Review Article

Narrative review of bovine milk exosomes as a potential therapeutic option for hepatic fibrosis

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Background and Objective: Exosomes and their microRNAs (miRNAs) are in the focus of recent research as they modify the homeostasis of hepatic stellate cells (HSCs) and play key roles in liver fibrogenesis as well as the resolution of hepatic fibrosis (HF). At present, no effective therapy of HF exists. Exosomes and their miRNA cargo are involved in the pathogenesis and resolution of HF. It is the intention of this narrative review to provide basic research evidence for oral administration of bovine milk-derived exosomes (BMEX) as a new treatment option of HF.

Methods: English literature published between 2000–2021 were searched using the PubMed database focusing on publications including HF, exosomes, miRNAs, fibrogenic and anti-fibrogenic miRNAs has been performed and related to known effects BMEX and their miRNAs.

Key Content and Findings: BMEX and their miRNAs accumulate in the liver after oral administration, are able to transit vascular endothelial cells and may thus reach the space of Disse, where HSCs reside. Dominant BMEX-derived miRNAs (miRNA-148a, miRNA-29s and let-7 family) are known suppressors of fibrogenic transcription factors and signaling components including DNMT1, DNMT3A, DNMT3B, TLR4, NF-κB, USP4, SMADs, PDGFB, CTGF, CCKBR, PTHLH, Hedgehog signaling, ATG7 and BECN1. Anti-fibrogenic effects of BMEX may be augmented by co-administration of metformin, a known suppressor of the miRNA sponge lncRNA-H19 further increasing the expression of anti-fibrogenic miRNA-148a, miRNA-29b and let-7 and suppressing the fibrogenic and oncogenic miRNA-21.

Conclusions: Presented basic research evidence suggests promising anti-fibrogenic effects of BMEX either alone or combined with other drugs increasing the expression of anti-fibrogenic miRNAs in the treatment of HF. Our narrative review should stimulate future experimental and clinical research.

Keywords: Anti-fibrogenic microRNA (miRNA); bovine milk-derived exosomes (BMEX); fibrogenic miRNAs; hepatic stellate cells; liver fibrosis

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Introduction

Hepatic fibrosis (HF) due to viral, toxic or metabolic chronic liver diseases is a major challenge of global health (1). Cirrhosis is currently the 11th most common cause of death in the world and the fourth most frequent cause of death in adults in central Europe (2-4). Progressive accumulation of extracellular matrix (ECM), which destroys the physiological architecture of the liver, is the pathological hallmark of HF (5). Toxic, viral, or metabolic factors lead to hepatocyte damage and infiltration of immune cells that activate trans-differentiation of hepatic stellate cells (HSCs) into collagen-producing myofibroblasts (6-9). HSC activation is considered as a pivotal event in HF (6-10). HSCs are mainly responsible for the excessive accumulation of ECM proteins in the liver associated with increased expression of type I collagen (COL1A1), smooth muscle α-actin (α-SMA) and increased cellular proliferation (10,11).

The liver's fate to either pass into an anti-fibrotic scar-dissolving stage or to proceed into an uninhibited fibrosis-promoting stage is mainly regulated by non-parenchymal cells, including Kupffer cells and other immune cells (12-16). Hepatocyte apoptosis and release of damage-associated molecular patterns (DAMPs) from hepatocytes activate Toll-like receptor 4 (TLR4), which activates HSCs directly and induces the recruitment and activation of lymphocytes and macrophages that contribute to the promotion of HSC trans-differentiation and myofibroblast activation by producing pro-inflammatory and fibrogenic cytokines (17-19). Distinct macrophage subpopulations on the other hand participate in the resolution of fibrosis through the expression of matrix metalloproteinases (MMPs) (20,21). Thus, on the molecular basis, a complex network of cytokine-induced signaling pathways orchestrates fibrogenic cell interactions. Transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and the inflammasome (NLRP3)-caspase 1 pathway, as well as WNT/β-catenin signaling have been suggested as key signaling pathways associated with HSC activation and fibrosis progression (22-25).

There is recent interest in the role of extracellular vesicles (EVs), in particular exosomes, in the regulation of HSC differentiation and HSC homeostasis. Exosomes are small discoid EVs originating from endosomes that are 30–150 nm in diameter and have an outer lipid bilayer membrane. They participate in immune responses, cell migration, cell differentiation, and tumor invasion and mediate intercellular communication, regulating the biological activity of receptor cells through proteins, nucleic acids including microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and lipids as integral constituents (26,27). Exosomes of various origin, i.e., hepatocytes, virally-infected hepatocytes, macrophages, lymphocytes, cholangiocytes, HSCs, adipocyte- and bone marrow-derived mesenchymal stem cells (MSCs), and blood plasma, all interfere with HSC homeostasis, activity and proliferation. Whereas certain exosomes induce HSC-mediated fibrogenesis (28-41), others contribute to the resolution and suppression of fibrogenesis offering new treatment options for HF (42-55). Thus, exosomes are considered as powerful tools for the treatment of HF (55,56), as long as an approved standardized drug for the therapy of HF does not exist (57). Recent evidence underlines the beneficial therapeutic effects of bovine milk-derived exosomes (BMEX) in the treatment of inflammatory bowel diseases and necrotizing enterocolitis (58-63). It is conceivable that BMEX and their miRNA cargo not only improve intestinal but also hepatic functions.

The objective of this narrative review is the presentation of experimental research data for potential anti-fibrotic and fibrotic effects of BMEX and their possible contribution for the alleviation or treatment of HF. We present the following article in accordance with the Narrative Review reporting checklist (available at https://dmr.amegroups.com/article/view/10.21037/dmr-21-79/rc).

Methods

Literature research was performed using the PubMed database between 2000–2021 selecting original research papers in English language including the search items: HF, liver fibrosis, liver cirrhosis, hepatic stellate cell, Ito cell, exosome, fibrosis, fibrogenesis, miRNA (miR), fibrogenic miRNA, anti-fibrogenic microRNA (miRNA, miR), bovine milk exosome (cow milk-derived exosome), bovine milk EV (cow milk-derived EV), milk miRNA, metformin, metformin-induced effects on miRNA expression. Original data of reported biological effects of miRNAs in HSC transformation or HF pathogenesis were extracted and provided in the corresponding miRNA section. Literature data of bovine milk exosome-derived miRNAs were inspected and related to their potential anti-fibrotic and fibrotic effects (*Table 1*).

Table 1 The search strategy summary

Items	Specification
Date of search (specified to date, month and year)	December 2021
Databases and other sources searched	PubMed
Search terms used (including MeSH and free text search terms and filters)	Exosome; hepatic stellate cell; miRNA, fibrogenesis
Timeframe	2000–2021
Inclusion and exclusion criteria (study type, language restrictions etc.)	Inclusion: Original research articles, English language; Exclusion: articles repeating published data
Selection process (who conducted the selection, whether it was conducted independently, how consensus was obtained, etc.)	First author conducted paper selection

Discussion

Hepatic stellate cells: potential targets of BMEX

BMEX and their miRNAs are bioavailable and reach the systemic circulation as well as peripheral tissues in humans and animal models (64-73). BMEX are regarded as promising biologics to reach distant organs for the treatment of human diseases (cross-species communication) (74-77). When fluorophore-labeled BMEX were administered retro-orbitally, BMEX accumulated in liver, spleen, and lungs in mice (69). Manca *et al.* (70) confirmed that BMEX labeled with fluorophores or fluorescent fusion proteins accumulated in the liver, spleen and brain following suckling, oral gavage and intravenous administration in mice and pigs. When synthetic, fluorophore-labeled miRNAs were transfected into BMEX and administered to mice, distinct species of miRNAs demonstrated unique distribution profiles and accumulated in the liver and other organs (70). The finding that human vascular endothelial cells transport BMEX by endocytosis is an important step for their delivery to organs and peripheral tissues (69).

Samuel *et al.* (73) recently demonstrated that 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR)-labeled bovine milk-derived EVs (30–150 nm, including TSG101-positive EV fractions in the density range of BMEX) preferentially accumulated in the liver of mice 24 h after oral EV gavage. Quantitative proteomic analysis confirmed the presence of bovine-specific proteotypic peptides in liver tissues of mice orally administered with milk-derived EVs confirming their bioavailability in the liver after oral uptake (73). Therefore, it is conceivable that BMEX may enter the space of Disse and approach HSCs (*Figure 1*). Betker *et al.* (75) have shown that antibodies are present in exosome preparations, and that DiR-labeled BMEX are absorbed as intact particles from the gastrointestinal tract and accumulated in the liver via the "neonatal" Fc receptor, FcRn. Notably, expression of Fc fragment receptors of IgG has been reported on rat HSCs (78). It has been shown in mice either fed a BMEX-supplemented or BMEX-deficient diet that BMEX supplementation significantly modified hepatic mRNA expression of genes involved in purine metabolism (79). Recently, López de Las Hazas *et al.* (80) confirmed in a mice model that BMEX-transported miRNAs are stable during gastrointestinal digestion, are bioavailable and reached the liver increasing the expression of miRNA-148a 1 h after BMEX administration. Thus, basic research evidence indicates that BMEX and their transported miRNAs reach the liver and may reach HSCs modifying their posttranslational gene expression.

Milk fat globule-EGF factor 8 in HSC regulation

Recent evidence indicates that milk fat globule-EGF factor 8 (MFG-E8) is a strong inhibitor of activation of human primary HSCs (81). Notably, levels of MFG-E8 are reduced in cirrhotic liver tissue from patients compared with controls (81). MFG-E8, a secretome member of MSCs, is secreted by umbilical cord-derived MSCs (UCMSCs). In mice with fibrosis, the accumulation of ECM proteins was significantly reduced 3 days after injecting secretomes from UCMSCs, and to a greater extent from hepatocyte-like UCMSCs (hpUCMSCs) (81). MFG-E8 down-regulated the expression of TGF-β type 1 receptor by binding to αvβ3 integrin on HSCs. In mice, injection of recombinant human MFG-E8 had anti-fibrotic effects comparable to those of the hpUCMSC secretome, reducing ECM deposition and HSC activation. Co-injection of an antibody

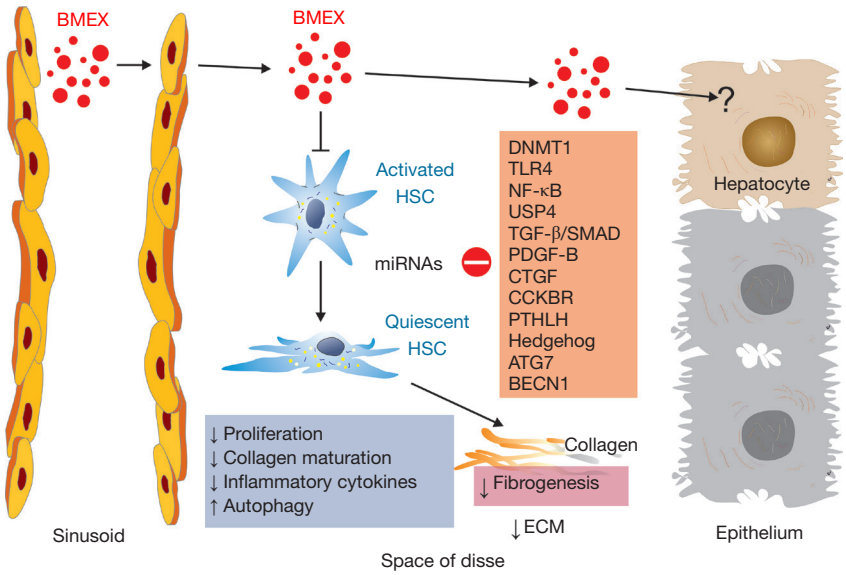

Figure 1 Illustration of the potential impact of bovine milk exosomes (BMEX) and their miRNAs on hepatic stellate cells (HSC). BMEX-derived miRNAs may target and suppress DNA methyltransferase 1 (DNMT1), toll-like receptor 4 (TLR4), nuclear factor-κB (NF-κB), ubiquitin-specific protease 4 (USP4), transforming growth factor-β (TGF-β), SMA- and MAD-related protein (SMAD), platelet-derived growth factor-β (PDGFB), connective tissue growth factor (CTGF), cholecystokinin receptor B (CCKBR), parathyroid hormone-like hormone (PTHLH), Hedgehog signaling, autophagy-related 7 (ATG7) and beclin 1 (BECN1), which synergistically may reduce hepatic fibrogenesis and attenuate HSC proliferation, collagen maturation and inflammation and reduce extracellular matrix (ECM) formation. The influence BMEX on hepatocytes remains uncertain.

against MFG-E8 reduced the anti-fibrotic effects of the hpUCMSC secretome in mice (81). In a mouse model of bleomycin-induced fibrosis and in the TSK mouse model (a genetic model of systemic sclerosis), deficient expression of MFG-E8 significantly enhanced both pulmonary and skin fibrosis, and administration of recombinant MFG-E8 significantly inhibited bleomycin-induced dermal fibrosis (82). In contrast, integrin α(v)-bound MFG-E8 associates with PDGFRB and focal adhesion kinase after PDGF-BB treatment, which results in cell surface retention of PDGFRB, delays receptor degradation and potentiates its downstream signaling (83).

MFG-E8 protein is a major constituent of MFG membranes (84), but is detectable only in minor amounts in milk EVs and BMEX (84-86). In contrast, MFG-E8 mRNA has been detected in BMEX (74) that may thus reach HSCs.

MiRNAs in HSC activation and liver fibrosis

Emerging evidence indicates that miRNAs are abnormally expressed in activated HSCs and play an important role in fibrogenesis (87-89). MiRNAs regulate HSC activation, proliferation, migration and apoptosis, as well as collagen production and ECM deposition (89). MiRNAs are of critical importance in the control of the Hedgehog (Hh) pathway of HSCs (90), which recently reached attention in the pathogenesis of HF (91).

Among various miRNAs studied in association with HSC activation and HF, certain anti-fibrotic miRNAs including miRNA-152, a homolog of miRNA-148a and member of the miRNA-148a/152 family (92), and miRNA-29 family members are down-regulated, whereas the fibrogenic miRNAs 34a/c, miRNA-125b and miRNA-21 were consistently up-regulated in activated HSCs (89).

DNMT1 and miRNA-148a in HSCs and hepatocytes

Epigenetic regulation plays an important role for HSC homeostasis and activation (93-96). Recent evidence indicates that DNA methyltransferase 1 (DNMT1) expression is increased in activated HSCs compared with quiescent HSCs (97). When rat HSC-T6 cells were incubated with PDGF-BB, they exhibited a time-dependent increase in protein expression of DNMT1, α-SMA and COL1A1, respectively, whereas siRNA-

treatment of DNMT1 or DNMT1 inhibition with 5-aza-2'-deoxycytidine significantly reduced α-SMA and COL1A1 expression (97). In addition, nuclear p-ERK1/2 expression was significantly down-regulated in DNMT1-siRNA cells and 5′-aza-2′-deoxycytidine treated cultures, which was associated with reduced cell proliferation (97). Moreover, DNMT1 controls the expression of the long noncoding RNA H19 (lncRNA H19) (97), which is dysregulated in HF and is a sponge of miRNA-148a-3p (98). The role of lncRNA H19 in HF is still controversial. One research group observed that lncRNA H19 was significantly down-regulated in HSCs and fibrosis tissues (97,99), whereas other authors reported that cholangiocyte-derived exosomal lncRNA H19 promotes cholestatic liver injury in mice and humans (34), promotes HSC activation and cholestatic HF (36), cholangiocyte proliferation and cholestatic HF in biliary atresia (37). Huang *et al.* (100) investigated the regulatory effects of the lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) on HF and HSC activation. Up-regulation of NEAT1 and cytohesin 3 (CYTH3) and down-regulation of miRNA-148a-3p and miR-22-3p were observed in mouse fibrotic liver tissues (100). Knockdown of NEAT1 or CYTH3 attenuated HF and collagen deposition *in vivo* and the activation of HSCs *in vitro*. MiRNA-148a-3p and miRNA-22-3p inhibitor facilitated HSC activation and collagen fiber expression (100). These data are in accordance with the known posttranslational inhibition of DNMT1 expression via miRNA-148a (101,102). MiRNA-148a-mediated suppression of DNMT1 may thus reduce α-SMA, COL1A1 and nuclear p-ERK1/2 expression (97).

Cytochrome P450 (CYP) enzymes are markedly decreased in alcoholic hepatitis (AH), a critical preclinical inflammatory condition resulting in HF (103). It has been demonstrated by Luo *et al.* (104) that miRNA-148a promotes CYP2B6 expression by increasing mRNA stability via directly binding to the 3'-UTR sequence of CYP2B6. In contrast, ethanol exposure of hepatocytes via suppressing hepatocyte nuclear factor 4α (HNF4α) decreases the expression of miRNA-148a and thus impairs CYP2B6 expression (104). As shown in cultured hepatocytes and mouse livers, alcohol exposure inhibits forkhead box protein O1 (FoxO1) expression, which correlates with decreased miRNA-148a levels (105). FoxO1 was identified as a further transcription factor for *MIR148A* transactivation (105). Alcohol-induced decline of miRNA-148a expression in hepatocytes facilitates over-expression of thioredoxin-interacting protein (TXNIP) and NLRP3 inflammasome activation (105). In HepG2 cells, miRNA-148a promotes alcohol dehydrogenase 4 (ADH4) expression by directly binding to the coding sequence of ADH4 and increasing the mRNA stability via an argonaute RISC component 1 (AGO1)-dependent manner (106). Luo *et al.* (106) suggested that the expressions of key alcohol-metabolizing enzymes are repressed in AH patients, and the non-canonical positive regulation of miRNA-148a on ADH4 reveals a new regulatory mechanism for ADH genes.

It has been shown in neonatal dermal fibroblasts that DNMT1 knockdown suppresses the expression of connective tissue growth factor (CTGF=CCN2) (107), which also plays a key role in the pathogenesis of HF (108,109) and activation of HSCs (110,111).

MiRNAs critically interact with the Hh pathway (90), which plays a pivotal role for HSC activation (91). Patched1 (PTCH1), a negative regulatory factor of the Hh signaling pathway, is down-regulated during HF and associated with hypermethylation of the PTCH1 promoter (112). Notably, salvianolic acid B (Sal B) suppressed the activation of HSCs in CCl_4-treated mice and mouse primary HSCs, leading to inhibition of cell proliferation, COL1A1 and α-SMA expression (112). Sal B-mediated up-regulation of PTCH1 was associated with decreased DNA methylation upon Sal B treatment resulting from decreased expression of DNMT1 (112). Interestingly, increased expression of miRNA-152 in Sal B-treated cells was responsible for the hypomethylation of PTCH1 by Sal B. DNMT1 was identified as a direct target of miRNA-152. Further studies showed that a miRNA-152 inhibitor reversed Sal B-mediated PTCH1 up-regulation and DNMT1 down-regulation. DNMT1 is a well-known target of miRNA-148a-3p, miRNA-148b-3p and miRNA-152-3p exhibiting (8mer) highly conserved binding sites (113). These data confirm the importance of the epigenetic DNMT1-mediated regulation of HSC homeostasis.

The uptake of bovine MEX by intestinal epithelial cells (IEC) resulted in an intracellular increase of miRNA-148a associated with a significant suppression of DNMT1 (60,109,110). Notably, miRNA-148a is the most abundant signature miRNA in cow milk and BMEX (114-117) and is highly conserved between mammals (117). The nucleotide stem loop sequence of human (*hsa*-miR-148a-3p) and bovine (*bta*-miR-148a-3p) miRNA-148a is identical (118,119). Importantly, miRNA-148a is one of the most highly expressed miRNAs in bovine milk, before and after pasteurization (114,120). These observations imply that the administration of BMEX miRNA-148a may attenuate DNMT1-induced HF.

MiRNA-148a attenuates TGF-β signaling

The TGF-β/SMAD signaling in cooperation with PDGF is considered as one of the most important pathways driving HSC activation and fibrogenesis (24,121). Inactive TGF-β molecules bind to the latency associated protein (LAP) and accumulate in the ECM and must be cleaved by specific proteases to become active. Endothelial cells participate in the conversion of TGF-β from the latent to the active form. Moreover, interactions with transmembrane integrins are considered as the principal activating mechanism for latent TGF-β (122). The active form binds to and activates TGF-β receptor type 2 (TGFBR2), which recruits the TGF-βreceptor type 1 (TGFBR1). The downstream canonical signaling of TGF-β1 converges on SMAD proteins. SMAD3 is crucial for inducing HSC activation and fibrogenic gene transcription such as α-SMA or COL1A1, whereas SMAD2 and SMAD7 are believed to be anti-fibrotic (1,24). However, recent evidence indicates that SMAD2 is up-regulated by endoplasmic reticulum stress related to HSC activation and HF (123). Kupffer cells secrete TGF-β1 promoting HSC activation via the SMAD2/BRD4/C-MYC/EZH2 pathway in HF (124). Furthermore, exosomes derived from human umbilical cord MSCs alleviated HF and decreased the expression of collagen type I and III, TGF-β1 and phosphorylation of SMAD2 (42). Notably, it has been demonstrated in human hepatocellular carcinoma (HCC) cell lines (HepG2, Huh-7, and MHCC97H) that miRNA-148a inhibits the TGF-β/SMAD2 signaling via targeting the SMAD2 3'-UTR decreasing the expression and function of SMAD2 (125).

In accordance with umbilical cord MSC-derived exosomes, BMEX may as well attenuate TGF-β/SMAD2 signaling in HSCs.

MiRNA-148a attenuates cholecystokinin signaling

Dietary fat stimulates growth of pancreatic cancer and promotes fibrosis of the tumor microenvironment through the cholecystokinin (CCK) receptor (CCK-R) (126), whereas a CCK antagonist halts progression of pancreatic cancer precursor lesions and fibrosis in mice (127). Although CCK-Rs have not been reported on HSCs, CCK-R blockade with proglumide could have a similar effect on HSCs as it does on pancreatic stellate cells, thereby preventing or even regressing HF in NASH (128). CCK-Rs have been reported on fibroblasts which when activated, can induce fibrosis (128). CCK-BR expression was markedly up-regulated in the liver and HCC cells of mice fed a saturated fat 75% choline-deficient-ethionine-supplemented (CDE) diet compared with normal hepatic parenchymal cells, whereas CCK-R blockade with proglumide decreased HF associated with NASH (128). In addition, CCK-BR expression was epigenetically suppressed by miRNA-148a (128), which directly targets the mRNA of CCK-BR (129). CCK-BR expression was significantly down-regulated in the Dt81Hepa1-6 cells that over-expressed miRNA-148a, confirming an epigenetic regulatory effect of CCK-BR mRNA expression by miRNA-148a (128). In addition, miRNA-148a was decreased in interstitial renal fibrosis and tubular atrophy (130) underlining the anti-fibrotic effect of miRNA-148a.

Let-7 family miRNAs suppress TGF-β signaling

Lin28 and let-7 play important roles in the regulation in liver diseases (131). The let-7 family of miRNAs is regarded as anti-fibrotic regulators (132). Notably, treatment of HSCs with lipopolysaccharide (LPS) and TGF-β significantly decreased the expressions of let-7a and let-7b. Conversely, over-expression of let-7a and let-7b suppressed the myofibroblastic activation of cultured human HSCs induced by LPS and TGF-β, as evidenced by repressed ACTA2 (α-actin 2), COL1A1, TIMP1 (tissue inhibitor of metalloproteinase 1), and FN1 (fibronectin 1), which supports the view that HSC activation is controlled by let-7 (133). Furthermore, Lin28B deficiency increased the expression of let-7a/let-7b as well as reduced HSC activation and HF in mice with alcoholic liver injury (133). Of note, Lin28 functions as a specific, post-transcriptional inhibitor of let-7 biogenesis (134). High expression of let-7 suppressed epithelial-mesenchymal transition (EMT) and TGF-β signaling (135), which is of key importance in the pathogenesis of HF (136-138). Importantly, let-7 decreases the mRNA expression of TGFBR1, TGF-β receptor type 3 (TGFBR3), and SMAD2 proteins (139,140).

Exosomal let-7 from menstrual blood-derived endometrial stem cells alleviated pulmonary fibrosis (141). Let-7 family members let-7a, let-7b, let-7f, as well as miRNA-148a belong to the most abundant and highly conserved miRNAs of milk EVs of various mammalian species (117). BMEX-derived let-7 and miRNA-148a-mediated suppression of TGF-β signaling may exert anti-fibrogenic activities. In contrast, TGF-β is an outer membrane component of BMEX (142). On the other hand, cow milk-derived EVs contain abundant miRNA-125b (143), which directly targets and inhibits

Lin28 mRNA, inhibiting the translation and expression of the Lin28 protein (144), a mechanism that may further enhance let-7-mediated suppression of TGF-β signaling. Recent evidence indicates that the addition of BMEX to IEC18 cells increased the ratio of TGF-β3 to TGF-β1 (145).

MiRNA-148a suppresses TLR4-NFκB signaling

TLR4 enhances TGF-β signaling and plays a crucial role in HF (146-157). Using TLR4-chimeric mice and *in vivo* LPS challenge, Seki *et al.* (148) demonstrated that quiescent HSCs, the main precursors for myofibroblasts in the liver, are the predominant target through which TLR4 ligands promote fibrogenesis. In quiescent HSCs, TLR4 activation not only up-regulates chemokine secretion and induces chemotaxis of Kupffer cells, but also down-regulates the TGF-β pseudoreceptor Bambi to sensitize HSCs to TGF-β-induced signals and allow for unrestricted activation by Kupffer cells. LPS-induced Bambi down-regulation and sensitization to TGF-β is mediated by a MyD88-nuclear factor κB (NF-κB)-dependent pathway. Accordingly, Myd88-deficient mice have decreased HF. Thus, modulation of TGF-β signaling by a TLR4-MyD88-NF-κB axis provides a novel link between pro-inflammatory and fibrogenic signals (148). Notably, miRNA-148a inhibits multiple regulatory checkpoints of the TLR4-MyD88-NF-κB axis (62). MiRNA-148a directly targets the 3'-UTR of TLR4 (158). Furthermore, over-expression of miRNA-148a using an agomiR markedly reduced the production of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) and suppressed NF-κB p65 activation by targeting the TLR4-mediated pathway (158). In addition, miRNA-148a directly targets calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) (159), which phosphorylates CARD-containing MAGUK protein 1 (CARMA1) involved in the activation of IκB kinase α (IKKα) and IκB kinase β (IKKβ). Furthermore, miRNA-148a mitigated hepatic ischemia/reperfusion injury by ameliorating TLR4-mediated inflammation via targeting CaMKIIα *in vitro* and *in vivo* (160,161). In addition, miRNA-148a directly targets IKKα and IKKβ, thereby enhancing the inhibitory effect of IκB on NF-κB (162). NF-κB directly activates Lin28 transcription and thus rapidly reduces let-7 levels (163).

There is convincing evidence in experimental models of colitis that MEX attenuate TLR4 expression and TLR4-induced NF-κB activation (62,164,165). Porcine MEX also decreased LPS-induced TLR4/NF-κB signaling pathway activation in IECs (164). Human MEX as well protected against intestinal organoid injury and decreased TLR4 expression (165). It is thus conceivable, that BMEX may also attenuate TLR4-mediated activation of HSCs.

Anti-fibrotic activity of miRNA-29s

PDGF signaling activates HSCs and portal fibroblast proliferation, chemotaxis, migration and cell survival (22,23,166). PDGFB is believed to be the most potent factor associated with early HSCs activation and is transiently increased during the early stage of activation (1,167,168). PDGFR is expressed on the membrane of HSCs and can therefore stimulate HSCs activation through autocrine mechanisms. Rapid induction of PDGFRB is a core feature of HSC activation (167). Depletion of PDGFRB in HSCs decreased injury and fibrosis *in vivo*, while its auto-activation accelerated fibrosis (167). The miRNA-29 family (miRNA-29a, miRNA-29b-1, miRNA-29b-2, miRNA-29c) is regarded to exert important anti-fibrogenic effects in various tissue fibroses including HF (169-176). In the healthy liver, miRNA-29 is highly expressed in hepatic HSCs and is responsible for repression of ECM proteins, in particular collagens, as well as PDGF-C and insulin-like growth factor 1 (IGF-1) (169). During fibrogenesis, fibrogenic growth factors are increased and stimulate HSCs in a paracrine and autocrine manner. Fibrogenic stimulation of TGF-β and PDGF-BB results in miRNA-29 repression. The resulting loss of miRNA-29 by TGF-β and PDGF-BB results in the abolished repression of fibrogenic expression of ECM, PDGF-C or IGF-1. Enhanced secretion of PDGF-C and IGF-1 stimulates HSC in an autocrine manner increasing HSC proliferation and ECM production (169). IGF-1 is a strong mitogen stimulating phosphatidylinositol 3-kinase (PI3K)/AKT signaling. Thus, the loss of miRNA-29 is regarded to be due to the response of HSCs to the exposure to the fibrogenic mediators TGF-β and PDGF-BB (169). Kwiecinski *et al.* (175) provided evidence that myofibroblastic transition of primary HSCs resulted in the loss of miRNA-29, but in a significant increase of PDGF-C and IGF-1. Compensation of reduced miRNA-29 levels by miRNA-29 over-expression in myofibroblastic HSCs was followed by a definitive repression of IGF-1 and PDGF-C synthesis (175). In contrast, Sanz *et al.* (176) reported that targeted over-expression of IGF-1 by activated HSCs restricts their activation, attenuates fibrogenesis, and accelerates liver regeneration.

Notably, BMEX enhanced IEC expression of glucose-regulated protein 94 (GRP94) (177). GRP94 is a critical

endoplasmic reticulum (ER) chaperone that enhances the secretion of properly folded mature IGF-1 (178).

Fu *et al.* (179) recently showed that miRNA-29a-3p suppresses HF pathogenesis by modulating HSC proliferation via targeting *PIK3R3* gene (phosphatidylinositol 3-kinase, regulatory subunit 3) modulating PI3K/AKT signaling. In miRNA-29a transgenic mice, miRNA-29a alleviated bile duct ligation with exacerbation of HF in mice through suppression of DNMT1 and DNMT3B lowering COL1A1 and SET domain containing 1A (SET1A) expression in HSCs (180). Gain of miRNA-29a signaling resulted in DNA hypomethylation and high PTEN expression. *in vitro*, miRNA-29a mimic transfection lowered COL1A1, DNMT1, DNMT3B and SET1A expression in HSCs (180). Thus, epigenetic action of miRNA-29a ameliorates the development of cholestatic HF. There is further evidence that miRNA-29a disrupts DNMT3B to ameliorate diet-induced non-alcoholic steatohepatitis (NASH) in mice (181). In accordance, a miRNA-29a mimic transfection reduced DNMT3B expression in primary HSCs (181).

An up-regulation of miRNA-29 and suppression of HSC activation has also been observed by class II histone deacetylase (HDAC) inhibition (182). In miRNA-29a transgenic mice, over-expression of miRNA-29a decreased COL1A1, histone deacetylases 4 (HDAC4) and activated HSC markers of glial fibrillary acidic protein expression compared to wild-type littermates. Over-expression of miRNA-29a and HDAC4 RNA-interference decreased the expression of fibrotic genes, HDAC4 signaling, and HSC migration and proliferation. In contrast, knockdown of miRNA-29a with an antisense inhibitor increased HDAC4 function, restored HSC migration, and accelerated HSC proliferation (183). Hepatic tissue in miRNA-29a transgenic mice displayed a weak fibrotic matrix concomitant with low fibrotic COL1A1 expression within the affected tissues compared to the wild-type mice fed a high-fat diet (184).

In comparison with patients without HF, miRNA-29b-3p level was remarkably reduced in those with HF (185). TGF-β1-induced HSC activation significantly decreased miRNA-29b-3p expression. However, miRNA-29b-3p over-expression repressed TGF-β1-induced COL1A1 protein and α-SMA expression. Furthermore, it was noted that miRNA-29b-3p directly binds to signal transducer and activator of transcription 3 (STAT3) and suppressed its expression (185). Wang *et al.* (186) demonstrated that miRNA-29b was significantly down-regulated in human and mice fibrotic liver tissues and in primary activated HSCs. MiRNA-29b down-regulation was directly mediated by SMAD3 through binding to the promoter of *MIR29B* in the HSC line LX1, whilst miRNA-29b could in turn suppress SMAD3 expression (186). Furthermore, miRNA-29b prevents liver fibrogenesis by inhibiting HSC activation and inducing HSC apoptosis through inhibiting PI3K/AKT signaling. These results provide further insights for the anti-fibrotic effect of miRNA-29b (186). Both miRNA-29b over-expression and SMAD3 silencing antagonized the effects of TGF-β1 on the expression of α-SMA and COL1A1 (187). Furthermore, infection with miRNA-29b mimics suppressed SMAD3 and TGF-β1 expression, suggesting that miRNA-29b inhibited LX-2 activation mediated by both SMAD3 and TGF-β1. Thus, Liang *et al.* (187) suggested a crosstalk between miRNA-29b and TGF-β1/SMAD3 during LX-2 activation. Homeobox transcript antisense RNA (HOTAIR), is an intergenic long non-coding RNA (lncRNA), which is up-regulated in HSCs in HF and is a target of miRNA-29b. HOTAIR epigenetically modulates PTEN expression via miRNA-29b, a further mechanism promoting HF (188). Transfection of a miRNA-29b precursor markedly attenuated the expression of COL1A1 and COL1A2 mRNAs and additionally blunted the increased expression of α-SMA, DDR2, FN1, ITGB1, and PDGFRB, which are key genes involved in the activation of HSCs (189). Over-expression of miRNA-29b led HSCs to remain in a quiescent state (189). Remarkably, the nucleotide sequences of human PDGFB 3'-UTR (UGGUGCU) and bovine PDGFB 3'-UTR (UGGUGCU) as well as the seed sequences of human miRNA-29b-3p (ACCACGA) and bovine miRNA-29b-3p (ACCACGA) are identical (190,191). Human and bovine let-7-5p are further miRNAs (7mer-m8) with higher probability for targeting human and bovine PDGFB (190,191). Furthermore, PDGFRB is a reported target of miRNA-29-3p (192). Thus, miRNA-29b effectively targets PDGFB-PDGFRB signaling, which represents a key mechanism activating HSCs. To make use of the anti-fibrotic activity of miRNA-29b as a novel miRNA tool, a MRI-visible nanocarrier system targeting HSCs has been developed (193). This pH-sensitive and vitamin A (VA)-conjugated copolymer VA-polyethyleneglycol-polyethyleneimine-poly(N-(N',N'-diisopropylamino-ethyl)-co-benzyl-amino) aspartamide (T-PBP) micelle efficiently transports the miRNA-29b and miRNA-122 to HSC in a magnetic resonance imaging-visible manner, resulting in a synergistic anti-fibrosis effect via down-regulating the expression of fibrosis-related genes, including COL1A1, α-SMA, and TIMP1 (193).

CTGF (also known as CCN2) is critically involved in HSC activation and HF (108,109,111,194). Notably, fibrogenic signaling is suppressed in HSCs through targeting of CTGF by cellular or exosomal miRNA-199a-5p (195). The basic helix-loop-helix transcription factor, Twist1, binds to the promoter of *MIR214* and drives miRNA-214 expression resulting in the suppression of CTGF (29). MiRNA-214 in LX-2 cells was shuttled by exosomes to recipient LX-2 cells or human HepG2 hepatocytes, resulting in suppression of CTGF and its downstream targets, α-SMA and collagen (196). Exosomal transfer of miRNA-214 thus regulates CTGF-dependent fibrogenesis and identifies fibrotic pathways as targets of intercellular regulation by exosomal miRNAs (196). MiRNA-214-enriched exosomes derived from endometrial stromal cells inhibited endometriosis fibrosis (197). Of note, miRNA-29b treatment exhibited an inhibitory effect against skin scar formation via inhibition of the TGF-β1/SMAD/CTGF signaling pathway (198). In accordance, miRNA-29b inhibited endometrial fibrosis by regulating the Sp1-TGF-β1/SMAD-CTGF axis in a rat model (199). Thus, miRNA-29-mediated suppression of CTGF may also attenuate HF. Remarkably, CTGF binds to fibroblast growth factor receptor 2 (FGFR2) and modulates its signaling (200). FGFR2-IIIb is exclusively expressed by hepatocytes, but not by activated HSCs (201).

Runt-related transcription factor 1 (RUNX1) and RUNX2 are fibrogenic transcription factors that are induced at the post-transcriptional level during activation of HSC and suppress TIMP1 via interaction with UTE-1, a regulatory DNA motif on the TIMP1 promoter (202). In the CCl_4-induced rat cirrhotic model, selective p38 mitogen-activated protein kinase (MAPK) inhibition exerted salutary effect on liver cirrhosis through down-regulation of RUNX2 (203). However, Baier *et al.* (64) demonstrated that the expression of RUNX2 increased by 31% in blood mononuclear cells 6 h after consumption of commercial cow milk in adult healthy volunteers compared with baseline. During osteoblast differentiation, miRNA-29b decreased the activity of COL1A1, COL5A3, and COL4A2 3'-UTR sequences in reporter assays, as well as endogenous gene expression but increased RUNX2 expression (204).

In contrast, miRNA-29b targets and negatively regulates parathyroid hormone-like hormone (PTHLH) (205), which is involved in the pathogenesis of HF (206,207). In cultured human HSCs, mRNA and protein levels of α-SMA, COL1A1, MMP-2, and TGF-β1 were increased by PTHLH treatment. A similar increasing pattern was also observed in LX-2 cells. Moreover, PTHLH significantly increased TGF-β1 secretion in cultured media from HSCs (207). Recent evidence links PTHLH to Hh pathway activation (208). PTHLH-mediated parathyroid hormone 1 receptor (PTH1R)-protein kinase C θ (PKC θ) pathway activation was identified as key event in the pro-fibrotic Hh-dependent activation of HSCs (208). As shown in IECs, PTHLH-PTH1R signaling can propagate protein kinase A (PKA) pathway activating downstream nuclear transcription factors, including RUNX2 (209). Thus, miRNA-29b-mediated suppression of PTHLH may suppress RUNX2 expression in HSCs.

Recently, autophagy has attracted much attention in HF pathogenesis. However, the role of autophagy in the course of HF is still controversial (210). Long non-coding small nucleolar RNA host gene 7 (lncRNA-SNHG7) was significantly increased in liver tissue and HSCs of liver fibrosis model of mice, whereas inhibition of SNHG7 expression in liver fibrosis mice could reduce HF (211). SNHG7 binds to miRNA-29b in HSCs and inhibits its expression (211). SNHG7 controls the expression of DNMT3A, which is a downstream target of miRNA-29b. In TGF-β-stimulated normal HSCs, knockdown of SNHG7 expression could restrain DNMT3A and HSCs activation factors α-SMA, COL1α and autophagy-related factors LC3I/II and Beclin1 (211).

Cow milk contains bovine miRNA-29b (64,212). A dose-dependent increase in miRNA-29b plasma levels has been observed with a peak maximum at 6 h after intake of commercial milk by healthy human volunteers (64). MiRNA-29 family members (miRNA-29a, miRNA-29b, miRNA-29c and miRNA-29d) have all been detected in BMEX and milk-derived EVs (73,213,214). It is thus conceivable that BMEX miRNA-29s may reach HSC after oral administration and exert anti-fibrogenic activities.

MiRNA-223

Hepatocyte TAZ/WWTR1 promotes inflammation and fibrosis in NASH (215). A key mechanism linking hepatocyte TAZ to NASH fibrosis is TAZ/TEA domain (TEAD)-mediated induction of Indian hedgehog (Ihh), a secretory factor that activates fibrogenic genes in HSCs (215). It has recently been shown that exosomes enriched in miRNA-223 attenuate non-alcoholic fatty liver disease (NAFLD)-associated fibrosis (54). Furthermore, miRNA-223, which is highly expressed in exosomes of natural killer (NK) cells inhibited TGF-β-induced HSC activation (210). Autophagy-related 7 (ATG7)

was confirmed as a direct target of miRNA-223. ATG7 over-expression in LX-2 cells abolished the suppressive effect of NK-derived exosomes on HSC activation (216). Inhibition of autophagy via miRNA-96-5p targeting ATG7 has also been shown to prevent HSC activation (217). It has been shown that autophagy releases lipid species that promote fibrogenesis by activated HSCs in mice and in human tissues (218). In addition, miRNA-223 targets the transcriptional activator with PDZ-binding motif (TAZ), a well-known factor promoting NASH fibrosis (54). After activation, neutrophils can deliver miRNA-223 via exosomes into macrophages (219,220) or hepatocytes (221) thereby inhibiting NASH and HF. Hepatic expression of miRNA-223 targets fibrotic genes including TAZ/WWTR1, NLRP3, IGF1R, and CXCL10 (54,221,222). Transcription factor TAZ/WWTR1 is increased in mouse and human NASH and silencing TAZ can prevent or reverse NASH features, notably fibrosis (218). Interestingly, Liu *et al.* (223) reported that up-regulation of circular RNA PWWP2A promotes HF via sponging miRNA-223 and miRNA-203. Circ-PWWP2A was up-regulated in both TGF-β- and LPS-activated HSCs and in mouse fibrotic liver tissues and was positively correlated with HSC activation and proliferation (223). In addition, FGFR2 is a direct target of miRNA-223 (224,225).

Izumi *et al.* (226) reported that miRNA-223 belongs to the group of dominant immune development-related miRNAs of cow milk. Benmoussa *et al.* (143) found miRNA-223 in BMEX but more abundant quantities in 200 nm EVs sedimenting at a centrifugation speed lower than that for BMEX. They observed that incubation of bovine milk EVs with human cultured HeLa cells led to cellular enrichment in miRNA-223, which was concomitant with decreased expression of a reporter gene placed under the control of miRNA-223, thereby demonstrating the functionality of miRNA-223. Their results suggest that bovine milk EVs transfer their miRNAs to human cells and regulate recipient cell gene expression (227).

Thus, miRNA-223 can be added to the list of potential anti-fibrogenic miRNAs of bovine milk EVs.

MiRNA-125b

You *et al.* (228) observed increased expression of miRNA-125b in activated HSCs but not in hepatocytes. Inhibition of miRNA-125b suppressed the expression of fibrogenic genes in culture-activated primary HSCs and reduced the basal TGF-β-induced α-SMA expression and cell contraction of the immortalized HSC cell line, whereas ectopic expression of miRNA-125b promoted α-SMA expression and HSC contraction (228). Antagonizing miRNA-125b *in vivo* significantly alleviated HF in CCl_4-treated mice. Mechanistically, over-expression of miRNA-125b in HSCs enhanced RhoA activity by directly targeting StAR-related lipid transfer (START) domain containing 13 (STARD13), a RhoA-specific GTPase-activating protein, whereas knockdown of miRNA-125b abrogated RhoA activation (228). Furthermore, inhibition of RhoA or its downstream molecules, MRTF-A and SRF, attenuated the miRNA-125b-induced α-SMA expression and HSC contraction. Thus, these findings identify a miRNA-125b-STARD13-RhoA-α-SMA signaling cascade in HSCs promoting HF.

In contrast, Hyun *et al.* (229) reported that miRNA-125b attenuated Hh signaling and promoted liver regeneration by chorionic plate-derived MSC (CP-MSC)-derived exosomes loaded with miRNA-125b. Notably, miRNA-125b is highly expressed in CP-MSCs, but not in LX2 HSCs. MiRNA-125b targeting SMO (Smoothened, frizzled class receptor) was retained in exosomes of CP-MSCs, whereas CP-MSCs with miRNA-125b inhibitor failed to attenuate the expression of Hh signaling and fibrotic genes in the activated HSCs. Therefore, the authors concluded that exosomal miRNA-125b derived from CP-MSCs suppressed the activation of Hh signaling and contributed to liver regeneration. Zhou *et al.* (230) reported that miRNA-125b directly targets SMAD2 and SMAD4, which inhibits EMT process in HCC cells. Because EMT is closely associated with HSC activation, it is possible that miRNA-125b exerts its anti-fibrotic effect through targeting SMAD4 and other EMT-related genes (230), including Hh signaling, in CCl_4-induced HF (90). FGFR2 was identified as a direct target suppressed by miRNA-125b (231,232).

Bovine milk-derived EVs and BMEX, which contain miRNA-125b (143), thus exert controversial effects in the pathogenesis of HF.

MiRNA-155

Zhu *et al.* (89) reported that miRNA-155 is up-regulated in HF, whereas Kitano *et al.* (88) provided evidence for reduced expression of miRNA-155 in activated HSCs. According to Csak *et al.* (233), miRNA-155 affects the progression of HF by regulating the SMAD3 signaling pathway. In particular, miRNA-155 deficiency attenuates steatosis and fibrosis in a mouse model of steatohepatitis (233). In a rat model of AH, miRNA-155 via targeting suppressor of cytokine signaling 1 (SOCS1) activated MAPK signaling and promoted HSC

viability and cycle progression and reduced cell apoptosis by silencing SOCS1, whereas inhibition of miRNA-155 up-regulated SOCS1 and inactivated the MAPK signaling pathway, thereby inhibited the proliferation of alcoholic HSCs (234). Bala *et al.* (235) observed that fibrogenic genes were attenuated in miRNA-155 KO mice after alcohol diet or CCl_4 treatment. Furthermore, they showed that TLR4 signaling regulates miRNA-155 as TLR4 KO mice showed no induction of miRNA-155 after alcohol exposure (235). Dai *et al.* (236) observed that miRNA-155 attenuates activation of HSCs by simultaneously preventing EMT and ERK1 signaling. Bone morphogenetic protein 9 (BMP9) is the most recently discovered member of the BMP family involved in HF (237). Both, RUNX2 and bone morphogenetic protein receptor type 2 (BMPR2) are two direct target genes of miRNA-155 (238). Accordingly, over-expressed miRNA-155 in MSCs reduced the expression of RUNX2 (238).

Both bovine colostrum and mature cow milk contain immune-modulating miRNA-155 (226). Pre-treatment of IEC-6 cells with BMEX increased intracellular miRNA-155 levels (239). Notably, miRNA-155 was enriched in exosomes released from HCC cells, suppressed the expression of PTEN and stimulated HCC cell proliferation (240). In fact, miRNA-155-5p promoted proliferation, invasion and migration, but inhibited apoptosis in HCC by directly targeting the 3'-UTR of PTEN (241). Furthermore, hepatitis C virus (HCV)-induced up-regulation of miRNA-155 promotes hepatocarcinogenesis by activating WNT signaling (242). In addition, miRNA-155 mediates hepatitis B virus (HBV) replication by reinforcing SOCS1 signaling-induced autophagy (243).

These data indicate, that BMEX-derived miRNA-155 may exert unfavorable effects especially in states of HF associated with HBV- and HCV infection and HCC.

MiRNA-30a

TGF-β1 down-regulated the expression of miRNA-30a in activated HSCs and LX-2-exosomes, whereas over-expression of miRNA-30a suppressed α-SMA, TIMP-1, COL1 expression, and cell viability in HSCs (244). MiRNA-30a was significantly down-regulated in HF mice and over-expression of miRNA-30a prevented BDL-induced fibrogenesis, concomitant with the down-regulation of ECM. MiRNA-30a inhibited HSCs autophagy via targeting its downstream effector beclin1 (244). Furthermore, miRNA-30a directly targets the mRNA of growth/differentiation factor 2 (GDF2=BMP9) (245). It has been shown in murine multilineage cells that BMP9, a fibrinogenic transcription factor (237), enhanced the expression of miRNA-21 (246).

MiRNA-30a belongs to the top 50 miRNAs expressed in whey-derived BMEX (213) and counterbalance fibrogenic miRNA-21 signaling.

MiRNA-34a

MiRNA-34a as well as miRNA-155 have been associated with the pathogenesis of NAFLD (247). Li *et al.* (248) confirmed a direct interaction of peroxisome proliferator-activated receptor-γ (PPARγ) with miRNA-34a and miRNA-34c. Notably, the expression of PPARγ was negatively correlated with the expression of miRNA-34a and miRNA-34c during the activation of HSCs. In activated human HSCs, inhibitors of miRNA-34a and miRNA-34c up-regulated the expression of PPARγ and down-regulated the expression of α-SMA (248). These data suggested that the miRNA-34 family may be involved the process of HF by targeting PPARγ. Furthermore, miRNA-34a appears to play an important role in the process of HF by targeting acyl-CoA synthetase long-chain family member 1 (ACSL1) ACSL1 (249). MiRNA-34a-silenced HSCs showed higher ACSL1 and lower α-SMA, COL1A1, and desmin expression than that of matched negative controls and non-transfected cells (249). Tian *et al.* (250), using a CCl_4-induced rat HF model found that the miRNA-34a/SIRT1/p53 signaling pathway was activated in hepatocytes but not in HSCs. They suggested that the activation of this pathway in hepatocytes results in the apoptosis of hepatocytes and thus activates HSCs (250).

In contrast, Feili *et al.* (251) observed decreased expression of miRNA-34a-5p in patients with HBV-activated HF and HCC, as well as in CCl_4-induced HF model mice. The TGF-β1/SMAD3 pathway was significantly augmented in $CC1_4$-induced mice compared with normal control, whereas an inhibitor of TGF-β1 significantly attenuated HF and TGF-β1/SMAD3 activation. Administration of the miRNA-34a-5p mimic de-activated TGF-β1/SMAD3 pathway in human HSCs (LX-2). Moreover, the target gene for miRNA-34a-5p, SMAD4, was predicted and verified in LX-2 cells. Taken together, these data demonstrated that over-expression of miRNA-34 in HSCs ameliorated the development and progression of HF by targeting SMAD4 and regulating TGF-β1/SMAD3 pathway (251). Liu *et al.* (252) reported that miRNA-34a and miRNA-29c were significantly reduced and Sirt1 protein was increased in PU box binding protein PU.1+/- HSCs compared with WT

HSCs. Notably, PU.1 directly binds to both the promoter regions of miRNA-34a and miRNA-29c. PU.1 suppresses Sirt1 translation via transcriptional promotion of miRNA-34a and miRNA-29c, thus promoting Sirt1-mediated HSC activation and thioacetamide-induced HF.

In comparison to bovine colostrum, mature milk contains lower quantities of mRNA-34a (226). Gao *et al.* (253) presented evidence that under hypoxic conditions BMEX decreased hypoxia inducible factor-1α (HIF-1α). However, bovine miRNA-34a was found to be an effective regulator for alleviating hypoxic injury of IEC-6 via up-regulation of HIF-1α (254). HIF-1α was significantly increased in hepatic fibrotic tissues and activated HSCs. Furthermore, knockdown of HIF-1α expression inhibited the proliferation and activation of HSCs. In addition, HIF-1α-dependent genes and the extensive network of signaling cascades focus on HIF-1α have been reported to associate with the development of HF, suggesting that HIF-1α might play a crucial role in HF (255,256).

BMEX-mediated suppression of HIF-1α may thus exert beneficial effects in HF.

Fibrogenic miRNA-21

MiRNA-21 is up-regulated in fibrogenesis. Wei *et al.* (257) used PDGF-BB to stimulate HSCs (LX-2 cells) and found that the levels of COL1A1 and α-SMA were reduced while miRNA-21 levels were increased. Remarkably, decreasing the expression of miRNA-21 by transfecting a miRNA-21 inhibitor into LX-2 cells prevented PDGF-BB induced LX-2 cell activation (257). The mRNA level of PTEN was reduced in LX-2 cells by over-expression of miRNA-21, leading to activation of AKT signaling (257). The authors concluded that miRNA-21 induced HSC activation by regulating the PTEN/AKT signaling pathway (257). Moreover, the auto-regulatory feedback loop of miRNA-21/programmed cell death protein 4 (PDCD4)/activation protein-1 (AP-1) was considered a driving force for the development of HF (257). Disrupting this feedback loop by using either miRNA-21- or AP-1 inhibitors significantly diminished fibrogenesis while blocking PDCD4 enhanced fibrogenesis in HSCs (258). MiRNA-21 also modulates extracellular signal-regulated kinase 1 (ERK1) during fibrogenesis by regulating HNF4α and Sprouty 2 (SPRY2) expression (259). Methyl helicterate (MH) has been reported to have protective effects against CCl_4-induced hepatic injury and fibrosis in rats. Huang *et al.* (260) reported that MH treatment significantly decreased miRNA-21 expression and inhibited the activation of the ERK and TGF-β1/SMAD2/3 pathways in liver tissues. MH strongly inhibited HSC-T6 cell activation and reduced collagen accumulation. Notably, miRNA-21 over-expression significantly promoted HSC-T6 cell proliferation, reduced HSC apoptosis, and increased collagen formation, while these abnormal changes induced by miRNA-21 over-expression were significantly reversed by MH treatment. Over-expressed miRNA-21 activated the ERK and TGF-β1/SMADs pathways via repressing SPRY2 and SMAD7, respectively (261). In addition, 3,3'-diindolylmethane (DIM), a natural autolytic product in plants that can down-regulate miRNA-21 expression, suppressed the central TGF-β signaling pathway underlying HSC activation by down-regulating miRNA-21. Decreased miRNA-21 expression was achieved by inhibiting the activity of the transcription factor AP-1 (261). Of note, miRNA-21 is transcriptionally up-regulated in response to SMAD3 rather than SMAD2 activation after TGF-β stimulation. In addition, TGF-β promotes miRNA-21 expression by formation of a microprocessor complex containing SMAD proteins. Elevated miRNA-21 may then act as a fibrogenic miRNA by its repression of the TGF-β inhibitory SMAD7 protein (169). Anti-miRNA-21 treatment reduced liver tumor growth and prevented tumor development. These effects were accompanied with a decrease in HF and a concomitant reduction of $CD24^+$ liver progenitor cells and $S100A4^+$ cancer-associated stromal cells (262). In contrast, Caviglia *et al.* (263) recently reported that miRNA-21 antisense inhibition did not suppress the activation of murine or human HSCs in culture or in liver slices. Moreover, genetic deletion of miRNA-21 in two independently generated knockout mice or miRNA-21 antisense inhibition did not alter HSC activation or HF in models of toxic and biliary liver injury. As inhibition of the most up-regulated miRNA did not affect HSC activation, HF, or fibrosis-associated liver cancer. Remarkably, Dicer deletion, which decreased HSC miRNA expression only exerted negligible effects on HSC activation and HF (263). On the other hand, Ning *et al.* (264) reported that miRNA-21 mediates angiotensin II-activated NLRP3 inflammasome and resultant HSC activation via targeting SPRY1 and SMAD7. Hypoxia-responsive miRNA-21-5p promoted RUNX2 expression (at least in part) by targeting the 3'-UTR and down-regulating SMAD7 expression (265), which represses RUNX2 expression (266).

Makhmudi *et al.* (267) showed that miRNA-21 expression was significantly increased in patients with biliary atresia compared to controls, whereas miRNA-21 expression was significantly lower in cirrhosis than non-cirrhosis patients

with biliary atresia. Up-regulated expression of miRNA-21 in liver samples from biliary atresia patients negatively correlated with PTEN expression and downstream activated AKT pathway provoked HF by enhancing α-SMA levels (268).

HCC cells exhibit a great capacity to convert normal HSCs to cancer-associated fibroblasts (CAFs). They secrete exosomal miRNA-21 that directly targeted PTEN, leading to activation of PDK1/AKT signaling in HSCs (269). Knockout of miRNA-21 attenuates AH through the VHL/NF-κB signaling pathway in HSCs (270). Recent evidence indicates that miRNA-21, acting via the HIF-1α/VEGF signaling pathway, is also involved in arsenite-induced HF through mediating aberrant cross-talk of hepatocytes and HSCs (271). In addition, oncogenic virus (HBV, HCV, HPV, and EBV) infections up-regulated host miRNA-21 to evade host immune responses and to promote viral replication (272). Taken together, accumulated evidence supports adverse effects of miRNA-21 in HSC activation, HF and development of HCC.

MiRNA-21 is a dominant miRNA (signature RNA) of commercial cow milk (116) and component of BMEX (58,72,273). In human volunteers, increased plasma miRNA-21 levels have been reported 6 h after milk consumption (68). *Table 2* summarizes the anti-fibrogenic and fibrogenic miRNAs in relation to prominent signature miRNAs transported by BMEX.

Gut-liver axis

The gut-liver axis refers to the bidirectional relationship between the gut and its microbiota, and the liver, resulting from the integration of signals generated by dietary, genetic and environmental factors (274,275). Cirrhosis by itself is associated with profound alterations in gut microbiota and damage at the different levels of defense of the intestinal barrier, including the epithelial, vascular and immune barriers (274,275). The deterioration of intestinal barrier integrity and the consulting increased intestinal permeability in cirrhotic patients play a pivotal pathophysiological role in the development of severe complications (276,277). Bacterial translocation is the migration of viable or nonviable microorganisms or their pathogen-associated molecular pattern (PAMPs), such as LPS, from the gut lumen to the mesenteric lymph nodes, systemic circulation and other normally sterile extraintestinal sites (276). Via portal circulation, the liver is the first organ in the body to encounter not only absorbed nutrients, but also gut-derived bacteria and PAMPs. Chronic exposure to increased levels of PAMPs has been linked to disease progression during early stages and to infectious complications during late stages of liver disease (cirrhosis) (277). There are indications for intestinal epithelial barrier dysfunction in patients with chronic liver diseases and especially in patients with cirrhosis, which can be caused by various factors affecting both the small and large intestine (278,279). It has been demonstrated that human liver cirrhosis induces significant alterations in enterocytes' tight junctions (277). Patients with decompensated and compensated cirrhosis presented significantly reduced expression of occludin and claudin-1 as compared to controls (280).

A shortage of autochthonous non-pathogenic bacteria and an overgrowth of potentially pathogenic bacteria are common findings in cirrhotic patients. The ratio of the amounts of beneficial autochthonous taxa (*Lachnospiraceae* + *Ruminococaceae* + *Veillonellaceae* + *Clostridiales Incertae Sedis XIV*) to those of potentially pathogenic taxa (*Enterobacteriaceae* + *Bacteroidaceae*) was low in those with early death and organ failure (281). Recently, increased levels of systemic LPS-positive bacterial EVs in patients with intestinal barrier dysfunction have been detected (282). Apparently, intestinal barrier dysfunction opens the door for bacterial EV-associated LPS that may trigger TLR4 activation of HSCs. Thus, emerging therapeutic strategies targeted at restoring intestinal eubiosis, augmenting gut barrier function and ameliorating the mucosal and systemic immune deficits that characterize and define the course of decompensated cirrhosis are in the focus of recent research (283).

There is accumulating evidence from experimental models of colitis and necrotizing enterocolitis that human, bovine, yak, porcine and rat MEX and EVs improve intestinal mucin synthesis, up-regulate the expression of tight junction proteins improving intestinal barrier function (58-61,164,165,177,258,284-293), recently reviewed elsewhere (62). In a murine model of ulcerative colitis (kindlin2 KO mice), Stremmel *et al.* (61) observed strong anti-inflammatory effects of orally given BMEX. In accordance, BMEX prevented colon shortening, reduced intestinal epithelium disruption, inhibited infiltration of inflammatory cells and intestinal tissue fibrosis (63). Mechanistically, BMEX attenuate inflammatory responses via inhibiting TLR4-NF-κB signaling and NLRP3 inflammasome activation (63).

Furthermore, the disturbed gut microbiota in dextran sulfate sodium-induced colitis recovered partially upon treatment with bovine MEX (63). In addition, Yu *et al.* (294)

Table 2 Potential effects of milk exosomal miRNAs on hepatic stellate cells (HSCs)

miRNAs	Effect on HSCs	Mode of action	References
miRNA-148a	Anti-fibrogenic	Inhibition of DNMT1	Yang *et al.* (97)
miRNA-148a	Anti-fibrogenic	Inhibition of SMAD2	Jiang *et al.* (125)
miRNA-148a	Anti-fibrogenic	Inhibition of CCKBR	Tucker *et al.* (128)
miRNA-148a	Anti-fibrogenic	Inhibition of TLR4	Jiang *et al.* (158)
miRNA-148a	Anti-fibrogenic	Inhibition of CAMK2A	Liu *et al.* (159)
miRNA-148a	Anti-fibrogenic	Inhibition of IKBKB	Patel *et al.* (162)
let-7	Anti-fibrogenic	Inhibition of TGFBR1 and TGFRB3	Bronevetsky *et al.* (139)
let-7	Anti-fibrogenic	Inhibition of SMAD2	Shen *et al.* (140)
miRNA-29a	Anti-fibrogenic	Inhibition of DNMT1	Yang *et al.* (180)
miRNA-29a	Anti-fibrogenic	Inhibition of DNMT3B	Yang *et al.* (181)
miRNA-29a	Anti-fibrogenic	Inhibition of PIK3R3	Fu *et al.* (179)
miRNA-29b	Anti-fibrogenic	Inhibition of STAT3	Gong *et al.* (185)
miRNA-29b	Anti-fibrogenic	Inhibition of SMAD3	Wang *et al.* (186)
miRNA-29b	Anti-fibrogenic	Inhibition of SMAD3	Liang *et al.* (187)
miRNA-29b	Anti-fibrogenic	Inhibition of CTGF	Guo *et al.* (198)
miRNA-29b	Anti-fibrogenic	Inhibition of CTGF	Li *et al.* (199)
miRNA-29b	Anti-fibrogenic	Inhibition of PTHLH	Dou *et al.* (205)
miRNA-29b	Anti-fibrogenic	Inhibition of PTHLH	He *et al.* (208)
miRNA-29b	Anti-fibrogenic	Inhibition of DNMT3B	Xie *et al.* (211)
miRNA-29	Anti-fibrogenic	Inhibition of PDGFB	Targetscan 7.2 (190)
miRNA-29	Anti-fibrogenic	Inhibition of PDGFC and IGF1	Kwiecinski *et al.* (175)
miRNA-223	Anti-fibrogenic	Inhibition of ATG7	Wang *et al.* (216)
miRNA-223	Anti-fibrogenic	Inhibition of TAZ/WWTR1	Hou *et al.* (54)
miRNA-223	Anti-fibrogenic	Inhibition of TAZ/WWTR1	He *et al.* (221)
miRNA-223	Anti-fibrogenic	Inhibition of NLRP3	Jimenez Calvente *et al.* (222)
miRNA-125b	Anti-fibrogenic	Inhibition of SMO and Hedgehog	Hyun *et al.* (229)
miRNA-125b	Anti-fibrogenic	Inhibition of SMAD2 and SMAD4	Zhou *et al.* (230)
miRNA-125b	Fibrogenic	Inhibition of STARD13	You *et al.* (228)
miRNA-155	Anti-fibrogenic	Reduced EMT and ERK signaling	Dai *et al.* (236)
miRNA-155	Fibrogenic	Inhibition of SOCS1	Liu *et al.* (234)
miRNA-155	Fibrogenic	HSC activation	Csak *et al.* (233)
miRNA-30a	Anti-fibrogenic	Inhibition of BECN1	Chen *et al.* (244)
miRNA-30a	Anti-fibrogenic	Inhibition of BMP9	Targetscan 7.2 (245)
miRNA-34a	Anti-fibrogenic	Inhibition of SMAD4	Feili *et al.* (251)
miRNA-34a	Fibrogenic	Suppression of PPARγ	Li *et al.* (248)

Table 2 (*continued*)

Table 2 (*continued*)

miRNAs	Effect on HSCs	Mode of action	References
miRNA-34a	Fibrogenic	Inhibition of ACSL1	Yan *et al.* (249)
miRNA-34a	Fibrogenic	Sirt1-mediated HSC activation	Liu *et al.* (252)
miRNA-21	Fibrogenic	Inhibition of PTEN	Wei *et al.* (257)
miRNA-21	Fibrogenic	Increased PI3K-AKT signaling	Shen *et al.* (268)
miRNA-21	Fibrogenic	Inhibition of PDCD4	Zhang *et al.* (258)
miRNA-21	Fibrogenic	Inhibition of HNF4A and SPRY2	Zhao *et al.* (259)
miRNA-21	Fibrogenic	SPRY2 and SMAD7	Zhang *et al.* (261)
miRNA-21	Fibrogenic	Inhibition of SRRY1 and SMAD7	Ning *et al.* (264)

showed that BMEX modify bacterial growth and could promote the growth of *Escherichia coli* K-12 MG1655 and *Lactobacillus plantarum* WCFS1. Zhou *et al.* (295) studied the influence of dietary BMEX on bacterial communities in C57BL/6 mice. At the OTU level, four OTUs from the family of *Lachnospiraceae* were more than two times more abundant in mice fed BMEX compared with mice fed BMEX-deficient diet at age 7 and 47 wk. The authors concluded that BMEX modify the gut microbiome across species boundaries (295).

BMEX co-administration with anti-fibrogenic drugs

BMEX-mediated anti-fibrogenic miRNA signaling may be augmented by co-administration of drugs that further enhance anti-fibrogenic miRNA signaling such as salvianolic acid (Sal B) (112) and metformin (296). In mice, metformin could mitigate CCl_4-induced HF, beneficial effects which might result from suppressed TGF-β1/SMAD3 signaling (296). Metformin directly targets and suppresses the expression of miRNA-21 (297-304), a major oncogenic and fibrogenic miRNA and component of BMEX. In C57/BL6 mice, long-term treatment with metformin reduced hepatic p-STAT3 and miRNA-21 levels and protected mice from aging-dependent hepatic vesicular steatosis (305). Importantly, metformin down-regulates the expression of lncRNA-H19, a sponge of anti-fibrotic miRNAs including miRNA-148a (98,306), let-7 (307-309), and miRNA-29b (310), respectively. Recent evidence underlines that the H19/miRNA-148a/USP4 axis facilitates HF by enhancing TGF-β signaling in both HSCs and hepatocytes (311). In fibrotic liver tissue of CCl_4-induced mice, H19 over-expression significantly activated HSCs and EMT of hepatocyte both by stimulating the TGF-β pathway. H19 via sponging miRNA-148a sustained the level of ubiquitin-specific protease 4 (USP4), a direct target of miRNA-148a (312,313) stabilizing TGF-β receptor 1 signaling (311) (*Figure 2*).

It has been proposed that metformin via AMPK activation may also inhibit the expression or activity of Lin28/Lin28A proteins, which act post-transcriptionally to decrease the levels of all let-7 family members (314-317). In a rat model of polycystic liver disease, metformin, an indirect AMPK activator and suppressor of mechanistic target of rapamycin complex 1 (mTORC1) (318), reduced liver cyst formation and HF (319). Metformin showed also preventive effects on HCC development in animal models and diabetic patients (320,321). Thus, the combination of BMEX with metformin may not only have synergistic effects in the treatment of HF but might reduce the risk of oncogenic BMEX-derived miRNAs involved in the hepatocellular cancerogenesis. Due to the fact that HF and cirrhosis enhance the risk of HCC (322), HF treatment with BMEX prior to the occurrence of HCC may decrease the risk of HCC development. Conversely, there is a risk of pre-existing hidden HCC and HCC progression that may be promoted by BMEX administration as shown in a recent rodent tumor model (73). In addition, epidemiological and translational evidence identified cow milk consumption as a nutritional risk factor of HCC (323). However, in contrast to the cancerogenic effects of the complex system milk, BMEX-derived miRNA-148a via targeting CCK-BR and USP4 may reduce the susceptibility for the development of HCC (311,324).

Thus, a combined treatment with BMEX and metformin may prevent both HF and HCC development.

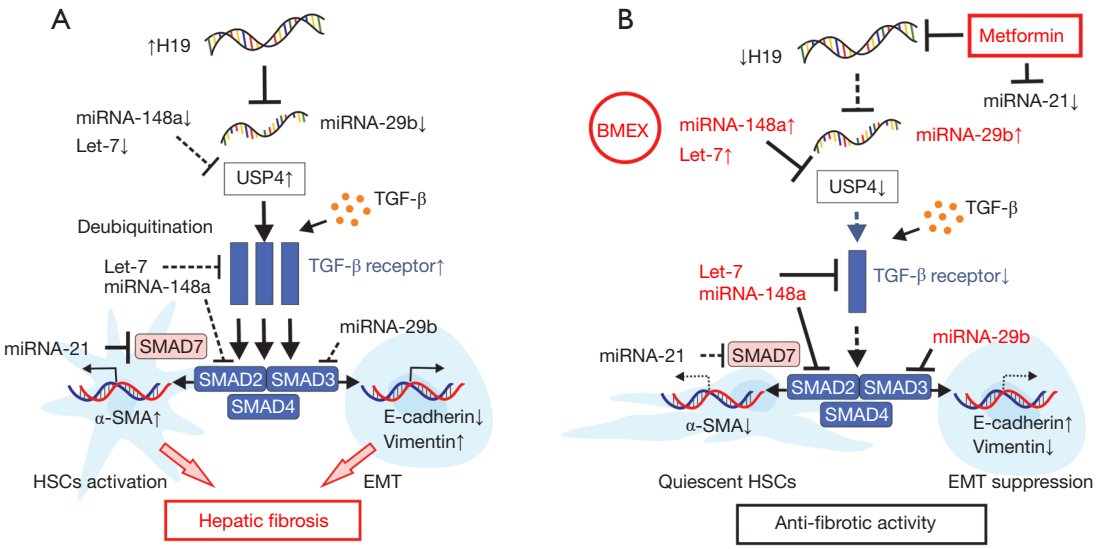

Figure 2 MiRNA signaling in the pathogenesis and treatment of hepatic fibrosis. (A) Illustration of fibrogenic miRNA signaling in hepatic fibrosis. Enhanced expression of lncRNA H19 suppresses the activity of miRNA-148a, let-7 and miRNA-29b. Reduced miRNA-148a expression enhances the expression of ubiquitin-specific protease 4 (USP4) resulting in reduced proteasomal degradation of TGF-β receptor 1. Reduced expression of let-7 further enhances the expression TGF-β receptor 1. Reduced expression of miRNA-29b enhances the expression of SMAD3. Increased miRNA-21 expression attenuates the expression of SMAD7, a negative regulator of TGF-β-SMAD signaling. (B) Potential therapeutic intervention in hepatic fibrosis with milk exosomes co-administered with metformin. Bovine milk-derived exosomes (BMEX) may increase the availability of miRNA-148a, let-7 family members and miRNA-29b in hepatic stellate cells. Metformin suppresses H19 and thus further enhances the levels of these anti-fibrogenic miRNAs. Furthermore, metformin reduces the expression of fibrogenic miRNA-21, enhancing the expression of SMAD7. Increased expression of let-7, miRNA-148a, miRNA-29b reduce the expression of TGF-β receptor 1, SMAD2 and SMAD3, respectively, thus synergistically reduce TGF-β signaling, critically involved in the pathogenesis of hepatic fibrosis as well as epithelial-mesenchymal transition (EMT) in hepatocellular carcinoma.

Conclusions

Recent research interest focuses on siRNA- and miRNA-based therapeutics for HF (325). MiRNAs let-7b, let-7a, let-7c, miRNA-148a, let-7f, miRNA-29c, miRNA-30d, miRNA-30a and miRNA-125b, miRNA-29a and let-7d belong to the top 50 miRNAs expressed in whey-derived BMEX (211). The great majority of these miRNAs exerts anti-fibrotic effects (*Table 2*). The net signaling effect of all MEX-derived miRNAs should be anti-fibrogenic, because fibrogenic signaling during postnatal milk intake and the development of liver fibrosis would restrict appropriate liver growth during early life. The majority of dominant miRNAs delivered by BMEX such as miRNA-148a, let-7 and miRNA-29s are key anti-fibrotic miRNAs that inhibit HSC activation. However, there is concern about fibrogenic and oncogenic miRNAs transferred via BMEX such as miRNA-21, which could be silenced by specific RNAs loaded to BMEX prior to delivery (74,326) or co-administration of miRNA-21-suppressing drugs such as metformin (297-304).

Experimental evidence indicates that BMEX and their miRNA cargo reach the liver (70,73,75), and presumably the space of Disse including HSCs. Recent bioinformatic evidence supports the view that bovine miRNAs are able to interact with human genes (327). Future studies in rodent systems as well as HSC cell cultures exposed to BMEX are required to obtain further experimental support. The stabilization of intestinal barrier function by BMEX, the anti-inflammatory effects of BMEX and the modification of the microbiome by BMEX may be additional beneficial effects for the treatment of patients with HF. Thus, BMEX may have a direct impact on HSC homeostasis and the gut-liver axis, which is in accordance with recent views promotes the therapeutic use of BMEX per se or for drug delivery (74-77,328,329).

There is accumulating evidence that exosomes and their cargo derived from MSCs and NK cells are

effective inhibitors of HSC activation and fibrogenesis (42,81,195,330). Our predictions of BMEX as potential treatment option of HF is still limited by the fact that experimental and clinical studies proving beneficial therapeutic effects of BMEX in HSC cultures, animal models of HF and in patients with HF are still missing. However, it has recently been shown in a rat model of cardiac fibrosis that administration of BMEX over a period of 7 days alleviated cardiac fibrosis and reduced the over-expression of COL1 and α-SMA (331). Exosome-encapsulated miRNA-29 decreased renal fibrosis, TGF-β, α-SMA, fibronectin, and COL1A1 in the kidney of unilateral ureteral obstruction mice (332). Accumulated basic research evidence presented in this narrative review should thus stimulate future research work to elucidate the therapeutic efficacy of oral BMEX administration for the treatment of HF.

Taken together, basic research evidence presented in this narrative review identifies BMEX as promising therapeutic agents for the treatment of HF either native or supplemented with siRNAs (antagomiR-21) or co-administered with other anti-fibrotic drugs that modify miRNA expression in HSCs.

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