Mesoderm/mesenchyme homeobox l as a potential target that orchestrates hepatic stellate cell activation

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Abstract

Aim: Hepatic stellate cell (HSC) activation is considered as a key event during the occurrence of liver fibrosis. Recent studies have shown that Mesoderm/mesenchyme homeobox 1 (Meox1) can promote organ fibrosis, such as in the skin and heart. We aimed to elucidate a potential role of the Meox1 during Hepatic stellate cell (HSC) activation.

Methods: The human HSC cell line Lx-2 was employed as model to evaluate the function of Meox1 in HSC activation. The expression of fibrotic genes was measured, and proliferation, migration, cell cycle assays were performed.

Results: In this study, Meox1 was recognized as a positive regulated factor of HSC activation. Following transforming growth factor-β1 (TGF-β1)-treated Lx-2 cells, the expression level of Meox1 was upregulated significantly. Meox1 knockdown mediated by small interference RNA (siRNA) was observed to inhibit TGF-β1-induced expression of HSC activation markers and fibrotic genes, including α smooth muscle actin (α-SMA), collagen type I (collagen-I) and matrix metalloproteinase 2 (MMP-2). Overexpression of Meox1 promoted HSC activation, as demonstrated by an increase in specific markers, including α-SMA, collagen-I and MMP-2 and enhancement of proliferation and migration. We also found that phosphorylation of Smad3 was elevated with forced expression of Meox1 in Lx-2 cells, whereas the TGF-β1 and total Smad3 protein levels were not changed. In addition, Meox1 could induce extension of the G1 phase and expression of the $p21^{CIP1/WAF1}$ in Lx-2 cells.

Conclusions: Our results indicate that Meox1 could play a crucial role in HSC activation and potential participation in the canonical TGF-β1/Smad pathway.

Keywords: Fibrosis, hepatic stellate cell, mesoderm/mesenchyme homeobox 1, transforming growth factor-β1.

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Introduction

Liver fibrosis is a common pathological change in varieties of chronic hepatic diseases, including alcohol liver disease, nonalcoholic steatohepatitis, chronic Hepatitis C Virus (HCV) and Hepatitis B Virus (HBV) infection, and autoimmune diseases, eventually progressing to cirrhosis and even hepatocellular carcinoma (HCC)(Ebrahimi et al., 2016; Parola and Pinzani, 2019; Schuppan et al., 2018; Weiskirchen and Tacke, 2016). The major feature of liver fibrosis is massive accumulation of extracellular matrix (ECM) that is sustainably synthesised by myofibroblasts, resulting in abnormal proliferation of connective tissue. Recent studies have found that the major origin of myofibroblasts is activated hepatic stellate cells (HSCs), which are considered to play a crucial role during the occurrence of liver fibrosis(Elpek, 2014; Moreira, 2007; Puche et al., 2013).

HSCs account for approximately one-third of the normal liver resident nonparenchymal cells (Dewidar et al., 2019; Moreira, 2007; Puche et al., 2013; Yan et al., 2021). Under liver exposure to pathological stimulation, quiescent HSCs can be activated and transdifferentiated to myofibroblasts to produce ECM for tissue regeneration repair during wound healing. Sustained, continuous HSC activation leads to an imbalance between the synthesis and degradation of ECM, resulting in the production and accumulation of large amounts of ECM, including collagen type I (collagen-I), tissue inhibitors of metalloproteinases (TIMP), and matrix metalloproteinase (MMP), contributing to liver fibrosis and organ dysfunction. Inhibition of HSC activation could contribute to ameliorating liver fibrosis(Coppola et al., 2018; Lu et al., 2015; Luo et al., 2022; Roehlen et al., 2020; Zhang et al., 2018). Therefore, exploring the molecular mechanism involved in HSC activation can further clarify liver fibrosis and identify a novel druggable target to reverse this process.

Mesoderm/mesenchyme homeobox 1 (Meox1) belongs to the homeobox transcription factor subfamily (Candia et al., 1992; Candia and Wright, 1996; Samuel and Naora, 2005). Previous studies have pointed out that Meox1 is necessary for organ and cell differentiation and development during embryonic development, such as somite differentiation(Mankoo et al., 2003), and axial skeleton(Bayrakli et al., 2013; Stamataki et al., 2001), muscle(Nguyen et al., 2017; Sutcu and Ricchetti, 2018) and vessel(Dong et al., 2018; Nguyen et al., 2014) formation. In a pathological state, Meox1 could promote tissue repair, including neointima formation for vascular injury(Wu et al., 2021) and osteoblastic differentiation for skeletal diseases(Huang et al., 2021). To date, more and more studies have played attention to the function of Meox1 in orchestrating the pathogenesis of organ fibrosis. In

the skin, Meox1 obviously enhanced the proliferation and migration of human dermal fibroblast cells and participated in hypertrophic scar formation in burned patients(Stelnicki et al., 1997; Wei et al., 2020). In vascular diseases, Meox1 can induce endothelial cell senescence, contributing to the progression of atherosclerosis(Douville et al., 2011). Another study on vascular diseases indicated that Meox1 was increased in balloon injury-induced arterial smooth muscle cells (SMCs) and promoted vascular SMCs phenotypic transformation and pathological vascular remodeling(Wu et al., 2018). Meox1 expression was markedly upregulated in the heart tissue of hypertrophic cardiomyopathy patients, and overexpression of Meox1 accelerated hypertrophy decompensation via interaction with the transcriptional target gene Gata4(Lu et al., 2018). Importantly, a recent study revealed that Meox1 is a central regulator from fibroblasts to profibrotic myofibroblasts in myocardial fibrosis and is required for TGFβ-induced fibroblast activation(Alexanian et al., 2021). With respect to idiopathic pulmonary fibrosis patients, Meox1 has been found to be upregulated in lung tissue(Sivakumar et al., 2019). However, information about the role of Meoxl, especially its relationship with HSC activation, is limited.

In this study, we decided to explore the possible influence of Meoxl on HSC activation. An immortalized human hepatic stellate cell (HSC) lines, Lx-2 cells, possess typical primary HSCs features which expressing glial acidic fibrillary and desmin protein and the ability to respond to Platelet-Derived Growth Factor BB and transforming growth factor-β (TGF-β), is used extensively for studying liver fibrosis (Xu et al., 2005). We found that Meox1 expression was upregulated in TGF-β1-induced Lx-2 cells, while Meox1 knockdown dramatically inhibited the expression of HSC activation markers and fibrogenesis-related genes induced by TGF-β1. Furthermore, Meox1 overexpression remarkably promoted HSC phenotypic alteration, proliferation and migration. This suggests that Meox1 plays a crucial role in orchestrating the activation of HSCs and could be recommended as a possibility antifibrotic drug target for the therapy of hepatic fibrosis.

Methods

Cell culture and reagent

Lx-2 cell lines (Procell CL-0560) authenticated by short tandem repeat (STR) profiling were kindly provided by Procell Life Science & Technology Co., Ltd. (Wuhan, Hubei, China) and cultured in Dulbecco′s Modified Eagle Medium (Thermo Fisher, CA, USA) supplemented with 10% fetal

bovine serum (Lanzhou Minhai Bio-Engineering Co., Ltd., Gansu, China) at 37℃ in a humidified atmosphere containing 5% CO_2. The TGF-β1 was purchased from Abcam (ab50036, Abcam, UK) and stored in -20℃. For TGF-β1 stimulation experiments, cells were starved overnight and subsequently treated with increasing concentration of TGF-β1 (0, 0.5, 1, 5 10, 20 ng/ml) for 72 h to collect protein(Coppola et al., 2018; Guimaraes et al., 2015; Wei et al., 2020).

The full-length peptide coding sequence of Homo sapiens Meox1 was synthesized by Sangon, and inserted into the multiple cloning site of pCDNA3.1(+) to construct the expression plasmid pcDNA3.1-Meox1, which was designed and synthesized by Sangon (Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China). The Lx2-Meox1 cell lines were established by stably transfecting pcDNA3.1-Meox1 into Lx-2 cells with hygromycin B selection. The transfection of plasmids was achieved by Lipofectamine 3000 reagent according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, USA).

RNA interference

For Meox1 inhibition experiments, we transfected small interference RNA (siRNA) for Meox1 or a negative control into Lx-2 cells using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, USA). The siRNA used to conduct experiments was synthesized by Genepharma Co, Ltd. (Shanghai, China). The sequence for negative control siRNA was as follows: 5'-TTC TCC GAA CGT GTC ACG TTT-3' (sense) and 5'-ACG TGA CAC GTT CGG AGA ATT-3' (antisense). The sequence for Meox1 siRNA was as follows: 5'-CTG CCA ATG AGA CAG AGA ATT-3' (sense) and 5'-TTC TCT GTC TCA TTG GCA GTT-3' (antisense)(Wei et al., 2020).

Quantitative reverse transcription PCR (qPCR)

The extraction of cells total RNA was performed using TRIzol Reagent according to the manufacturer's instructions (TaKaRa, Otsu, Japan). Next, the reverse transcription was performed using the PrimeScript™ RT reagent kit according to the manufacturer's instructions (TaKaRa, Otsu, Japan). Quantitative reverse transcription PCR (qPCR) was performed using the TB Green® Premix Ex Taq™ II kit (TaKaRa, Otsu, Japan) on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, USA). All primers were synthesized by Sangon (Sangon Biological

Engineering Technology & Services Co., Ltd., Shanghai, China), and the sequence was as follows:

α-SMA forward 5′-CTTCGTTACTACTGCTGAGCGTGAG-3′,

α-SMA reverse 5′-CCCATCAGGCAACTCGTAACTCTTC-3′,

collagen-1(1A1) forward 5′-TGATCGTGGTGAGACTGGTCCTG-3′,

collagen-1(1A1) reverse 5′-CTTTATGCCTCTGTCGCCCTGTTC-3′,

MMP-2 forward 5′-CACCTACACCAAGAACTTCCGTCTG-3′,

MMP-2 reverse 5′-GTGCCAAGGTCAATGTCAGGAGAG-3′,

TGF-β1 forward 5′-TACAGCAACAATTCCTGGCGATACC-3′,

TGF-β1 reverse 5′-CTCAACCACTGCCGCACAACTC-3′,

Meox-1 forward 5′-AAGGATGAAGTGGAAGCGTGTGAAG-3′,

Meox-1 reverse 5′-TCTGAACTTGGAGAGGCTGTGGAG-3′,

GAPDH as an internal reference primer was purchased from Sangon (B661104-0001, Shanghai, China).

Western blotting

The extraction of cells total protein was performed using RIPA lysis buffer according to the manufacturer's instructions (Solarbio, Beijing, China) containing freshly added PMSF (Solarbio, Beijing, China) and protein phosphatase inhibitor (Solarbio, Beijing, China), and the concentrations were measured by a BCA protein assay kit according to the manufacturer's instructions (Solarbio, Beijing, China). Equal weights of protein were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane (Millipore, USA) or polyvinylidene difluoride membrane (Millipore, USA) that was blocked with 5% BSA or 5% nonfat dry milk separately. The primary antibodies used to conduct the experiments were as follows: anti-Meox1 (1:1000, ab105349, Abcam), anti-α-SMA (1:500, ab5694, Abcam), anti-collagen type I (1:2000, 66761-1-Ig, Proteintech), anti-MMP-2 (1:1000, 66366-1-Ig, Proteintech), anti-TGF-β1 (1:1000, 21898-1-AP, Proteintech), anti-Smad3 (1:1000, 66516-1-Ig, Proteintech), anti-phospho-Smad3 (1:1000, 9520T, Cell Signaling Technology), anti-p53 (1:5000, 60283-2-Ig, Proteintech), anti-p21 (1:1000, 10355-1-AP, Proteintech), anti-p16 (1:2000, 10883-1-AP, Proteintech), and anti-GAPDH (1:10000, ab181602, Abcam). Secondary antibodies included HRP-conjugated Affinipure goat anti-rabbit IgG (H+L) (1:5000, SA00001-2, Proteintech) and HRP-conjugated

Affinipure goat anti-mouse IgG (H+L) (1:5000, SA00001-1, Proteintech). The protein expression levels were detected with an enhanced chemiluminescence kit (New Cell & Molecular Biotech Co., Ltd., Suzhou, China) using the ChemiDoc™ Touch imaging system (Thermo Fisher Scientific, NY, USA).

Cell proliferation assay

Cell Counting Kit-8 was employed to analyze cell proliferation (CCK-8; Dojindo Lab, Kumamoto, Japan). Cell lines were seeded into 96-well plates at indicated density. Adding ten microliters of CCK-8 into each well at indicated time (0, 24, 48, 72 and 96 h) and then incubating for 2 h at 37 °C in a 5% humidified CO_2 incubator. The OD value was measured at a wavelength of 450 nm every 24 h using a microplate reader (Bio-Tek; Instruments, Winooski, VT, USA).

Cell migration assay

Eight-micrometer Transwell chambers containing polycarbonate filters (Corning, NY, USA) were employed to perform the cell migration assay. The cell lines were seeded into the Transwell chamber at indicated density. After incubation for 24 h, the filters were fixed with methyl alcohol for 1 h and stained with 0.5% crystal violet for 20 min. The stained cells were counted under an inverted microscope in five randomly selected fields at a magnification of 100×.

Flow cytometry assay

Flow cytometry was used to assess the cell cycle. Cell Cycle Assay Kit Plus (C6078, Shanghai Bioscience Technology Co. Ltd., Shanghai, China) was employed to accomplish the cell cycle assay according to the manufacturer's instructions. The processes were performed using a fluorescence-activated cell sorting Aria II flow cytometer (BD Biosciences, Pharmingen, San Diego, CA, USA).

Statistical analysis

Statistical analysis was performed using SPSS software (version 26.0; SPSS Inc. Chicago, IL, USA). All experiments are performed three times and values are expressed as the mean ± standard deviation. Data were evaluated using unpaired Student's *t* test or one-way analysis of variance (ANOVA), and was considered statistically significant at $p < 0.05$.

Results

Meox1 was upregulated in TGFβ1-induced HSC activation

To observe whether Meox1 was upregulated in activated HSCs, Lx-2 cells were employed as a model, which are widely used for exploring fibrogenesis and HSC activation(Xu et al., 2005). TGFβ1 is well known to induce HSC activation(Caja et al., 2018; Dewidar et al., 2019; Dooley and ten Dijke, 2012; Fabregat et al., 2016). Thus, we detected the effects of TGFβ1 on stimulating Meox1 expression in Lx-2 cells. Following treat with increasing concentration of the TGFβ1, the expression of Meox1 could be upregulated in a dose-dependent manner, stimulation of Lx-2 cells up-to 10 ng/ml TGFβ1 caused Meox1 protein levels to increase remarkably (Fig. 1A and B). Meanwhile, we evaluated the levels of HSC activation markers and fibrosis-related factors in TGFβ1-induced Lx-2 cells. Comparable result reveal in these markers. The α smooth muscle actin (α-SMA) (Fig. 1A and C), collagen-1 (Fig. 1A and D) and MMP-2 (Fig. 1A and E) protein expression level gradually increase with the increasing concentration of the TGFβ1 stimulation, consistent with previous reports(Coppola et al., 2018; Guimaraes et al., 2015; Li et al., 2015; Zhang et al., 2012), indicating that Meox1 was induced during HSC activation.

Meox1 was essential for TGFβ1-induced HSC activation

To further explore the influence of Meox1 on TGFβ1-stimulated HSC activation, the Meox1 was knocked down by treating Lx-2 cells with its specific siRNA (si-Meox1). As result shown in Fig. 2A and B, the expression of Meox1 and HSC activation markers were reinforced synchronously by TGFβ1 stimulation, and Meox1 was efficiently depressed by its specific siRNA. Upon Meox1 knockdown, the expression of TGF-β1-stimulated HSC activation markers α-SMA (Fig. 2A and C), collagen-1(Fig. 2A and D) and MMP-2 (Fig. 2A and E) was attenuated and, importantly, appeared to affect expression at the basal level without TGF-β1 stimulation. These results confirmed that TGF-β1 could enhance the HSC activation markers expression, however, this activation effect can be blocked by Meox1 knockdown, suggested Meox1 could be essential for TGFβ1-induced HSC activation.

Overexpression of Meox1 promoted HSC activation

As Meox1 was upregulated during HSC activation, implying a role in transdifferentiation, we

observed the participation of Meox1 alone in HSC activation. Thus, we stably transfected the Meox1 gene into Lx-2 cells and analyzed the HSC activation markers expression levels. The results shown that the Meox1 mRNA (Fig. 3A) and protein (Fig. 3E) expression level were significantly upregulated compared with the control, confirming that we successfully established Lx-2-Meox1 cell lines with stably expressed Meox1. In Lx-2-Meox1 cell lines, the mRNA levels of α-SMA (Fig. 3B), collagen-1 (Fig. 3C) and MMP-2 (Fig. 3D) were remarkably upregulated. Western blot analysis also showed that the Meox1 overexpression significantly enhanced the protein level of α-SMA, collagen-1 and MMP-2 (Fig. 3E). Indicated that Meox1 could promote these HSC activation markers expression in transcriptional level. Cell proliferation and migration are important functional characteristics of HSC activation(Dewidar et al., 2019). Hence, we further evaluated the effect of Meox1 on HSC proliferation via CCK-8 assay (Fig. 3F). The data showed that the growth rate of Lx-2-Meox1 cells was faster than that of the control group at 48, 72 and 96 h, indicating that Meox1 accelerates cell proliferation. We also ascertained the role of Meox1 in HSC migration through a transwell assay (Fig. 3G). The data showed that the number of migrating cells was increased compared with that in the control group when Meox1 was upregulated. All the results suggest that Meox1 promoted HSC phenotypic transformation and subsequent proliferation/migration.

The Smad3 phosphorylation level increased in Lx-2-Meox1 cells

We first tested whether there was loop regulation between Meox1 and TGF-β1 during activation of HSC. The result shown in Fig. 4A, the TGF-β1 mRNA level was not remarkably altered after stably transfection of the Meox1 gene into Lx-2 cells. Additionally, the protein level of TGF-β1 was not upregulated via Meox1 overexpression in Lx-2 cells (Fig. 4B). There were lots of studies noted that the canonical receptor-activated Smad pathway plays a crucial role in mediating TGF-β1 signaling(Dewidar et al., 2019; Matsuzaki, 2012), so we detected whether Meox1 participated in the canonical Smad-dependent TGF-β1 pathway. Smad3 is considered crucial for mediating the TGF-β1 signaling on HSC activation and profibgenic gene expression including collagen-I and α-SMA(Dewidar et al., 2019; Xu et al., 2016). Thus, we focused on the influence of Meox1 on Smad3. As detected by western blot analysis, after forced expression of Meox1, the protein level of total Smad3 did not change in Lx-2 cells; however, the phosphorylation of Smad3 was elevated (Fig. 4B). These data suggested that Meox1 may be located downstream of TGF-β1 without a feedback loop with

TGF-β1 and potential participation in TGF-β1/Smad3 pathway.

Meox1 induced Lx-2 cells population redistribution

To assess whether Meox1 could alter cell cycle progression, we employed flow cytometry to detect cell population distribution. The proportion of cells in G2 phase was remarkably reduced in Lx-2-Meox1 cells, while the proportion of cells in G1 phase was raised compared with that in Lx-2 cells group (Fig. 5A). These data suggested that Meox1 could induce cell population redistribution, lengthening the G1 phase and shortening the G2 phase.

Meox1 activated $p21^{CIP1/WAF1}$ expression in HSCs

Meox1 is a transcriptional inducer of the $p16^{INK4a}$ and $p21^{CIP1/WAF1}$ in vascular endothelial cells(Douville et al., 2011). Hence, we also assessed the effect of Meox1 on stimulate the expression of $p16^{INK4a}$ and $p21^{CIP1/WAF1}$ in HSCs. As showed by western blot analysis, compared to the control, the protein expression of $p21^{CIP1/WAF1}$ was significantly increased in Lx-2-Meox1 cells, however, this phenomenon was not observed at the $p16^{INK4a}$ protein expression level (Fig. 5B). p53 serve as a well-known transcriptional inducer of the $p21^{CIP1/WAF1}$ gene(el-Deiry et al., 1994); however, this protein expression level was no difference in either Lx-2-Meox1 cells or control cells (Fig. 5B). This finding implied that the increased expression of $p21^{CIP1/WAF1}$ during Meox1-induced HSC activation was independent of p53.

Discussion

Accumulated evidence has confirmed that HSC activation and transdifferentiation into myofibroblasts are central steps in hepatic fibrosis, and inhibiting HSC activation is a great meaningful treatment strategy for ameliorating the fibrosis process(Dewidar et al., 2019; Yan et al., 2021). However, the safety and effectiveness of this therapeutic strategy is unknown. Hence, new studies are needed to discover therapeutic targets.

Some gene expression profiles are altered during fibrosis and are essential for myofibroblastic phenotype transdifferentiation and fibrogenesis(Parola and Pinzani, 2019; Tu et al., 2019; Yan et al., 2021). Recently, numerous studies have considered Meox1 as a key inducer of fibroblast activation connected with organ fibrosis, including skin(Wei et al., 2020), vessel(Wu et al., 2018),

heart(Alexanian et al., 2021; Lu et al., 2018) and lung(Sivakumar et al., 2019) fibrosis; more importantly, Meox1 is specifically expressed in activated fibroblasts to regulate an extensive fibrotic gene expression program, importantly, is necessary for TGFβ-stimulated fibroblast activation(Alexanian et al., 2021). Therefore, we evaluated the effect of Meox1 in the activation of human HSC. TGF-β1, as an inflammation mediator, is considered to be the principal profibrogenic cytokine in induced HSC activation and ECM production (Dewidar et al., 2019; Xu et al., 2016). The hallmark of HSC activation is high expression of α-SMA, which can reflect the level of HSC activation(Dewidar et al., 2019; Yan et al., 2021). In addition, both collagen-1 and MMP-2 are important components of the ECM and are widely adopted to evaluate the fibrosis severity(Dewidar et al., 2019; Yan et al., 2021). Our study found that Meox1 was upregulated along with the expression of α-SMA and accumulation of collagen-1 and MMP-2 in TGF-β1-treated Lx-2 cells. Interestingly, inhibiting the Meox1 expression was able to block the synthesis of α-SMA, collagen-I and MMP-2 induced by TGF-β1, further reducing ECM accumulation. These results suggest that Meox1 could participate in TGF-β1-induced HSC activation and subsequent ECM deposition.

HSC activation is a pleiotropic process, and alteration of HSC phenotype and function is a major characteristic involved in ECM production, proliferation and migration(Yan et al., 2021). Our study found that following Meox1 overexpression, the α-SMA level was significantly raised in Lx-2 cells. Similarly, both collagen-I and MMP-2 expression level was strongly enhanced under this condition, indicating that Meox1 could promote quiescent HSC transformation into a myofibroblastic phenotype. Previous research has suggested that approximately 80% of total fibrillary collagen-I is synthesized by activated HSCs and regulated by MMP-2 (Yan et al., 2021). MMP-2 is a profibrotic mediator secreted by activated HSCs that can degrade the liver extracellular matrix and increase activation and migration of HSCs(Coppola et al., 2018). However, inhibiting Meox1 could decrease both collagen-I and MMP-2 expression at basal levels. These data indicate that Meox1 could act as a collagen-I and MMP-2 inducer to regulate ECM production in HSCs. We also observed that the proliferation and migration of Lx-2 cells were remarkably promoted by Meox1. These data suggest that Meox1 plays a crucial role in HCSs activation, which is consistent with previous researches that Meox1 promotes the modulation of human dermal fibroblast cell(Wei et al., 2020) and smooth muscle cell (SMC)(Dong et al., 2018; Wu et al., 2018) phenotypes and function.

The TGF-β1/Smad signaling pathway plays a critical role during liver fibrosis through activation

of HSCs(Caja et al., 2018; Dewidar et al., 2019). Since TGF-β1 induced the expression of Meox1 and blockade of Meox1 inhibited TGF-β1-induced ECM synthesis, we further observed that the TGF-β1 expression was not significantly variation whether Meox1 was overexpressed in Lx-2 cells, suggesting that Meox1 was likely located downstream of TGF-β1 and without a feedback loop between Meox1 and TGF-β1. Active TGF-β binding to the TGF-β type II receptor results in phosphorylation of TGF-β type I, inducing TβRI catalytic activity. Next, the signaling pathway is initiated through phosphorylation of members of the Smad family(Caja et al., 2018; Dewidar et al., 2019). Several studies have confirmed that Smad3 is the crucial inducer of the profibrogenic response of HSCs(Dewidar et al., 2019; Xu et al., 2016). More importantly, the progression of liver fibrosis strongly relies on Smad3 activity(Caja et al., 2018; Dufton et al., 2017), particularly in the induction of ECM expression. The activity of Smad3 can be modulated at multiple levels, including expression and phosphorylation, and phosphorylated Smad3 shift into the nucleus and interacts with the other transcription elements, resulting in inducing target genes transcription(Dong et al., 2018). Our study found that the Smad3 phosphorylation level was raised with forced Meox1 expression in Lx-2 cells, whereas the total Smad3 expression level was not significantly variation, suggesting that Meox1 may participate in Smad3 phosphorylation regulation. The Meox1 is located in the nucleus, directly promotes TGF-β-induced Smad3 phosphorylation in the cytoplasm is unlikely. One study on SMCs confirmed that Meox1 could maintain the nuclear Smad3 phosphorylation status by inhibiting PPM1A, which is a phosphatase that can dephosphorylate Smad3 and block TGF-β signaling(Dong et al., 2018). Hence, we hypothesized that TGF-β1-induced Meox1 could preserve the phosphorylation of Smad3 to further promote HSC activation. The more delicate mechanisms of Meox1 in the regulation of HSC activation are indistinct and need to be explored in detail.

In addition, we found that increasing the expression of Meox1 resulted in a lengthened G1 phase and shorter G2 phase. Previous research pointed out that $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ act as downstream transcriptional target genes of Meox1 and can be directly activated by Meox1 in vascular endothelial cells(Douville et al., 2011), and serve as cyclin-dependent kinase inhibitors that are essential for arrest of the G1 phase(Ekholm and Reed, 2000). In Lx-2 cells, Meox1 overexpression had a remarkable effect on inducing $p21^{CIP1/WAF1}$ expression, while the $p16^{INK4a}$ expression level was not remarkably variation from that of the negative control. We speculate that this discrepancy may be due to the different intracellular responses in different cell types. Increasing expression of $p21^{CIP1/WAF1}$ not only at

the protein level but also at the mRNA level and the expression of p53, which is a well-known $p21^{CIP1/WAF1}$ transcriptional activator(Ekholm and Reed, 2000), was not significantly affected by Meox1 expression in Lx-2 cells, suggesting that Meox1-induced expression of $p21^{CIP1/WAF1}$ at the transcriptional level was independent of p53 expression. This could be a plausible explanation for the effect of Meox1 on lengthening the G1 phase. There was the study indicated that $p21^{CIP1/WAF1}$ play an important role in hepatocyte development, differentiation and cell cycle, members of the Smad family mediate the activation of the $p21^{CIP1/WAF1}$ promoter, truncated Smad3 block the transactivation(Moustakas and Kardassis, 1998). In fact, previous studies have confirmed that Meox1 can orchestrate muscle tissue growth and differentiation via regulation of the cell cycle(Nguyen et al., 2017). The close connection between the cell fate decisions and cell cycle machinery has been demonstrated by a great deal of research(Alenzi, 2004; Blomen and Boonstra, 2007; Pauklin and Vallier, 2014), revealing insights into the mechanisms by which cells utilize distinct cell cycle states to regulate biochemical and physical changes, such as metabolism, gene expression profiles and differentiation. The extension of the G1 phase could not only allow the cell to more efficiently respond to signals, activate development gene transcription and initiate differentiation programs but also maintain the cell differentiation state(Calder et al., 2013; Clegg et al., 1987; Sela et al., 2012). A study demonstrated that the Meox1 was necessary for TGF-β1-induced SMC differentiation from mesenchymal progenitors(Dong et al., 2018), and Meox1 was also a central transcriptional switch that governs TGFβ1-induced fibroblast activation in myocardial fibrosis(Alexanian et al., 2021). Our aforementioned results suggested that Meox1 could mediate TGF-β1-stimulated quiescent HSC transdifferentiation into a myofibroblastic phenotype that accumulates plenty of fibrillary collagens. Interestingly, the previous research suggested that $p21^{CIP1/WAF1}$ could enhance the sensitivity of cell response to TGF-β(Li et al., 1995). Hence, we speculate that Meox1-induced G1 phase extension may serve as a temporal window to benefit HSCs' response to various profibrogenic cytokines, allowing them to be activated more efficiently and maintain the transdifferentiation state, further promoting fibrosis. These data indicate a more sophisticated relationship between Meox1 and HSC activation that needs further exploration.

In summary, our studies considered Meox1 as a novel player in TGF-β1-induced HSC activation that plays a crucial role in HSC phenotypic modulation. Although the definite mechanisms could not be confirmed, the alteration in Smad3 phosphorylation status implied that Meox1 might modulate HSC

activation via the TGF-β/Smad signaling pathway. Meox1 promotes HSC activation and deserves further research; more importantly, it may be a potential antifibrotic drug target for treating hepatic fibrosis.

Abbreviations List: Transforming growth factor-β1, TGF-β1; Mesoderm/mesenchyme homeobox l, Meox1; Hepatic stellate cell, HSC; Extracellular matrix, ECM; α smooth muscle actin, α-SMA; Collagen type I, collagen-I; Matrix metalloproteinase 2, MMP-2.

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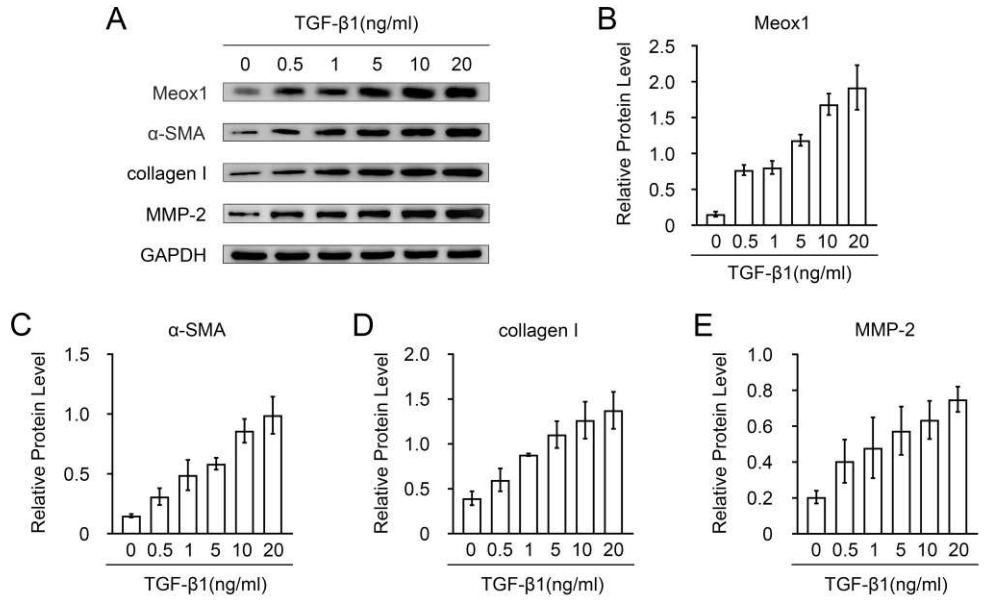

Fig. 1 TGF-β1 dose-dependently upregulated Meox1 and HSC activation markers expression in Lx-2 cells (A) Western blotting analysis the Meox1, α-SMA, collagen I, and MMP-2 expression. The Lx-2 cells were starved overnight, subsequently treated with increasing concentration of TGF-β1 (0, 0.5, 1, 5 10, 20 ng/ml) for 72 h to collect protein. (B-E) Quantification of Meox1 and HSC activation markers level shown in A by normalized to GAPDH level. Western blot scanning densitometry for three independent experiments was analyzed with ImageJ software (n=3, $p < 0.05$). TGF-β1, Transforming growth factor-β1; Meox1, Mesoderm/mesenchyme homeobox gene l; HSC, Hepatic stellate cell; α-SMA, α smooth muscle actin; collagen-I, Collagen type I; MMP-2, Matrix metalloproteinase 2; GAPDH, glyceraldehyde phosphate dehydrogenase.

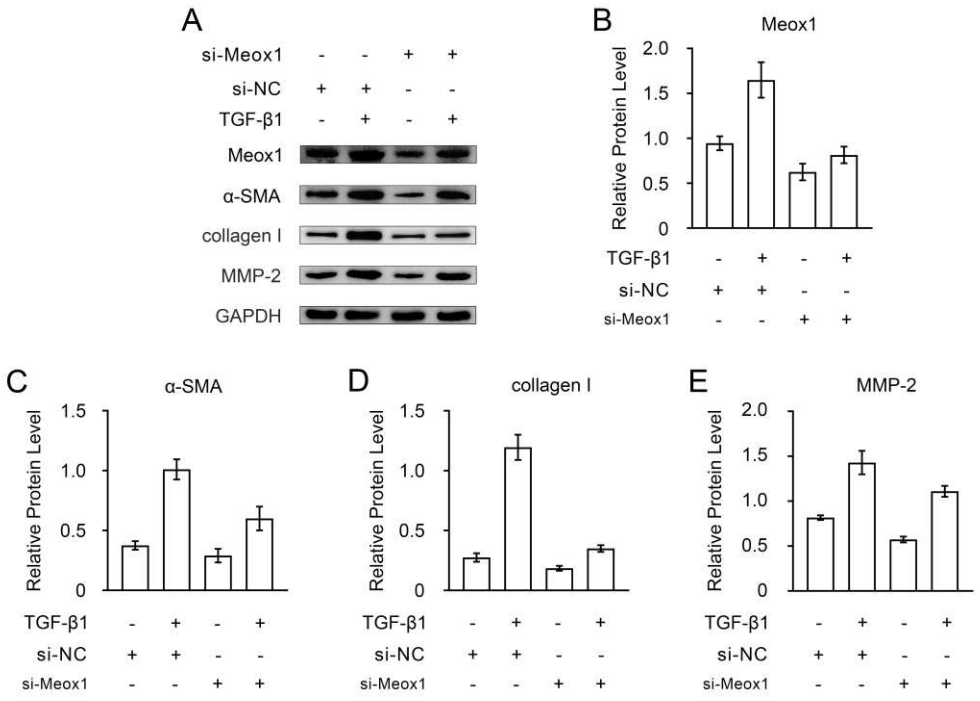

Fig. 2 Meox1 was essential for TGFβ1-induced HSC activation. (A) The Meox1 knock down inhibited TGF-β1-induced HSC activation markers protein expression in Lx-2 cells. Lx-2 cells were transfected with control (*si-NC*) or Meox1 siRNA (*si-Meox1*). Following incubation for 48 h, Lx-2 cells were treated with 10ng/ml TGF-β1 for 72 h to collect protein. The protein expression level of Meox1 and HSC activation markers were detected by Western blotting. (B-E) Quantification of protein levels shown in A by normalized to GAPDH for each individual protein. Western blot scanning densitometry for three independent experiments was analyzed with ImageJ software (n=3, $p < 0.05$). TGF-β1, Transforming growth factor-β1; Meox1, Mesoderm/mesenchyme homeobox gene l; HSC, Hepatic stellate cell; GAPDH, glyceraldehyde phosphate dehydrogenase.

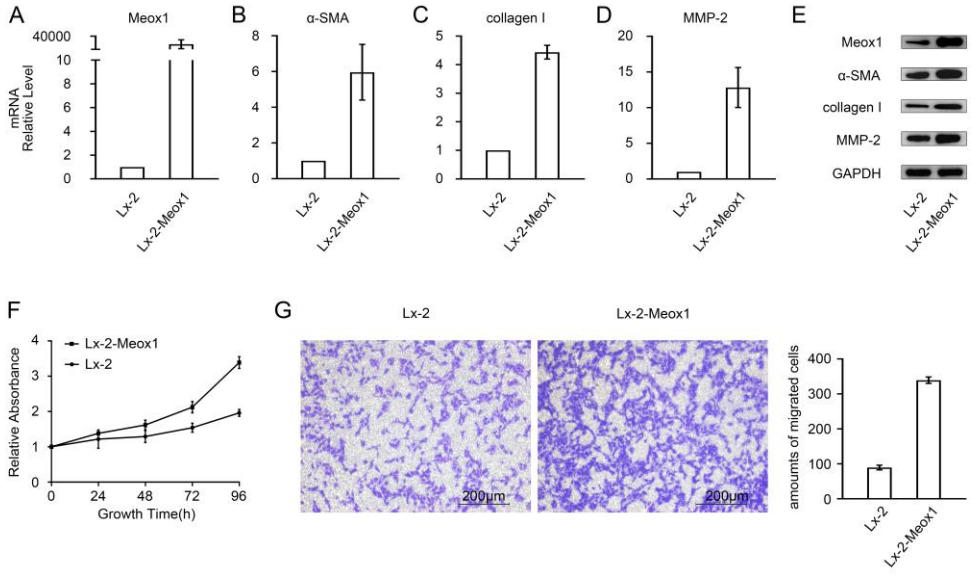

Fig. 3 Meox1 induced HSC activation markers expression and accelerated HSC proliferation/migration. The Lx2-Meox1 cell lines were established by stably transfecting pcDNA3.1-Meox1 into Lx-2 cells with hygromycin B selection. (A-D) Quantitative reverse transcription PCR analysis of Meox1, α-SMA, collagen I and MMP-2 mRNA expression level in Lx2-Meox1 cell lines (n=3, $p < 0.05$). (E) Western blotting analysis of Meox1, α-SMA, collagen I and MMP-2 protein expression level in Lx2-Meox1 cell lines (n=3). (F) Cell lines in the logarithmic growth phase were resuspended and seeded into 96-well plates at a density of 1×10^3 cells/well. Cell proliferation was detected at 0, 24, 48, 72and 96 h using the Cell Counting Kit-8 assay in Lx-2 and Lx-2-Meox1 cells (n=5, $p < 0.05$). (G) Representative phase contrast image and quantitative analysis of transwell migration assay in Lx-2 and Lx-2-Meox1 cells (n=3, $p < 0.05$). Cell lines in the logarithmic growth phase were resuspended in serum-free DMEM and seeded into the upper chamber at a density of 1×10^4 cells/well. Then, adding 600 μL DMEM containing 10% FBS into the lower chamber. TGF-β1, Transforming growth factor-β1; Meox1, Mesoderm/mesenchyme homeobox gene l; HSC, Hepatic stellate cell; α-SMA, α smooth muscle actin; collagen-I, Collagen type I; MMP-2, Matrix metalloproteinase 2.

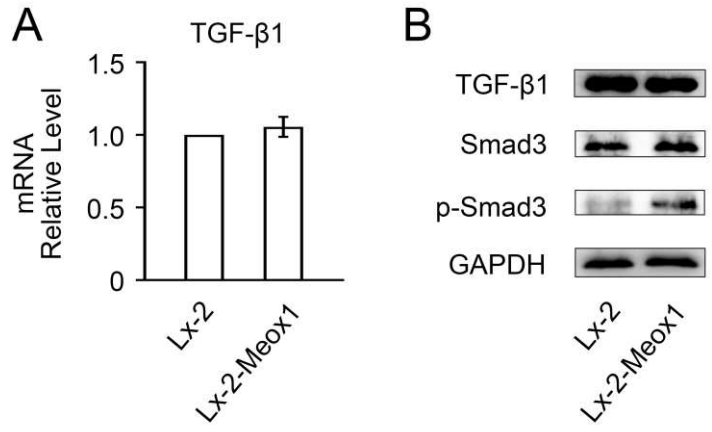

Fig. 4 The Smad3 phosphorylation level increased in Lx-2-Meox1 cells. (A) Quantitative reverse transcription PCR analysis of TGF-β1 mRNA expression level in Lx2-Meox1 cell lines (n=3, $p < 0.05$). (B) Western blotting analysis of TGFβ1, total Smad3 and phosphorylated Smad3 expression level in Lx2-Meox1 cell lines (n=3). TGF-β1, Transforming growth factor-β1; Meox1, Mesoderm/mesenchyme homeobox gene l.

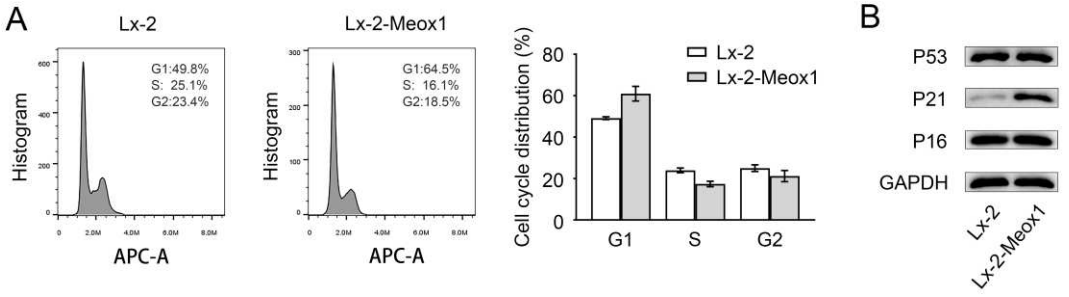

Fig. 5 Meox1 induced cell population redistribution and p21$^{CIP1/WAF1}$ expression in Lx-2 cells. (A) Effect of Meox1 on cell cycle distribution in Lx-2 cells was assessed via flow cytometry (n=3). (B) Western blotting analysis of p53, p21$^{CIP1/WAF1}$ and p16^{INK4a} protein expression level in Lx2-Meox1 cell lines (n=3). Meox1, Mesoderm/mesenchyme homeobox gene l.