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PM2.5-exposed hepatocytes induce hepatic stellate cells activation by releasing TGF-β1

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ABSTRACT :

The interaction between various types of hepatic cells is related to liver fibrosis. Recent studies demonstrated that fine particulate matter (PM2.5) exposure is an important risk factor for the occurrence of liver fibrosis, but its molecular mechanism is still obscure. In this study, we aimed to investigate whether transforming growth factor- β1 (TGF- β1) secreted from PM2.5-treated hepatocytes (L-O2) are shuttled to hepatic stellate cells (HSCs) and to establish their effects on HSCs. We have observed that the conditioned medium from L-O2 cells stimulated with PM2.5 induced the activation of LX-2 cells, and at the same time, the same results were obtained when we co-cultured LX-2 in PM2.5-exposed L-O2 cells. In addition, analysis of L-O2 cells stimulated with PM2.5 revealed significant increases in TGF-β1 expression. Moreover, we found that the TGF-β1 receptor inhibitor, SB-525334, decreases the proliferation and migration of LX-2 cells in the co-culture system. In addition, the expression of α-smooth muscle actin and type I collagen in LX-2 cells induced by PM2.5-treated L-O2 cells were also blocked by pretreated with SB-525334. These observations imply that PM2.5 induces TGF- β1expression in hepatocytes, which leads to HSCs activation.

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1. Introduction

With the increasingly serious air pollution in recent years, particulate matter has become an important threat to human health, especially PM2.5 whose aerodynamic diameter is less than 2.5 μm [1]. PM2.5 mainly comes from the combustion products of fossil fuel and biomass fuel, because the particle size is small, it can reach the alveoli, interfere with the respiratory system function, and even enter the circulatory system through the pulmonary capillaries and reach other organs of the whole body, causing diseases of multiple organs [2]. Several recent studies including epidemiological researches and laboratory-related assays revealed that PM2.5 was a vital key risk factor for hepatic disease [3,4]. Moreover, epidemiological studies demonstrated that PM2.5 exposure has a strong association with liver fibrosis [5]. In addition, animal studies also reported the potential role of PM2.5 in the progression of liver fibrosis [6,7]. Zheng et al. showed that inhalation of concentrated PM2.5 can induce liver fibrosis in normal mice [6]. The PM2.5-exposed mice in the Ding et al. study up-regulated TGF-β and COL-1 in liver tissues, which — as the authors inferred — would lead to liver fibrosis development [7]. However, its pathogenesis has not yet been completely known.

Liver fibrosis is a complex process of fibrosis and inflammation caused by chronic liver injury, characterized by increased deposition and altered composition of the extracellular matrix (ECM) [8]. It is generally accepted that Hepatic stellate cells (HSCs) play a central role in the process of fibrosis as they are the major source of ECM. Activated HSCs transdifferentiate to myofibroblasts become enhanced in abilities to proliferate, migratory and express a large number of α-smooth muscle actin (α-SMA) and extracellular matrix protein type I collagen (COL-1), which are the most prominent markers for identifying activated HSCs [9].

Indeed, activation of HSCs is a dynamic process initiated by

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paracrine stimulation of neighboring cells, composed mainly of hepatocytes, liver sinusoidal endothelial cells, and Kupffer cells [10]. In the interactive network, growth factors, chemokines, and molecular signals released by these cells directly or indirectly affect the state of HSCs and promote the transdifferentiation of HSCs [11]. Among them, the Transforming Growth Factor-β1 (TGF-β1) was considered as the most powerful fibrocytokine in the development of liver fibrosis [12]. And many of the factors that cause liver injury are associated with TGF- β1 level in varying degrees [13].TGF-β1 can activate quiescent HSCs to transdifferentiate into myofibroblasts and induce HSCs to synthesize excessive ECM [14]. There are several potential sources of TGF-β1 that may lead to fibrosis in the liver, such as hepatocytes [15].

Hepatocytes, the main liver cells, account for about 80% of the liver weight, are vulnerable to primary attack by external bacteria, viral infections, or poisons [16]. Persistently damaged hepatocytes secrete cytokines that attract HSCs to interact with them [17]. Then hepatocytes can modulate HSCs response and vice versa through direct cell-cell and paracrine interactions. Studies proved that the release of TGF-β1 by injured hepatocytes may be one of the first signals to activate adjacent quiescent HSCs [18].

In a word, excessive activation of HSCs is the core event in liver fibrosis, and direct stimulation of HSCs proliferation and collagen synthesis by TGF-β1 generated from damaged hepatocytes is a leading hypothesis to account for liver fibrosis. Our previous study demonstrated that PM2.5 has an effect on hepatocytes [19]. Thus, we hypothesize that PM2.5 may participate in the activation of HSCs by regulating the expression of TGF-β1 in hepatocytes.

In the present study, we found that PM2.5-treated hepatocytes have the potential to induce the activation of HSCs, as confirmed by up-regulation of cell proliferation, migration, and the expression of myofibroblast markers (α-SMA and COL-1). Moreover, our study indicates that TGF-β1 released by PM2.5-treated hepatocytes leads to the activation of HSCs. These findings contribute to a better understanding of the processes involved in liver fibrosis caused by PM2.5.

2. Materials and methods

2.1. Cells culture and reagents

Human normal hepatocytes (L-O2 cells) and immortalized HSCs (LX-2 cells) were purchased from SuyanBiotech (Shanghai, China). Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose containing 10% fetal bovine serum (FBS) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37 °C with 5% CO_2. The PM2.5 (Sigma, St. Louis, USA) was suspended in a certain amount of PBS to obtain a stock concentration of 5 mg/mL. The TGF-β1 receptor inhibitor (SB-525334, Selleck, Houston, USA) was dissolved DMSO to obtain a final concentration of 1 μM.

2.2. Hepatocyte-conditioned medium preparation

L-02 cells were exposed to PM2.5 (0,12.5,25,50 μg/mL) for 24h. After exposure, the medium was collected and centrifuged at 2000×*g* for 5min to obtain the conditioned medium. Conditioned medium that of L-O2 cells treated with PM2.5 referred to as PM2.5-CM. PM2.5-CM were transferred onto LX-2 cells for subsequent experimentation.

2.3. Co-culture of L-O2 cells with LX-2 cells

The method of cell co-culture in this experiment refers to the study of Xiangyu Dai et al. [20]. Briefly, PM2.5-exposed L-02 cells and LX-2 cells were co-incubated, by conducted using Transwell Permeable Supports 0.4-mm membranes (Corning, NY, USA). Before co-culture, L-02 cells were incubated in the serum-free medium containing different concentrations of PM2.5 (0,12.5, 25, 50 μg/mL) for 24h. For EdU assay and wound healing assay, LX-2 cells were seeded in 6-well plates, and the PM2.5-exposed L-02 cells were plated onto the 0.4-mm Transwell membranes. For the Tranwell migration assay, the LX-2 cells were plated on the insert and L-02 cells on the 6-well plate. Co-culture was started at 37 °C in the CO_2 incubator by setting the insert on the 6-well plate.

2.4. Cell proliferation analysis

The Cell Counting Kit-8 (CCK-8) assay was used to measure cell proliferation of LX-2 cells incubation with PM2.5-CM. Briefly, after treated with different concentrations of PM2.5-CM for 24h, LX-2 cells were incubated with CCK-8 solution (CCK-8; Meilunbio, Dalian, China) at 37 °C for 1h. Then optical density was measured at 450 nm (Molecular Devices, Biotek, Vermont, US).

For the non-contact co-culture system, the proliferation ability of LX-2 cells was determined using the 5-ethynyl-2′-deoxyuridine (EdU) proliferation assay. After 24h co-culture, LX-2 cells were incubated with 10 μM EdU (Beyotime, Shanghai, China) for another 2h. Then cells were fixed with 4% paraformaldehyde for 15mins and subsequently cultivated with Azide 594 staining solution for 30mins, and further incubated with Hoechst 33,342 for 10minis. The stained cells were observed using a laser confocal scanning microscope (Carl Zeiss, Jena, Germany).

2.5. Cell migration assay

Wound healing assay and transwell migration assay were performed to evaluate the migratory ability of LX-2 cells.

For wound healing assay, LX-2 cells (5×10^5 cells/well) were plated onto 6-well plates and cultivated until 90% confluence. The cell monolayer was head perpendicular scratched with a 10 μl fine pipette tip. After washed twice with PBS, LX-2 cells were incubated with PM2.5-CM or co-cultured with PM2.5-exposed L-02 cells for 24 h. The image of LX-2 cells was photographed at 0h and 24 h along the scrape line under a microscope (Olympus, Tokyo, Japan). The migration distances of LX-2 cells wound closure was measured using Image J software (version 1.80; NIH, Bethesda, MD, USA).

The migration abilities of the LX-2 cells were also assayed using Transwell chambers (8 μm pore size; Corning Costar, Cambridge, USA). A total of LX-2 cells (5×10^5 cells/well) were seeded into transwell chambers and incubated with L-O2 cells in the co-culture system. After cultivation for 24h, the cells of non-migrated were mechanically removed using cotton swabs, whereas the cells that had migrated to the underside of the membrane were fixed and stained with 0.1% crystal violet. The migrated cells were observed and counted by an optical microscope.

2.6. Myofibroblast markers assay

Quantitative real-time PCR (qRT-PCR) was used to measure the mRNA expression level of α-SMA and COL-1 in LX-2 cells incubated with PM2.5-CM or co-cultured with L-02. Total RNA isolated, complementary DNA (cDNA) synthesized, and qRT-PCR amplification was performed as our previous study described [19]. The qRT-PCR conditions of the amplification reaction included initial denaturation of 95 °C for the 30s, 95 °C for 5s, followed by 40 cycles of 95 °C for 5s, 60 °C for 30s, and then 95 °C for 10s. The relative expression of target genes was calculated based on the comparative $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: α-SMA (forward): 5′-GTT CCG CTC CTC TCT CCA AC-3′; α-SMA (reverse): 5′-ACG CTG GAG GAC TTG CTT TT-3′; COL-1 (forward): 5′-GTG CTC CTG

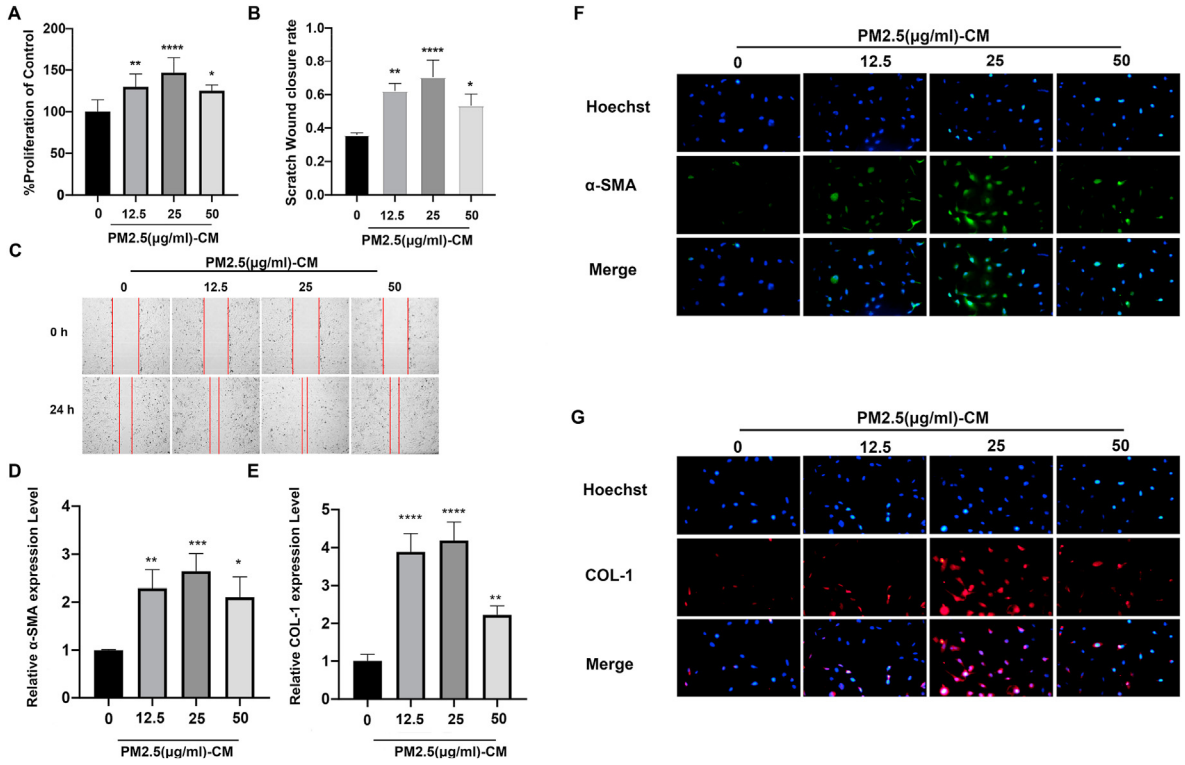

Fig. 1. PM2.5-CM induced the activation of LX-2 cells.
LX-2 cells showed a significant increase in proliferation, migration, and the expression of COL-1 and α-SMA in the presence of PM2.5-CM. A. CCK-8 assay was applied to explore the proliferation of LX-2 cells. B, C. Wound healing assay was to detect the migration of LX-2 cells. D, E. The mRNA levels of α-SMA and COL-1 were quantified by qRT-PCR. β-actin was used as an internal control. F, G. The immunofluorescence staining was used to detect the protein expression levels of α-SMA (green fluorescence) and COL-1(red fluorescence) in LX-2 cells.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. All data are expressed as the means ± SD from three independent experiments, each performed in triplicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

GTA TTG CTG GT-3′; COL-1 (reverse): 5′-ACC AGG TTC ACC GCT GTT AC-3′; TGF-β1 (forward): 5′-CTG TCC AAC ATG ATC GTG CG -3′; TGF-β1 (reverse): 5′-TGA CAC AGA GAT CCG CAG TC -3′; β-actin (forward): 5′- CAC TCT TCC AGC CTT CCT TCC-3′; β-actin (reverse): 5′-CGT ACA GGT CTT TGC GGA TGT C-3′.

Immunofluorescence staining was performed to further confirm the expression of α-SMA and COL-1 in LX-2 cells. After co-incubation for 24h, LX-2 cells were fixed with 4% paraformaldehyde for 20min, followed by blocked with 10% normal goat serum for 30min and incubated with primary antibodies against α-SMA (1:100, Affinity, Jiangsu, China) or COL-1 (1:100, Affinity, Jiangsu, China) overnight at 4 °C , and incubated with the Alexa 488 or Alexa 555 conjugated secondary antibody for 1h. Finally, the nuclei were stained with Hoechst 33,342 for 10min. Fluorescence images were photographed under the fluorescent microscope (Olympus, Tokyo, Japan).

2.7. Enzyme-linked immunosorbent assay(ELISA)

The levels of TGF-β1 in PM2.5-CM were determined with ELISA kits following the manufacturer's protocol (MEI MIAN, Jiangsu, China). And the light absorption value was measured at the wavelength of 450-nm.

2.8. Statistical analyses

Each group of experiments was performed independently more than three times. All data were expressed as the means ± standard deviation (SD) and were analyzed with one-way ANOVA by GraphPad Prism v7.0 software. Differences at $P < 0.05$ were considered statistically significant.

3. Results

3.1. PM2.5-CM activates LX-2 cells

HSCs activation is the key event in liver fibrosis [21]. To evaluate whether PM2.5-exposed hepatocytes lead to activation of HSCs, we first cultured HSCs with the PM2.5-CM as described above. Then the proliferation and migration ability of LX-2 cells were analyzed. Conditioned medium of 12.5,25,50 μg/ml of PM2.5 exposed L-O2 cells increased the proliferation rate by 29.8%, 46.8%, and 25%, respectively, in LX-2 cells compared with control (Fig. 1A). Moreover, the wound closure rate was significantly enhanced in LX-2 cells cultured in PM2.5-CM (Fig. 1B and C). We next investigated whether LX-2 cells expressed higher levels of myofibroblast markers (α-SMA and COL-1) after cultured with PM2.5-CM by qRT-PCR and Immunofluorescence staining. As shown in Fig. 1D and E, PM2.5-CM treatment significantly up-regulated the COL-1 and α-SMA expression. Furthermore, the results of immunofluorescence staining showed that PM2.5-CM markedly increased the fluorescence intensities of COL-1 and α-SMA (Fig. 1F and G). Following all these findings, PM2.5-CM activates LX-2 cells and indicates that PM2.5 can stimulate hepatocytes to release soluble factors to activate LX-2 cells.

3.2. Co-culture with PM2.5-treated L-02 cells induces the activation of LX-2 cells

A co-culture system was introduced to further confirm the interaction between PM2.5 exposed L-02 cells and LX-2 cells. Notably, the co-culture of LX-2 cells and PM2.5-treated L-O2 cells obtained a similar trend as in the conditioned medium that LX-

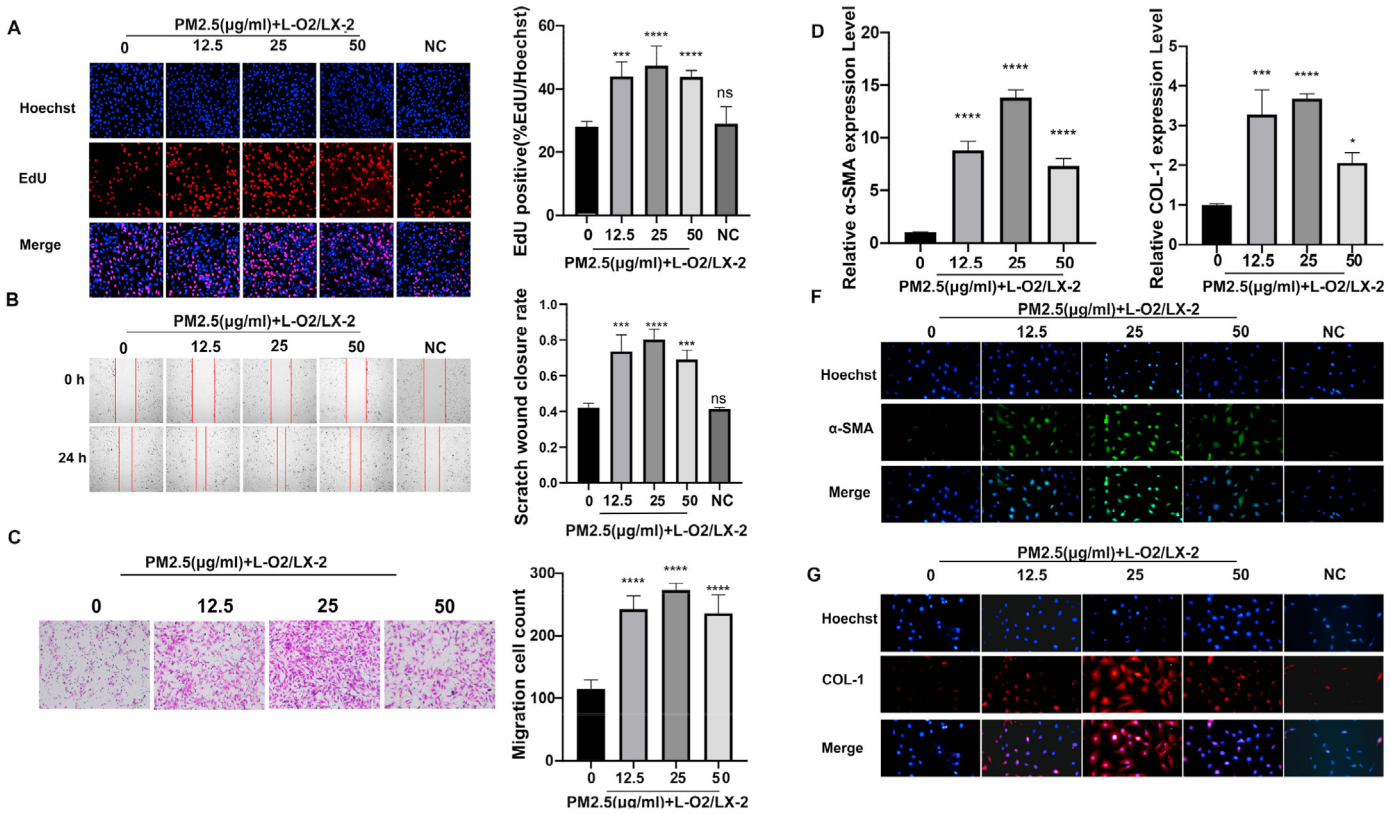

Fig. 2. The effect of PM2.5 on proliferation, migration, and the expression of α-SMA and COL-1 in HSCs co-cultured with hepatocytes.
LX-2 cells were co-cultured with PM2.5-treated L-O2 cells using a transwell system (L-O2/LX-2), and LX-2 cells were cultured alone as negative control (NC). A. EdU assay to detect the cell proliferation. EdU positive cells representing proliferation were stained in red with Azide 594. The nuclei of cells were stained in blue by Hoechst33342. B, C. The wound healing and transwell assays to test the cell migration. After co-cultured with PM2.5-treated L-02 cells for 24h, LX-2 cells showed enhanced cell migration ability. D, E. qRT-PCR to quantify the mRNA expression of α-SMA and COL-1 in LX-2 cells. β-actin was used as an internal control. F, G. The immunofluorescence staining to detect the protein expression levels of α-SMA (green fluorescence) and COL-1(red fluorescence). *p < 0.05; **p < 0.01; ***p < 0.001. All data are expressed as the means ± SD from three independent experiments, each performed in triplicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2 cells shown a significant enhancement in proliferation and migration ability. EdU assay was used for the measurement of cell proliferation. As shown in Fig. 2A, a sharp increase in the proliferation rate of LX-2 cells co-cultured for 24 h with PM2.5-treated L-O2 cells. Besides, the wound healing assay showed that exposure with PM2.5-treated L-02 cells accelerated the scratch closing of LX-2 cells (Fig. 2B). Furthermore, transwell assay results also showed that the number of migratory LX-2 cells was significantly increased

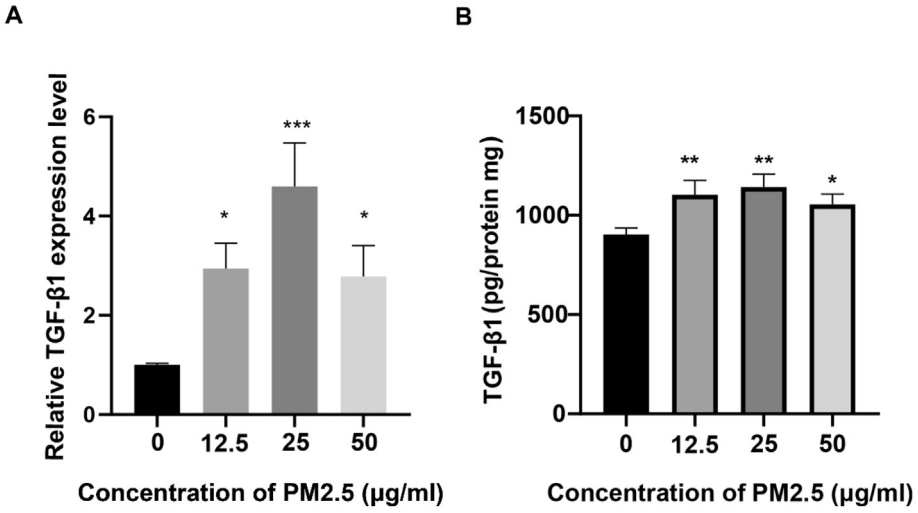

Fig. 3. PM2.5 induced TGF-β1 expression in L-O2 cells.
A. The expression of TGF-β1 in L-O2 cells was then determined by qRT-PCR. B. The concentrations of TGF-β1 in the conditioned medium of L-O2 cells were measured by ELISA. All the results were shown as mean ± SD (n = 3), which were three separate experiments performed in triplicate.*p < 0.05 and * *p < 0.01.

after being co-cultivation with PM2.5-treated L-02 cells (Fig. 2C). Then, the expression of myofibroblast markers of LX-2 cells was evaluated. Similar to the above tests, the results from qRT-PCR and immunofluorescence staining demonstrated a significantly increased expression of α-SMA and COL-1 in LX-2 cells after 24 h of co-cultivation with PM2.5-treated L-02 cells(Fig. 2D, E, 2F). Taken together, these results all demonstrated that co-incubated with PM2.5-treated L-02 cells induces LX-2 cell activation.

3.3. PM2.5 enhanced TGF-β1 secretion in hepatocytes

The above results suggested that PM2.5-treated hepatocytes actives HSCs through indirect paracrine interactions. TGF-β1 is the most common fibrosis factor secret by damaged hepatocytes leads to the activation of HSCs and the excessive accumulation of ECM, thus promoting the occurrence of liver fibrosis [2]. Therefore, the expression of TGF-β1 was evaluated in our study. As shown in Fig. 3, our qRT-PCR (Fig. 3A) and ELISA (Fig. 3B) results showed that the treatments of PM2.5 significantly increased the level of TGF-β1 expression in L-O2 cells. These results demonstrate a possible role for the TGF-β1 being released by PM2.5-treated L-O2 cells in the activation of HSCs.

3.4. TGF-β1 mediated HSCs activation in co-cultivation

Because high levels of TGF-β1 are observed in L-02 cells treated with PM2.5, the potential contribution of TGF-β1 in LX-2 fibro-transfiration was estimated with TGF-β1 receptor-specific inhibitor

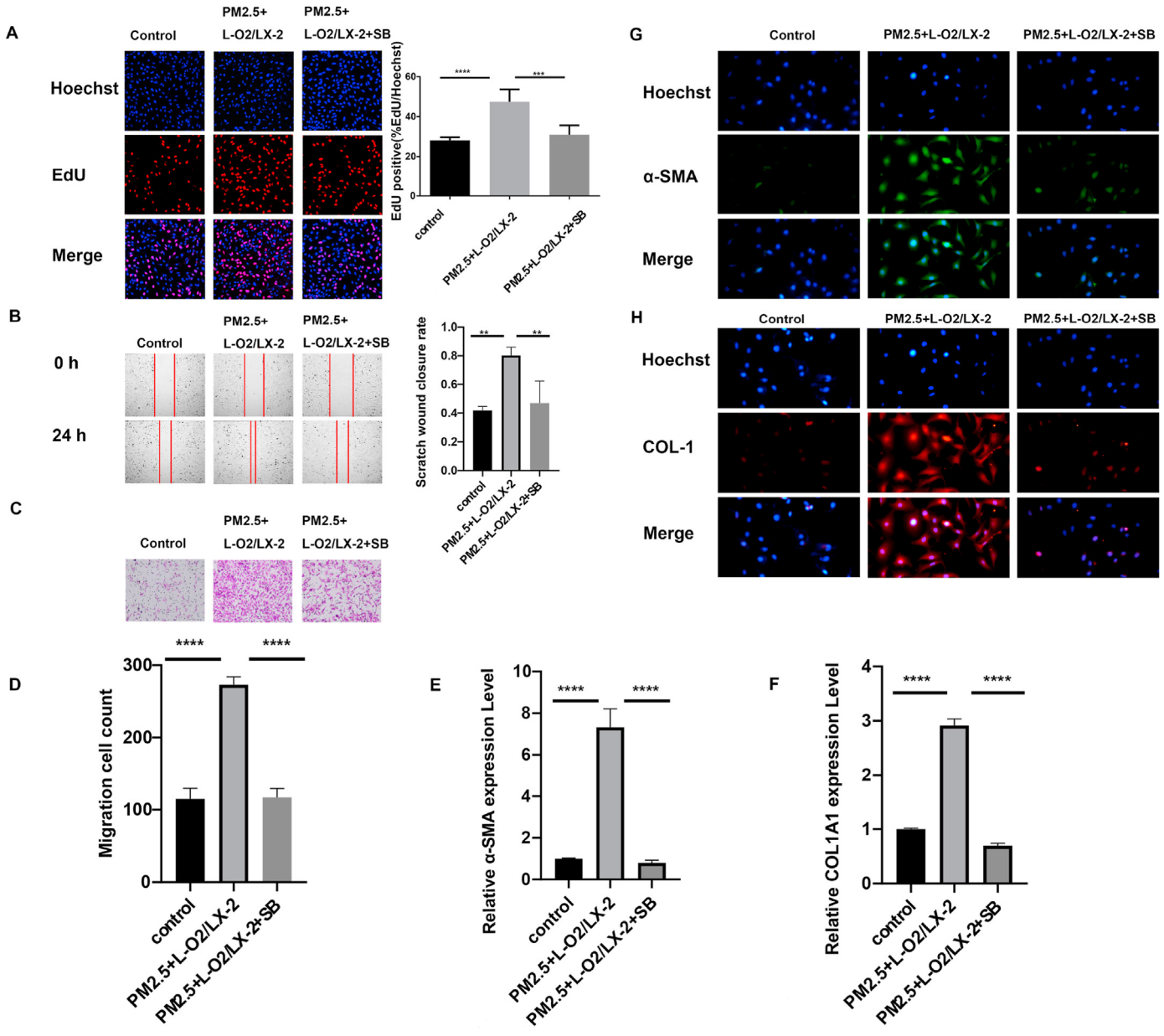

Fig. 4. TGF-β1 receptor inhibitor significantly reduced PM2.5-induced HSCs activation.
LX-2 cells were pretreated with SB525334 for 2h and then co-cultured with 25 μg/ml PM2.5-treated L-O2 cells (PM2.5+L-O2/LX-2+SB). A. The proliferation of LX-2 cells was determined by the EdU assay. B, C, D. The migration ability of LX-2 cells was determined by the wound healing and transwell assays. E, F. The mRNA levels of α-SMA and COL-1 were quantified by qRT-PCR. G, H. The immunofluorescence staining was used to detect the expression of α-SMA (green fluorescence) and COL-1(red fluorescence) *p < 0.05; **p < 0.01; ***p < 0.001. All data are expressed as the means ± SD from three independent experiments, each performed in triplicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in our present study. LX-2 cells were pretreated with TGF-β1 receptor inhibitor for 2h and then co-cultured with 25 μg/ml PM2.5-treated L-O2 cells. Later, the EdU assay uncovered that the proliferative ability of LX-2 cells was markedly weakened by blockage of the TGF-β1 signaling pathway with SB-525334 (Fig. 4A). Besides, the TGF-β1 receptor inhibitor significantly inhibited the migration of LX-2 cells (Fig. 4B, C, D). As shown in Fig. 4E and F, the mRNA expression level of α-SMA and COL-1 was significantly down-regulated by pre-treatment with SB525334. In addition, we also showed that the pretreatment with SB525334 decreased the fluorescence intensities of α-SMA and COL-1 (Fig. 4G and H). All these results proved that PM2.5 can induce excess TGF-β1 in L-O2 cells, resulting in the activation of LX-2 cells.

4. Discussion

The main finding of this study related to PM2.5 participation in HSCs activation. The results presented here showed that PM2.5 induced the expression of TGF-β1 in hepatocytes, which led to the activation of HSCs.

A great number of studies demonstrated that PM2.5 exposure has a great impact on human health [22]. It has been proved that the liver is a target organ and a key participant of PM2.5. Several studies show that PM2.5 is an important risk factor for liver fibrosis, for animals exposed to PM2.5, liver histology has obvious perisinusoidal fibrosis, and liver fibrosis markers are significantly up-regulated [6]. However, the molecular mechanism of PM2.5 induced liver fibrosis is still obscure, and the related research is limited.

Liver fibrosis is a complex pathological process, and the activation of HSCs is the pivotal event during the process [2]. A recent study also reported that PM2.5 actives HSCs by triggering ROS-mediated phagocytosis [23]. However, HSCs occupy only approximately 5–8% of the total liver cells. Hepatocytes, the most abundant cell type in the liver, account for about 70%–80% of the liver volume [24]. As the major components of the liver, parenchymal hepatocytes are the major cells faced with different types of liver damage. A growing number of studies demonstrated direct stimulation of HSCs proliferation and collagen synthesis by-products generated from hepatocytes is a leading hypothesis to account for liver fibrosis induced by hepatotoxins, including Bile acid [25], hepatitis C virus [26], arsenite [20].

Therefore, in the present study, we observed whether PM2.5 can induce HSCs activation through hepatocytes. Our results showed that cultured in PM2.5-CM results in increased proliferation and migration of HSCs. PM2.5-CM induced HSCs activation was also proved by up-regulation of the expression of fibroblast markers of type I collagen and α-SMA. To further confirm these results, a co-culture system was established, based on the co-incubation of the hepatocytes and HSCs. Interestingly, the same results could be obtained. This finding suggests PM2.5-treated hepatocytes could active HSCs, maybe by inducing the generation of soluble factors.

Several soluble factors, including growth factors, cytokines, chemokines, and oxidative stress products, derived from hepatocytes, play a role in the activation of HSCs. TGF-β1 is a significant profibrogenic cytokine [27]. A great number of studies demonstrated that TGF-β1 is a master regulator of extracellular matrix remodeling, which can promote HSCs to myofibroblast transition [28]. Notably, according to previous studies, TGF-β1 was investigated that can release from hepatocytes treated with AAP, EtOH, CCl4, and other hepatotoxins [29]. Moreover, according to our previous studies, PM2.5 is harmful to hepatocytes [19]. Therefore, we explored whether PM2.5 could induce hepatocytes to release TGF-β1. Our results showed that L-O2 cells expressed higher levels of TGF-β1 after PM2.5 treatments. Most importantly, we also observed that the migration and proliferation of HSCs co-cultured with PM2.5-treated L-O2 cells were perturbed in presence of the TGF-β1 receptor inhibitor, which indicated that the activation of HSCs interfered. In addition, as expected, TGF-β1 receptor inhibitors significantly reduced the expression of myofibroblast markers (α-SMA and COL-1). This indicates that TGF-β1 released by PM2.5-exposed L-O2 cells medias HSCs activation.

In conclusion, We found that PM2.5 increases the level of TGF-β1 in hepatocytes, and TGF-β1 is transmitted into HSCs, resulting in HSCs activation and transdifferentiation into myofibroblasts. In the meantime, the TGF-β1 receptor inhibitor significantly suppresses HSCs activation when co-cultured with PM2.5-treated L-O2 cells. These results provide a new mechanism link between HSCs and hepatocytes in liver fibrosis caused by PM2.5. Moreover, this research is of great significance for the prevention and treatment of liver diseases related to air pollution.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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