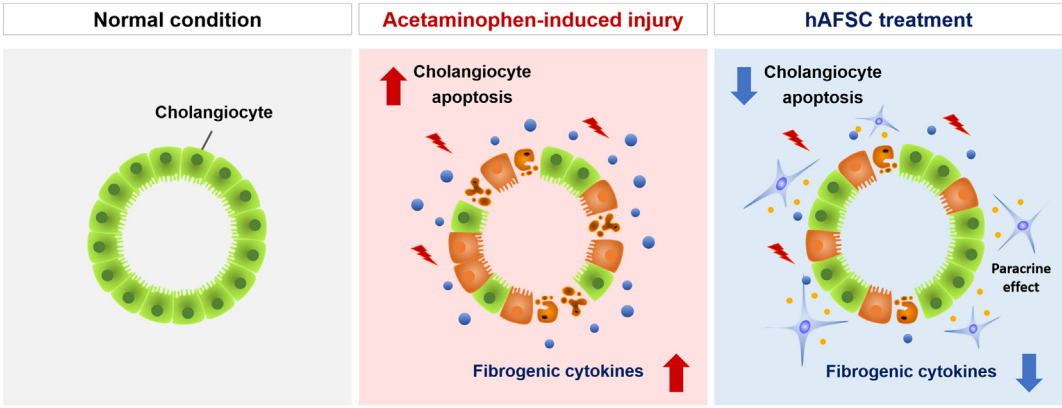

Fig. 4. Summary figure. After exposure to acetaminophen, the injured cholangiocytes underwent apoptosis and increased the expressions of potent fibrogenic cytokines that can initiate the fibrogenesis process. Co-culture of hAFSCs with liver ductal organoids attenuates the cholangiocytes from injury by decreasing cell apoptosis and promoting cell proliferation, which leads to inhibition of the fibrogenic cytokine expressions in injured cholangiocytes.

by decreasing collagen deposition in the liver [30]. This indicates that AFSCs may play different roles in different stages of liver fibrosis. They can minimize cholangiocyte apoptosis and the fibrogenic cascade at an early stage and decrease collagen deposition in the later stage of liver fibrosis.

A recent study by Babu et al. has demonstrated that liver organoids from BA patients exhibited abnormal growth and decreased expression of cell proliferation marker Ki67 [31]. This finding indicates that the proliferation of intrahepatic bile ducts of BA patients is impaired. Our study found that hAFSCs could increase proliferation in the injured organoids. Therefore, hAFSCs may also have the potential to stimulate proliferation of intrahepatic bile ducts in BA.

We believe that our study is the first step towards the application of hAFSCs as a treatment to prevent the progression of biliary atresia into liver cirrhosis. We also created the indirect co-culture system of hAFSCs with liver ductal organoids using transwell membrane insert to demonstrate the paracrine effect of hAFSCs. In this indirect co-culture system, we found that hAFSCs can interact with the organoids without direct contact and reduce the fibrogenic response of injured organoids through a paracrine effect. These findings are essential for further investigation of the application of hAFSC in *in vivo* disease models, including the administration route of hAFSCs and the possibility of using hAFSC conditioned medium and extracellular vesicles as a treatment for BA.

4. Conclusions

Our study demonstrated that hAFSCs attenuate the injury of liver ductal organoids and its fibrogenic response through a paracrine effect by decreasing apoptotic cholangiocytes and promoting cholangiocyte proliferation (Fig. 4). hAFSCs have the potential to diminish bile duct injury and its fibrogenic cascade that leads to the progression of liver fibrosis in cholangiopathic diseases such as BA.

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Declaration of competing interest

The authors declare no conflict of interest.

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