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Induced Pluripotent Stem-cells Inhibit Experimental Bleomycin-induced Pulmonary Fibrosis through Regulation of the Insulin-like Growth Factor Signaling

Paria Bayati[1,2], Marjan Taherian[1,2], Mohammad-Ali Assarehzadegan[1,2], Mansoureh Soleimani[3], Hadi Poormoghim[4], and Nazanin Mojtabavi[1,2]

[1] Department of Immunology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[2] Immunology Research Center, Institute of Immunology and Infectious Diseases, Iran University of Medical Sciences, Tehran, Iran
[3] Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, Iran
[4] Scleroderma Study Group, Firuzgar Hospital, Iran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is among the illnesses with a high mortality rate, yet no specific cause has been identified; as a result, successful treatment has not been achieved. Among the novel approaches for treating such hard-to-cure diseases are induced pluripotent stem cells (IPSCs). Some studies have shown these cells' potential in treating IPF. Therefore, we aimed to investigate the impact of IPSCs on insulin-like growth factor (Igf) signaling as a major contributor to IPF pathogenesis.

C57BL/6 mice were intratracheally instilled with Bleomycin (BLM) or phosphate-buffered saline; the next day, half of the bleomycin group received IPSCs through tail vein injection. Hydroxyproline assay and histologic examinations have been performed to assess lung fibrosis. The gene expression was evaluated using specific primers for *Igf-1*, *Igf-2*, and insulin receptor substrate 1 (*Irs-1*) genes and SYBR green qPCR master mix. The data have been analyzed using the $2^{-\Delta\Delta CT}$ method.

The mice that received Bleomycin showed histological characteristics of the fibrotic lung injury, which was significantly ameliorated after treatment with IPSCs comparable to the control group. Furthermore, gene expression analyses revealed that in the BLM group, Igf1, Igf2, and Irs1 genes were significantly upregulated, which were returned to near-normal levels after treatment with IPSCs.

IPSCs could modulate the bleomycin-induced upregulation of *Igf1*, *Igf2*, and *Irs1* genes. This finding reveals a new aspect of the therapeutic impact of the IPSCs on IPF, which could be translated into other fibrotic disorders.

Keywords: Idiopathic pulmonary fibrosis; Induced pluripotent stem cells; Insulin-like growth factor Igf; IgfIrs1 protein

Corresponding Author: Nazanin Mojtabavi, PhD; Department of Immunology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran. Tel/Fax: (+98 21) 8862 2652, E-mail: mojtabavi.n@iums.ac.ir

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is among the rheumatologic and inflammatory diseases characterized by excessive release of extracellular matrix (ECM) components from activated fibroblasts in the lung connective tissue, causing progressive breathing difficulties and pneumonitis.[1] The global epidemiologic data on IPF are contradictory. Still, the prevalence of IPF is estimated to be 24-29.8 per 10^5 individuals in the USA, with an annual incidence of 16.3-17.4 in 10^5.[2,3] No effective treatment has been developed for this disease so far, and the majority of the therapies are those anti-inflammatory agents commonly employed in various rheumatologic conditions with different adverse effects;[4-6] since in the majority of the patients, the outcome of the available treatments is poor, the disease progresses into a state where the lungs respiration capacity would substantially decrease.[7] The 5-year survival rate of the patients with IPF is as low as nearly 50%; This partly results from the poor understanding of the IPF pathogenesis, which hinders the development of effective therapies.[8] Several attempts have been made to discover a novel potential treatment by researchers in the field. Among the possible therapeutic approaches is taking advantage of the regenerative and anti-inflammatory properties of the induced pluripotent stem cells (IPSCs). The use of IPSCs for treating fibrotic disorders such as IPF has shown promising results in vitro and in vivo studies using animal models;[9-13] but still, there are many unknown aspects surrounding the use of IPSCs in the treatment of fibrosis. To gain a complete insight into the therapeutic potential of IPSCs, it is necessary to investigate their possible effects on those aspects of the IPF pathogenesis identified so far.[14] Various studies have addressed the role of insulin-like growth factor (IGF) signaling in the pathogenesis of fibrotic disorders, including IPF;[15-21] it has been shown that various components of the IGF signaling pathway, along with other signalling pathways, contribute to the pathogenesis of IPF. It is speculated that persistent activation of the healing processes in response to chronic lung damage results in the scarring lesions of fibrosis which progress over time until most parts of the lungs are affected.[1] The cardinal mediator in healing responses is the transforming growth factor β (TGF-β) and its downstream signaling pathways, which eventually contribute to the activation of a process called epithelial to mesenchymal transition (EMT);[22] this process leads to the differentiation of fibroblasts into myofibroblasts.[23] Myofibroblasts produce excessive amounts of the ECM components for healing the lesions, thereby enhancing the integrity of the affected tissue; they are also capable of contracting like muscle cells, which contract the affected tissue.[24] From the previous knowledge regarding the EMT in cancers, in which the EMT is responsible for survival, invasion, and metastasis of the cancerous cells;[25] several signaling pathways were identified to be progressing in EMT.[26] Briefly, the TGF-β released in response to chronic damage to the tissue elicits several downstream signaling pathways such as the SMAD, phosphoinositide 3-kinases (PI3K)/ Ak strain transforming (AKT)/ Mammalian target of rapamycin (mTOR), and Wingless-related integration site (WNT)/β-Catenin, which eventually contribute to transcription of the epithelial repressors zinc finger e-Box binding homeobox (ZEB), SNAIL, and TWIST.[27,28] These alterations in the gene profile of the fibroblasts render the EMT, resulting in fibrosis. Given the previous studies regarding the role of IGF signaling in fibrosis and the fact that it is affected by TGF-β and other associated pathways;[15,17,20,29] we aimed to assess the therapeutic potential of the IPSCs in a mouse IPF model by evaluating their impact on the expression of *IGF1*, *IGF2*, genes which are cardinals the of IGF signaling.[30] We also decided to assess the gene expression of Insulin response substrate1 (IRS1) as a pivotal contributor to signal transduction in the IGF pathway; upon engagement of IGF1/2 with IGF receptors, this molecule binds to the cytoplasmic terminals of the receptors and provides a docking site for SH2 bearing signaling adaptor molecules such as the PI3K which mediates the IGF signaling by recruiting AKT.[31,32] The AKT is subsequently phosphorylated by phosphoinositide-dependent kinase 1 (PDPK1), and this eventually leads to increased transcription of epithelial repressors such as ZEB, SNAIL, and TWIST, which are required for initiating the EMT process.[33-37]

MATERIALS AND METHODS

Animal Model

C57BL/6 male mice 6-8 weeks old were obtained from the center for experimental studies, Iran University of Medical Sciences (IUMS), Tehran, Iran.

All methods and experimental protocols were approved by the ethics committee of the IUMS (ethics code: IR.IUMS.REC.1396.960). Mice were randomly allocated into three individual groups of control, Bleomycin (BLM) and BLM+IPSCs. The mice in BLM and BLM+IPSCs groups received 5mg/kg Bleomycin dissolved in phosphate-buffered saline (PBS) via the surgical installation of the BLM into the trachea; the control group received PBS by the same root. Forty-eight hours later, the BLM+IPSCs received 2×10^6 IPSCs per kg suspended in 500 µL PBS via tail vein injection, while the other two groups only received PBS[12] All the three groups were sacrificed on day 21, the lungs were removed, and the lobes were divided for further processing.

Cell Culture and IPSCs Preparation

Mice embryos were taken from 13 weeks pregnant C57BL/6 mice, and they were minced physically using surgical blades in PBS under sterile conditions./ Subsequently, they underwent enzymatic dissociation using a combination of collagenases, trypsin, and DNAse1 (Thermo Fisher, USA). After that, they were cultured in the Dulbecco's Modified Eagle Medium (DMEM) medium containing 20% (Fetal bovine serum) FBS and 0.1mM β-mercaptoethanol (passage zero). The cells were passaged three times, and then the passage two cells were transduced with lentiviral particles carrying either OCT4, SOX2, KLF, and c-myc or eGFP in the presence of polybrene transfection reagent overnight (with a multiplicity of infection (MOI) of 15.[38] The eGFP was used to assess the transduction efficacy as well as for the calculation of MOI. Cells were cultured in DMEM complete-plus medium (Merck, Germany) containing 1% recombinant leukemia inhibitory factor (LIF) (R&D Systems, USA) for about 20 days; the transformed cells showed. the characteristic phenotypes of the stem cells and formed raised colonies were picked physically, and they were enzymatically dissociated; using Accutase cell detachment solution (Merk, Germany); the characteristic gene profile of the IPSCs was confirmed using qPCR with specific primers recognizing *OCT4*, *SOX2*, and *KLF4* genes which were included in the lentiviral vectors, as well as Nanog which was absent in lentiviral particles. gene expression data were compared with non-transduced MEFs (data not shown). Afterward, they were expanded to reach enough cells for treating the BLM+IPSCs group.

Histologic Examinations

Some lung lobes which were kept in formalin 10% were paraffinized and sectioned for Hematoxylin & Eosin (H&E) and Masson-trichrome staining (subsequent staining with hematoxylin, ponceau acid fuchsin red, and aniline blue) to evaluate the inflammation and visualize collagen depositions, respectively.

Hydroxyproline Assay

To assess the collagen content of the study groups, a colorimetric assay was conducted by measuring the color results from the reaction of chloramine-T with hydroxyproline residues of the collagen fibers. Briefly, lung specimens were first digested in HCL at 120°C overnight. Then they were transferred into a 96-well plates were treated with the chloramine-T solution and dimethyl-benzaldehyde+ perchloric acid. After the incubation time (60 minutes), the resulting yellow color was assessed using an ELISA plate reader at a wavelength of 550 nm. The results were reported as µg hydroxy-proline/mg lung specimen.

Real-time qPCR

Lung specimens that were kept frozen in RNALater solution were first minced. Then they were lysed with a homogenizer in Trizol lysis buffer (SinaClon, Iran) according to the manufacturer's protocol. RNA isolation was carried out using RNXplus (SinaClon, Iran) according to the manufacturer's instructions. The quality and the quantity of the resulting RNA were assessed by gel electrophoresis and NanoDrop spectrophotometer (Thermo Fisher, USA). Then the RNA samples were reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Fermentase, USA) according to the manufacturer's protocol. Eventually, the gene expression of IGF1, IGF2, and Irs1 was assessed utilizing specific primers and the RotorGeneQ real-time PCR instrument (Qiagen, Germany) with SYBR green master mix (Amplicon, England) (Supplementary Table1). Mouse GAPDH-specific primer was used as the reference gene for data normalization. Gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Statistical Analyses

All the statistical analyses were performed using the GraphPad PRISM software. One way-ANOVA was employed to compare the data with Tukey's post hoc

for multiple comparisons. $p \leq 0.05$ were considered statistically significant.

RESULTS

Histological Comparison of the Study Groups

H & E-stained lung sections showed remarkably higher inflammation and cell infiltration in the BLM group than in the control group; also, significant lung tissue remodeling characterized by decreased alveolar spaces was evident (Figure 1 A, B). The inflammation, cell infiltration, and alveolar spaces were significantly improved following treatment with IPSCs (BLM+IPSCs group) compared to the BLM group (Figure 1C).

Furthermore, In the Masson-trichrome staining of the lung sections, remarkably increased collagen depositions identified by blue color and dense alveolar spaces as a result of fibrotic changes were observed in the lungs of the BLM group compared with the control group (Figure 2A and 2B). In contrast, treatment with IPSCs significantly decreased the collagen depositions in the lungs of the BLM+IPSCs group, which was comparable to the control group (Figure 2C).

Hydroxyproline colorimetric assay is routinely performed to indirectly determine the collagen content of the tissues quantitatively by measuring the absorbance of light at 550nm wavelength; at this wavelength, the intensity of yellow color results from the interactions between chloramine-T and hydroxy-proline residues within the collagen fibers could be assayed. In line with the previous results, it was found that the hydroxyproline content of the lung specimens from the BLM group was remarkably increased compared with the control group. At the same time, it was significantly decreased back to the normal levels after treatment with IPSCs in the BLM+IPSCs group ($p \leq 0.0001$) (Figure 3).

Gene Expression Assessment by qPCR

The current study was designed to evaluate whether the previously reported therapeutic potential of IPSCs in IPF mice models impacts the IGF signaling. The IGF signaling is considered a prominent contributing factor in the EMT process, which is regarded as the principal underlying mechanism in the pathogenesis of both fibrotic disorders such as IPF and malignancies. After analysis of the gene expression data, it was demonstrated that the expression of IGF1, IGF2, and *Irs1* genes was significantly increased in the BLM group, compared with the control group (Figure 4); whereas treatment with IPSCs (BLM+IPSCs) considerably decreased the expression of these genes $p \leq 0.0001$).

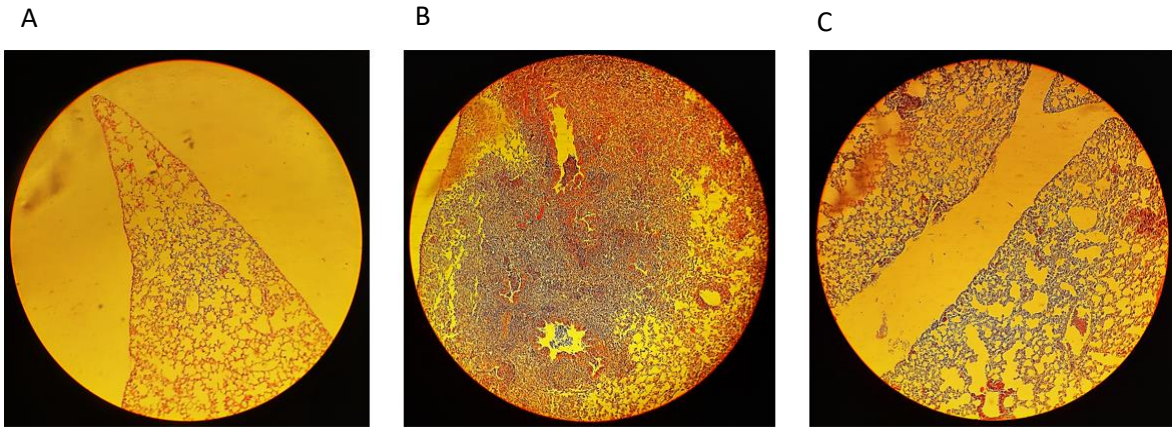

Figure 1. Hematoxylin and Eosin staining of the lung specimens from one mouse representative of each group. The lung sections were prepared after deparaffinization followed by Hydration; then underwent histological staining with hematoxylin and eosin; the sections were observed under the microscope. The airway remodeling, as well as increased inflammation identified by decreased alveolar spaces and immune cell infiltrations in the Bleomycin (BLM) group in the middle (B), compared with the control group in the left (A). The alveolar spaces were remarkably increased, and the immune cell infiltrates significantly decreased after treatment with induced pluripotent stem cells (IPSCs) at the right (C).

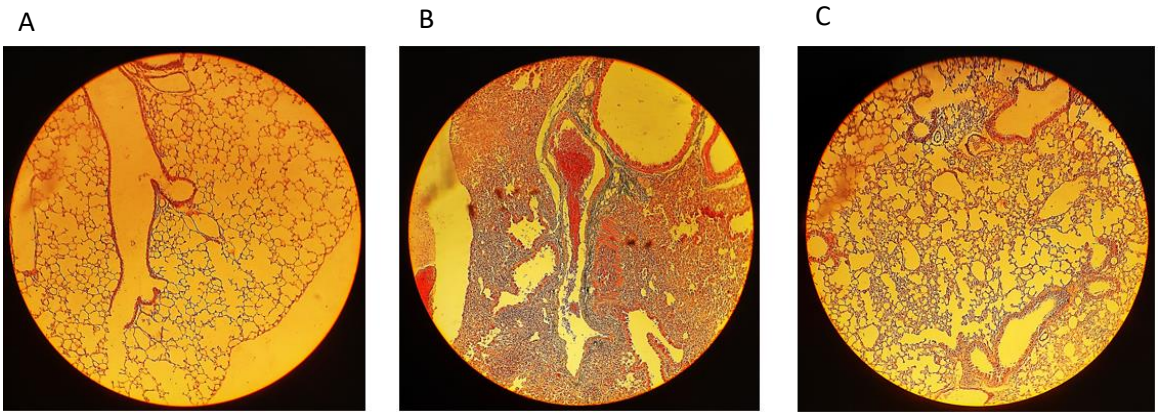

Figure 2. Masson-trichrome staining of the lung specimens from one mouse representative of each group. The sections were prepared and stained with hematoxylin, Ponceau Fuchsin, and aniline blue. The airway remodeling and fibrotic changes are evident in the substantially decreased alveolar spaces and increased collagen depositions characterized by the blue color in the middle image (B) in comparison with the control group at the left (A). the airways significantly improved after treatment with induced pluripotent stem cells (IPSCs) with markedly enhanced alveolar spaces and reduced collagen depositions at the right (C).

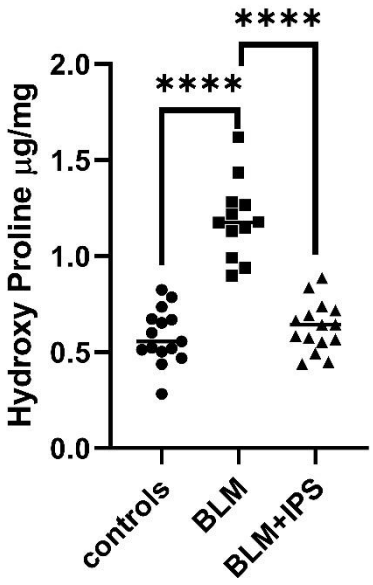

Figure 3. Hydroxy proline colorimetric assay results as a measure for assessing collagen content and fibrosis. The lung specimens were lysed; using HCL at 120°, then transferred to a 96 well plate and treated with chloramine-T; then, the resulting yellowish color was measured by a plate reader at 550 nm. The hydroxyproline content of the lung tissues from the Bleomycin (BLM) group (n=12) was significantly increased compared with the controls (n=15). At the same time, it was remarkably reduced to the normal level following treatment with IPSCs (n=15). $p \leq 0.05$ were regarded statistically significant; **** $p \leq 0.0001$.

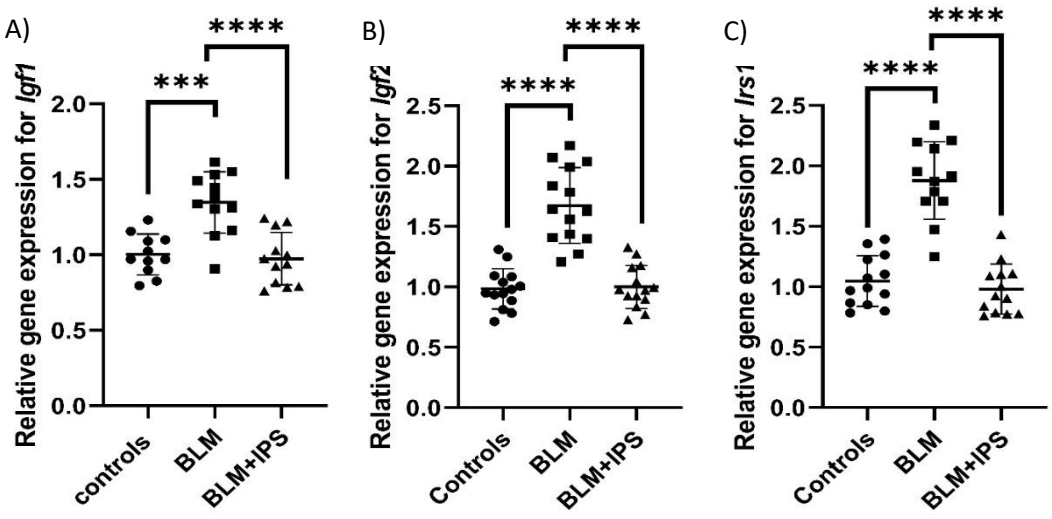

Figure 4. Comparison of the relative gene expression of insulin-like growth factor (IGF) 1, *2*, and *Insulin response substrate 1* among the three study groups. After RNA isolation from the lungs of mice and reverse transcribing it into cDNA, qPCR was carried out using specific primers and SYBR green master mix. A) Relative expression of IGF-1 was significantly increased in the Bleomycin (BLM) group (n=12) compared with the control group (n=11), and treatment of IPF mice with IPSCs (n=12) resulted in the reduced expression of IGF-1 in comparison to the BLM group; B) Relative expression of IGF-2 was significantly increased in the BLM group which was downregulated to the control level after treatment with IPSCs; C) Relative expression of Irs-1 was significantly increased in the BLM group, and it was shown to be downregulated back to normal levels after being treated with IPSCs. Relative expression of the *Gapdh* gene was used as the reference gene for data normalization. $p \leq 0.05$ were considered statistically significant; ***$p \leq 0.001$, ****$p \leq 0.0001$

DISCUSSION

The current study investigated the therapeutic potential of the IPSCs in regulating the three cardinal components of the IGF pathway. The IPSCs were prepared by transducing mouse embryonic fibroblasts with lentiviral particles carrying pluripotency factors. After induction of the IPF mouse models by direct intratracheal instillation of bleomycin, half of them received IPSCs via the tail vein injection the next day. All the three study groups were assessed on day 21. Histologic examinations and hydroxyproline assay demonstrated successful development of the Bleomycin-induced IPF mouse model; since the fibrotic measures such as inflammation, immune cell infiltrations, collagen depositions on the lung sections, and hydroxyproline content were significantly increased in the BLM group, indicating the fibrotic changes and remodeling of the lung tissues. Furthermore, it was found that the treatment of IPF mice models with IPSCs significantly attenuated the overexpression of Igf1, Igf2, and Irs1 to a level comparable to the control group. Consistent with our data, a few other studies have reported the therapeutic value of IPSCs in IPF mice models; for instance, How C.K, et al, have shown that treatment of IPF mouse models with intravenous administration of IPSCs resulted in the alleviation of lung inflammation and fibrosis through downregulation of inflammatory cytokines and chemokines such as interleukin-1 (IL-1), TNF-α and MCP-1 as well; they concluded that the antifibrotic effect of IPSCs is mainly due to the overexpression of interferon-γ-induced protein 10, which is known to impede fibro*gene*sis.[10] Also, Gazdhar et al, demonstrated that the IPSCs conditioned medium obtained from IPSCs cultures is capable of inhibiting fibrosis in bleomycin-induced lung fibrosis of rats; they have attributed this anti-fibrotic feature of the IPSCs conditioned medium to the release of hepatocyte growth factor (HGF) into the medium.[9] In a similar study by Ren, et al, it was found that IPSCs conditioned medium significantly decreased

differentiation of cultured human dermal fibroblasts into activated myofibroblasts as well as their contraction ability in response to treatment with TGF-β1.[39] Zhou Yan et al, further explored the mechanism underlying the anti-fibrotic effect of IPSCs conditioned medium on human and mouse fibroblast cultures; they concluded that the exhibited anti-fibrotic potential of IPSCs conditioned medium results from inhibition of the TGF-β and Smad2/3 phosphorylation, which is crucial for the fibrogenesis.[12,13] The same authors have also shown in a separate study that EMT markers such as vimentin, fibronectin, and α-smooth muscle actin are remarkably reduced in response to I.V. administration of IPSCs to the IPF mouse models; besides, it was observed that the expression of matrix metalloproteinases, MMP-2 and MMP-9, which are responsible in ECM remodeling, as well as inflammatory mediators such as Interleukin-6, Inducible nitric oxide synthase (iNOS), and prostaglandin E2 were significantly repressed.[12,40] Altogether, these data are consistent with our findings in the current study, where the anti-fibrotic effect of IPSCs was confirmed in the IPF mouse model. Some studies have progressed into further steps by differentiating the IPSCs into other cells capable of attenuating fibrosis or other inflammatory conditions which result in airway remodeling; instances are mesenchymoangioblasts, macrophages, airway epithelial cells, and alveolar cells, which contribute to inhibition of inflammatory responses or directly heal the damaged airways.[41-47] Furthermore, the secretory products of IPSCs such as exosomes have been shown to exert anti-fibrotic effects in pulmonary fibrosis.[11,48] To our knowledge, this is the first study that has assessed the anti-fibrotic effect of IPSCs through regulating the IGF signaling pathway. No previous studies were found in the literature that has addressed this issue in vivo or in vitro. Some prominent studies will be summarized to elucidate the role of IGF signaling in IPF. In this regard, it was observed that Igf1 is upregulated in IPF lung specimens from clinical and animal studies; besides, its downstream PI3K/AKT/mTOR pathway is capable of inducing the secretion of TGF-β1, CTGF, and MMP9 in a positive feedback loop; all of which are contributing factors in fibrogenesis and specifically in IPF.[17,19,20,49] On the other hand, IGF-1 was shown to be upregulated in response to Th2 major cytokines, IL-4 and IL-13, both of which are implicated in healing responses overactivated in IPF pathogenesis; the overexpression of Igf1 subsequently results in inhibition of apoptosis in myofibroblasts, leading to their maintenance and survival.[50] In support of our data regarding the therapeutic value of IGF signaling, two similar but independent studies have shown that metformin, a common anti-diabetic agent, and somatostatin analogs which inhibit the IGF1 receptor signaling, significantly improve the IPF-associated markers and symptoms in experimental mouse models.[18,21] Also, in one study considering the IGF2 role in IPF and scleroderma-associated pulmonary fibrosis, it was demonstrated that increased IGF2 binds to IGF1 receptor or insulin receptor and contributes to overexpression of TGF-β2 and TGF-β3, which leads to evoking the fibrogenic Smad signaling.[15] Another crucial component in IGF signaling is the Insulin receptor substrate-1 (IRS-1) which mediates IGF1/2 signaling by providing a docking site for recruitment and activation of PI3K and other SH2-containing signaling adaptors;[51] therefore, it could be implicated in EMT and fibrotic disorders such as IPF as well.[36,52] Accordingly, we evaluated the gene expression of *IRS1* in the bleomycin-induced IPF mouse model for the first time, as there are no previous reports regarding the assessment of Irs1 in the literature. Here we demonstrated for the first time that the Irs1 is upregulated in the IPF mouse model, which suggests a role for this gene in the pathogenesis of IPF. Furthermore, we have shown that *Irs1* gene expression returns to near-normal levels post-treatment with IPSCs. Altogether, our data revealed another aspect of the anti-fibrotic potential of IPSCs, which has never been investigated. additionally, we could propose targeting IGF signaling, especially the IRS1, as a possible therapeutic approach for IPF. Our study had some limitations which we are willing to be addressed in future studies; several signaling molecules cooperate in the IGF signaling pathway. Therefore, assessment of other mediators downstream of this pathway could remarkably increase the scientific merit of our study. Besides, assessing the antifibrotic effect of IPSCs only by gene expression is the major limitation of our work; assessing the corresponding expression of IGF1/2 and IRS1 at the protein level could result in a more robust conclusion. Also, utilizing an IGF signaling inhibitor or modulator such as Metformin could be included in our study as an individual study group. Furthermore, we could include another BLM+IPSCs group that would receive the IPSCs treatment a few days after the BLM

instillation. Despite all these limitations, our study could contribute to a better understanding of a treatment option with many unknown aspects, which has led to useful results, that other enthusiastic researchers could follow in the field.

Our data suggest that IPSCs could attenuate fibrotic lesions and symptoms associated with IPF, possibly through regulating the gene expression of *Igf1*, *Igf2*, and *Irs1*. Though further evaluations are required, the ease of access, safety, and efficacy of IPSCs in treating IPF or other fibrotic disorders makes them an appropriate choice.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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