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Corresponding Author: Jong-Hoon Kim

Authors: Yu Jin Jang[1], Su Yeon An[1], Jong-Hoon Kim[1,*]

Institution: [1]Laboratory of Stem Cells and Tissue Regeneration, Department of Biotechnology, College of Life Sciences & Biotechnology, Korea University, Seoul 02841, Republic of Korea,

Title Identification of MFGE8 in mesenchymal stem cell secretome as an anti-fibrotic factor in liver fibrosis

Author: Yu Jin Jang, Su Yeon An, and Jong-Hoon Kim*

* Corresponding author; E-mail: jhkim@korea.ac.kr

Laboratory of Stem Cells and Tissue Regeneration, Department of Biotechnology, College of Life Sciences & Biotechnology, Korea University, Seoul 02841, Republic of Korea

Keywords: MFGE8, MSC, liver fibrosis, secretome

Abbreviations: MFGE8, Milk fat globule-EGF factor 8; MSCs, Mesenchymal stem cells; HSCs, Hepatic stellate cells; ECM, Extracellular matrix; UCMSCs, Umbilical cord-derived MSCs; TGFβ, Transforming growth factor beta

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Beneficial paracrine roles of mesenchymal stem cells (MSCs) in tissue repairs promise potential therapeutic strategies against different diseases. However, key therapeutic factors secreted from MSCs and their exact molecular mechanisms of action are not well defined. We found that the cell-free secretome of umbilical cord-derived MSCs had significant anti-fibrotic activity in mouse models of liver fibrosis. Our study demonstrated that the reduction in fibrosis was achieved by perturbing hepatic stellate cell activation by directly inhibiting TGFβ/Smad-signaling. Importantly, the anti-fibrotic effects of MSC secretome were blocked *in vitro* and *in vivo* by antagonizing milk fat globule-EGF factor 8 (MFGE8) activity. Furthermore, we showed that MFGE8 was secreted by MSCs not only from umbilical cord, but also from other tissues, including teeth and bone marrow. We proved that administration of recombinant MFGE8 protein alone had a significant anti-fibrotic effect using two different models of liver fibrosis. Additionally, the MFGE8 downregulated TGFβ type I receptor expression by binding to αvβ3 integrin on HSCs. These findings uncover a potential new role of MFGE8 in modulating TGFβ-signaling and suggest that MFGE8 could serve as a novel therapeutic agent for liver fibrosis.

Tissue regeneration and repair necessitate compensatory restitution of lost tissues and reorganization of the extracellular matrix (ECM) framework. Many previous studies have shown that tissue reconstruction by grafts of stem cells or their progeny promotes functional recovery of injured tissues, including the liver. MSCs are promising candidates for cell replacement therapy due to their ability to differentiate into osteoblasts, chondrocytes, and adipocytes. Furthermore, they may differentiate possibly into broader lineages of cell types such as neurons, pancreatic cells, and hepatic cells, although this ability is still under debate. Recent accumulating evidence show that MSCs secrete a wide spectrum of soluble factors and may promote host tissue regeneration by paracrine actions rather than differentiation into functional somatic cells. However the therapeutic agents released by MSCs and their exact mode of action are largely unknown.

Liver fibrosis is one of the major medical problems with significant morbidity and mortality worldwide. Liver fibrosis is caused by excess deposition of ECM in tissues and can develop into cirrhosis or liver cancer. In the early stages of hepatic fibrosis, macrophages were recruited into the liver by chemokines produced from both damaged hepatocytes and endothelial cells, and produce a various inflammatory cytokines, including TGFβ1. TGFβ is the most potent stimulating factor for procollagen I & III gene transcription. TGFβ/Smad signaling activates quiescent HSCs into myofibroblast-like cells, a key player in ECM production. Activated HSCs (myofibroblast-like cells) also produce TGFβ1, thus establishing both autocrine and paracrine loops for the abnormal accumulation of collagen in the liver.

A series of recent findings have raised the possibility that grafted MSCs may be able to reduce hepatic, cardiac, and peritoneal fibrosis through paracrine actions. Umbilical cord-derived

MSCs (UCMSCs) are known to reduce fibrosis of bleomycin-induced lung injury. Particularly, direct injection of exosomes isolated from UCMSCs reduced collagen accumulation in fibrotic mouse livers, suggesting that UCMSCs may secrete anti-fibrotic factors. However, the precise mechanisms of secretome-mediated tissue regeneration have remained unclear. In our study, we demonstrated that a single injection of the secretome obtained from UCMSCs significantly reduced liver fibrosis without cell grafting. We found that injection of the secretome into mice with liver fibrosis markedly decreased fibrillary collagen deposition and reduced the both expression of fibrosis-related genes and activation of HSCs in the liver. Our *in vitro* study also showed that secretomes diminished HSC activation without inducing apoptosis or senescence, and decreased the phosphorylation of Smad2 in the presence of TGFβ1. Accordingly, these data indicated that the primary action mechanism of secretome was achieved by inhibiting the TGFβ signaling pathway.

Using LC/MS and network analyses, we analyzed the UCMSC secretome and selected MFGE8 as a potential anti-fibrotic factor that contribute to the secretome-mediated reduction of liver fibrosis. MFGE8 is a soluble glycoprotein composed of an N-terminal notch-like EGF domain with a highly conserved RGD motif and a C-terminal discoidin-like factor 5/8 factor domain. Our *in vitro* studies demonstrated that anti-MFGE8 neutralizing antibodies reduced the antagonistic activity of UCMSC secretome against HSC activation. In addition, we found that recombinant MFGE8 protein downregulated the expression of TGFB type I receptor (*TGFBR1*) at mRNA and protein levels, and decreased activation of HSC in the presence of TGFβ1. The MFGE8-mediated suppression of *TGFBR1* was counteracted by anti-αvβ3-integrin antibody, indicating that MFGE8 acts through αvβ3 integrin. These data suggest a potential new role for MFGE8 as a modulator of TGFβ signaling in HSCs. In addition, *in vivo* studies showed that the anti-fibrogenic activity of the secretome nearly diminished when the anti-MFGE8 antibody was administered. Furthermore, when recombinant MFGE8 protein alone was administered into mice with liver fibrosis, fibrotic area was significantly reduced. Histological studies using clinical samples demonstrated that the expression of MFGE8 was profoundly decreased in liver tissues of patients with cirrhosis, compared to normal liver. Therefore, we speculate that MFGE8 may play an anti-fibrogenic roles in endogenous regulation of liver fibrosis as well as in UCMSC secretome-mediated reduction of fibrosis.

In conclusion, our data demonstrated that secretome of MSCs has a potent anti-fibrotic power that significantly reduce fibrosis in the liver. The anti-fibrotic effect was mediated by inhibition of HSC activation by perturbing TGFβ signaling. We also identified MFGE8 as a novel key anti-fibrotic factor in MSC secretome that strongly diminish liver fibrosis and its therapeutic mechanism was validated *in vitro* and *in vivo.*

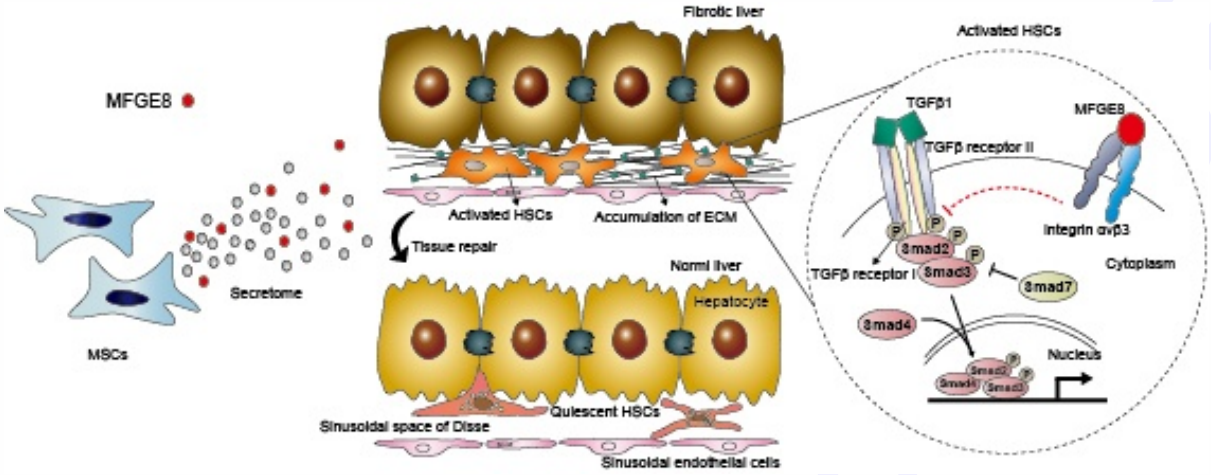

Fig. 1. Schematic model for MSC secretome-induced fibrotic regression in the liver. MSCs obtained from different tissues secrete many soluble factors, including MFGE8. MFGE8 binds to integrin αvβ3 on hepatic stellate cells (HSCs) and downregulates the expression of TGFBR1. Perturbation of TGFβ signaling attenuates the activation of HSCs, a major source of ECM production, reducing the fibrogenic progression.