Original Research Article

Immunomodulatory effects of the induced pluripotent stem cells through expressing IGF-related factors and IL-10 in vitro

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Abstract

Background: Induced Pluripotent Stem Cells (IPSCs) represent an innovative strategy for addressing challenging diseases, including various rheumatologic conditions. Aside from their regenerative capacities, some studies have shown the potential of these cells in the modulation of inflammatory responses. The underlying mechanisms by which they exert their effects have yet to be fully comprehended. Therefore, we aimed to explore the gene expression linked to the IGF pathway as well as IL-10 and TGF-β, which are known to exert immunomodulatory effects.
Methods: A C57/Bl6 pregnant mouse was used for obtaining mouse embryonic fibroblasts (MEFs), then the IPSCs were induced using lentiviral vectors expressing the pluripotency genes (OCT4, SOX2, KLF1, and c-MYC). Cells were cultured for 72 h in DMEM high glucose plus leukemia inhibitory factor; Evaluating the gene expression was conducted using specific primers for Igf1, Igf2, Igfbp3, Igfbp4, Irs1, Il-10, and Tgf-β genes, as well as SYBR green qPCR master mix. The data were analyzed using the $2^{-\Delta\Delta CT}$ method and were compared by employing the *t* test; the results were plotted using GraphPad PRISM software. MEFs were utilized as controls.
Results: Gene expression analyses revealed that Igf-1, Igf-bp3, Igf-bp4, and Il-10 were significantly overexpressed ($p \le .01$), while Igf-2 and Tgf-b genes were significantly downregulated in the lysates from IPSCs in comparison with the control MEFs. The Irs1 gene expression was not altered significantly.
Conclusion: IPSCs are potentially capable of modulating inflammatory responses through the expression of various anti-inflammatory mediators from the IGF signaling, as well as IL-10. This discovery uncovers a previously unknown dimension of IPSCs' therapeutic effects, potentially leading to more advanced in vivo research and subsequent clinical trials.

Keywords

immunomodulation, induced pluripotent stem cells, IGF-1, IGF-2, IGFBP3, IGFBP4, IPSCs, IL-10, TGF-b

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Introduction

In the dynamic landscape of medical science, innovative strategies are continually being explored to combat challenging diseases. One such group of diseases that have proven particularly difficult to treat effectively is rheumatologic disorders. These disorders, which include conditions like rheumatoid arthritis, lupus, and scleroderma, are characterized by inflammation and loss of function in the joints, muscles, or connective tissues. They pose a

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significant challenge to both patients and healthcare providers due to their chronic nature, the complexity of their management, and the significant impact they have on patients' quality of life.[1] The current treatment strategies for autoimmune diseases aim to suppress the immune response or replace the damaged cells, but both approaches have limitations and side effects.[2] Non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids are commonly used to reduce inflammation and pain, but they can also cause harm to various organs.[3] Biological drugs are a new option that target specific immune cells or mediators, but they can also disrupt the natural immune balance and cause adverse effects.[4] Therefore, novel therapeutic strategies are needed to combat these disorders.

In recent years, Induced Pluripotent Stem Cells (IPSCs) have emerged as a promising therapeutic option for these disorders. IPSCs are derived from mature cells that have undergone genetic modifications to revert to a state similar to that of embryonic stem cells.[5] This reprogramming endows them with the ability to differentiate into any cell type in the body, thereby opening up the possibility of replacing damaged cells in various parts of the body. This regenerative capability offers hope for conditions that were previously considered irreversible. In addition to their regenerative capabilities, IPSCs have also shown the potential to modulate inflammatory responses in the context of several experimental conditions.[6–8] Inflammation is a key aspect of rheumatologic disorders, and the ability to control this process could potentially lead to more effective treatments for these disorders, reducing symptoms and improving patients' quality of life. However, despite the promise that IPSCs hold, the exact mechanisms through which they exert these effects remain elusive. Understanding these mechanisms is crucial, as it could lead to more effective utilization of IPSCs in treatment strategies and potentially open up new avenues for treating several diseases.

One of the possible factors that may affect the immunomodulatory properties of iPSCs is the insulin-like growth factor (IGF) pathway.[9] The IGF pathway is made up of two ligands (IGF1 and IGF2), a pair of receptors (IGF1R and IGF2R), along with six binding proteins (IGFBP1-6) that control the availability and function of these ligands.[10] The IGF pathway activates various signaling cascades, such as the PI3K/AKT/mTOR and MAPK/ERK pathways, which are responsible for controlling cellular growth, viability, specialization, and energy processes.[11] A potential role for the IGF pathway in the control of immune reactions has been suggested, making it a particularly interesting target for investigation in the context of rheumatologic disorders.[9,12] Also, the components from the IGF pathway are known to contribute to the induction of immunosuppressive effects by mesenchymal stem cells.[13–16] However, the expression and function of the IGF pathway in iPSCs and their immunomodulatory effects have not been explored so far.

In the current study, our objective was to shed light on these mechanisms by investigating the gene expression of the Insulin-like Growth Factor (IGF) signaling components (Igf1, Igf2, Igfbp3, Igfbp4, and Irs1 genes). We also investigated the expression of Interleukin-10 (Il-10) and Transforming Growth Factor-beta (Tgf-β), two cytokines known for their immunomodulatory effects.[17] Both IL-10 and TGF-β are known to play crucial roles in the regulation of immune functions, and alterations in their expression are linked to various inflammatory diseases. By understanding how the expression of these genes is altered in IPSCs, we hope to gain insight into the cells' immunomodulatory capabilities, potentially paving the way for new therapeutic strategies for rheumatologic disorders. In summary, this study represents an important step toward understanding the mechanisms through which IPSCs can modulate inflammatory responses and potentially contribute to the treatment of rheumatologic disorders. The findings of this study could offer opportunities for establishing novel treatment approaches, not only for rheumatologic disorders but also for a wide range of other diseases where inflammation plays a key role.

Methods

Animal experiments and cell isolation

Every procedure involving animals was conducted following the guidelines of the Declaration of Helsinki and received approval from the Institutional Animal Care and Use Committee at the Iran University of Medical Sciences, under the ethics code: IR.IUMS.REC139632474. All procedures were performed in accordance with the ARRIVE guidelines, as per the instructions provided. A healthy pregnant female C57/Bl6 mouse obtained from the Center for experimental studies, (Iran University of Medical Sciences (IUMS), Tehran, Iran) was euthanized by cervical dislocation on day ≈12. Multiple embryos from the single pregnant mouse were used to isolate mouse embryonic fibroblasts (MEFs), providing several biological replicates for the iPSC induction and subsequent analyses. Mouse embryonic fibroblasts (MEFs) were isolated from the dorsal region of the embryos by physical mincing and enzymatic digestion with 0.25% trypsin-EDTA (Sigma-Aldrich, USA), collagenase and Dnase1 for 15 min at 37°C. The cells were washed with phosphate-buffered saline (PBS) and resuspended in Dulbecco's modified Eagle's medium (DMEM) high glucose (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, USA). The cells were seeded in 10 cm culture dishes and incubated at 37°C in a humidified

atmosphere with 5% CO_2. The medium was changed every 2 days and the cells were passaged at 80% confluence. MEFs in passage three were used for iPSC induction.

iPSCs induction and culture

MEFs were transduced with two distinct lentiviral vectors either expressing the pluripotency genes OCT4, SOX2, KLF1, and c-MYC (Addgene, USA) or eGFP at a multiplicity of infection (MOI) of 15 in the presence of polybrene.[18] The transduction efficiency was assessed by fluorescence microscopy after 48 h. The cells were then cultured in DMEM high glucose plus 10 ng/mL leukemia inhibitory factor (LIF) (Millipore, USA) for 72 h. The medium was then replaced with DMEM complete-plus medium (Merck, Germany) supplemented with 15% knockout serum replacement (Gibco, USA), 2 mM L-glutamine (Gibco, USA), 0.1 mM non-essential amino acids (Gibco, USA), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, USA), and 10 ng/mL LIF (R&D). The cells were cultured on gelatin-coated dishes for about 20 days and the medium was changed daily. iPSC colonies were picked and expanded in the same medium.

Gene expression analysis

Whole RNA was isolated from iPSCs and MEFs using RNXplus (SinaClon, Iran) Following the guidelines provided by the manufacturer. The concentration and purity of RNA were measured by a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The RNA underwent reverse transcription to form cDNA by employing the High-Capacity cDNA Reverse Transcription Kit (Fermentase, USA) following the protocol provided by the manufacturer. The primary outcome measure was the quantification of gene expression levels associated with the IGF pathway (Igf1, Igf2, Igfbp3, Igfbp4, Irs1) and immune-modulating cytokines (Il-10, Tgf-β). The expression of genes associated with the IGF pathway (Igf1, Igf2, Igfbp3, Igfbp4, Irs1) and anti-inflammatory cytokines (Il-10, Tgf-β) was quantified by real-time PCR using SYBR green qPCR master mix (Amplicon, Denmark) and specific primers (Table 1). The qPCR analysis was carried out on a RotorGene Q Real-Time PCR System (Qiagen, Germany) with the following cycling conditions: 95°C for 15 min, this was succeeded by 40 cycles at 95°C for 15 s and 60°C for 1 min. Gene expression levels were standardized against the expression of the housekeeping gene GAPDH and calculated using the 2-ΔΔCT method. The data were presented as the average value plus or minus the standard deviation (SD) of three independent experiments. The differences between iPSCs and MEFs were compared by employing *t* test using GraphPad PRISM software (version 8.0, USA). *p*-values smaller than 0.05 were regarded as having statistical significance.

Public gene expression data retrieval

We retrieved publicly available gene expression data from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). We specifically used the dataset GSE19022, which includes mRNA expression profiles of mouse embryonic fibroblasts (MEFs) and induced pluripotent stem cells (iPSCs) at 7 and 11 days post-induction.[19]

Differential gene expression analysis (DGEA)

We employed the GEO2R online analytical tool (https://www.ncbi.nlm.nih.gov/geo/geo2r/) to analyze the differentially expressed genes (DEGs) between MEFs and iPSCs. GEO2R implements a linear model approach to identify genes with statistically significant changes in expression between groups. We used a Benjamini-Hochberg procedure to adjust *p*-values for multiple testing, considering genes with an adjusted p-value $<.05$ as significantly differentially expressed.

Pathway enrichment analysis

To explore the biological context of the identified DEGs, we performed pathway enrichment analysis using Enrichr-KG: (https://maayanlab.cloud/Enrichr/); We uploaded the DEG list to Enrichr-KG, a comprehensive resource for pathway enrichment analysis. Then we analyzed the DEGs for enrichment in functional annotation categories and biological pathways (KEGG pathways, Reactome). We focused on pathways related to our initial investigation of IGF signaling, IL-10, and TGF-β. This tool allowed us to explore the DEGs within the context of various gene sets and pathway databases, further corroborating the involvement of the aforementioned pathways in the observed gene expression changes.

Statistical analyses

All the statistical analyses were performed using the GraphPad PRISM software. The data were presented as the average value plus or minus the standard deviation (SD) of three independent experiments, and the 's *t* test was utilized to compare the data. *p*-values of 0.05 or lower were considered of statistical significance. The data analyst was blinded to the group allocations until the completion of the statistical analysis. To ensure that the data were normally distributed Shapiro-Wilk test was utilized.

Table 1. Primer sequences used for gene expression assays by qPCR.

	Gene	Primer sequence (5′→3′)	Length	Tm
1	**Igf-1**			
	Forward primer	GACCGGCACAACAGCAACATC	21	62.65
	Reverse primer	AATGGCACACGTTCCCGC	18	61.36
2	**Igf-2**			
	Forward primer	GATCAAGGACGGTGCCACTATG	22	61.06
	Reverse primer	GGCCGTAGTCGTTGTCCTCC	20	62.55
3	**Igfbp-3**			
	Forward primer	GCTTTCCCATCTTCCAACAACAGG	24	62.35
	Reverse primer	TGACACTATCCTGCCATCTTCACAC	25	62.59
4	**Igfbp-4**			
	Forward primer	TCCGTCGTGGATCTGACGTG	20	61.92
	Reverse primer	GATGCCTGCTTCACCACCTTC	21	61.55
5	**Irs**			
	Forward primer	TGGTTCAGTCTCTTCCTCATGCC	23	62.20
	Reverse primer	CGTCCAGAACCTCTCAGTCTCC	22	61.78
6	**Tgf-β**			
	Forward primer	AGAGCCCTGGATACCAACTATTGC	24	62.27
	Reverse primer	CACTTCCAACCCAGGTCCTTCC	22	62.79
7	**Il-10**			
	Forward primer	CGGGAAGACAATAACTGCACCC	22	61.51
	Reverse primer	CGGTTAGCAGTATGTTGTCCAGC	23	61.77

Results

Gene expression analysis of iPSCs and MEFs

First, the iPS cell induction was validated by qPCR for pluripotency genes and data are published elsewhere.[6] To investigate the gene expression of the IGF pathway components and anti-inflammatory cytokines in iPSCs and MEFs, we conducted real-time PCR analysis using specific primers.

It was observed that iPSCs significantly overexpressed Igf1, Igfbp3, and Igfbp4 genes in comparison to MEFs ($p <$ .05 for all comparisons) (Figure 1). These genes are responsible for control of cell proliferation, longevity, differentiation, metabolic processes, and immune responses as well. IGFBP genes encode for binding proteins that modulate the activity and availability of IGF ligands; aside from their classic role, they are implicated in the modulation of immune responses as well. On the other hand, iPSCs expressed significantly lower levels of the Igf-2 gene than MEFs ($p \leq .05$). The Irs1 gene was not observed to be altered; this may suggest that the iPSCs are not affected by the autocrine IGF. These results suggest that iPSCs have a distinct gene expression profile of the IGF pathway cardinal genes compared to MEFs and that they may modulate inflammatory responses through the IGF pathway.

We further assessed the gene expression of two prominent immunomodulatory cytokines TGF-β and IL-10 (Figure 2). We noted that while the Tgf-β gene was downregulated in IPSCs in comparison to MEFs, the IL-10 gene expression was upregulated in the iPSCs. This finding may further support the anti-inflammatory role of iPSCs through increased expression of IL-10 which has many diverse inhibitory effects across a wide variety of immune cells and mediators.

Differential gene expression analysis

To complement our initial investigation of gene expression using qPCR, we performed an in-silico analysis of publicly available data from the GEO dataset GSE19022. This dataset includes gene expression profiles of mouse embryonic fibroblasts (MEFs) and induced pluripotent stem cells (iPSCs) at 7 and 11 days post-induction.

We employed GEO2R to analyze differentially expressed genes (DEGs) between MEFs and iPSCs. The analysis revealed a significant alteration of the genes associated with anti-inflammatory effects in iPSCs compared to MEFs. These altered genes included IGF-1, IGF-2, IGFBP3, and IGFBP4, all key components of the IGF signaling pathway. Additionally, IL-10, and TGF-b as well-known anti-inflammatory cytokines, were also significantly altered in iPSCs (Figure 3).

Pathway enrichment analysis

To further explore the biological context of the identified DEGs, we utilized Enrichr-KG for pathway enrichment

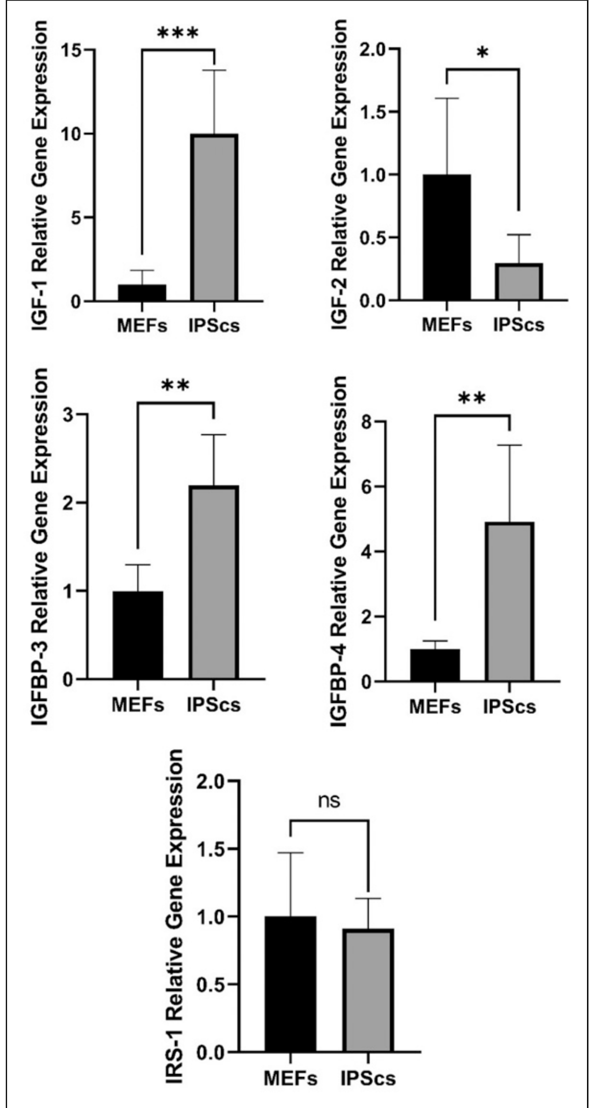

Figure 1. Gene expression analysis of iPSCs and MEFs by real-time PCR. The gene expression of IGF1, IGF2, IGFBP3, IGFBP4, IRS1, IL-10, and TGF-β was standardized against the expression of GAPDH and was obtained through the 2-ΔΔCT method. The results are presented as mean ± SD from three individual replicates. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ compared to MEFs by t test.

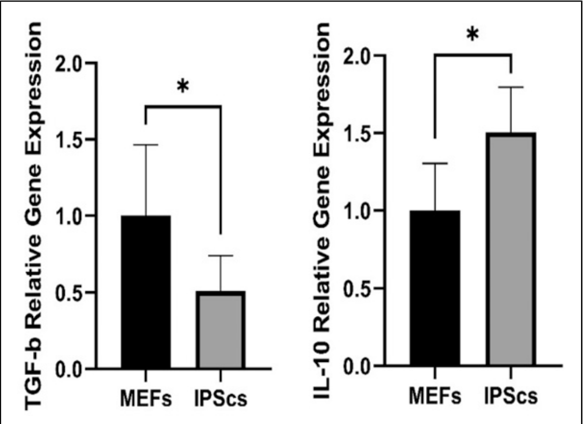

Figure 2. Comparison of Il-10 and Tgf-β genes expressed by iPSCs and MEFs. It was observed that iPSCs overexpress Il-10 gene while they express lower levels of Tgf-β gene, suggesting an IL-10 dependant anti-inflammatory role for them. Data were normalized to Gapdh gene expression ($^{*}p \leq .05$).

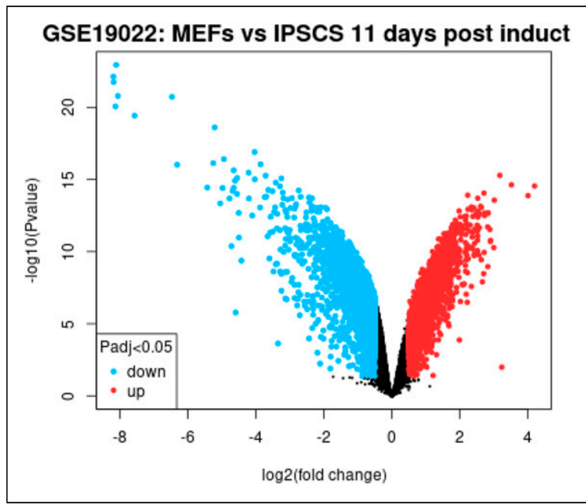

Figure 3. Volcano plot from GEO2R. this plot represents all data points associated with alterations in gene expression between MEFs and IPSCs; the blue points represent downregulated genes, while the red points represent upregulated genes. The black dots represent genes with constant expression between the two cell subsets.

analysis. This tool revealed a significant enrichment of genes associated with the IGF signaling pathway, the IL-10 signaling pathway, and the TGF-β signaling pathway among the DEGs. This finding further supports the involvement of these pathways in the observed gene expression changes between MEFs and iPSCs (Figure 4).

Discussion

Autoimmune and rheumatic diseases are chronic inflammatory disorders that affect various organs and tissues of the body and cause significant morbidity and mortality. The current treatments for these diseases are mainly based on reducing the symptoms and preventing the progression of the disease, but they are not curative and have many side effects and limitations. Therefore, there is an urgent need for novel and effective therapeutic strategies that can modulate the immune system and repair damaged tissues.

Stem cells are a promising source of therapy for autoimmune and rheumatic diseases, as they have the potential to transform into a range of cell types and secrete

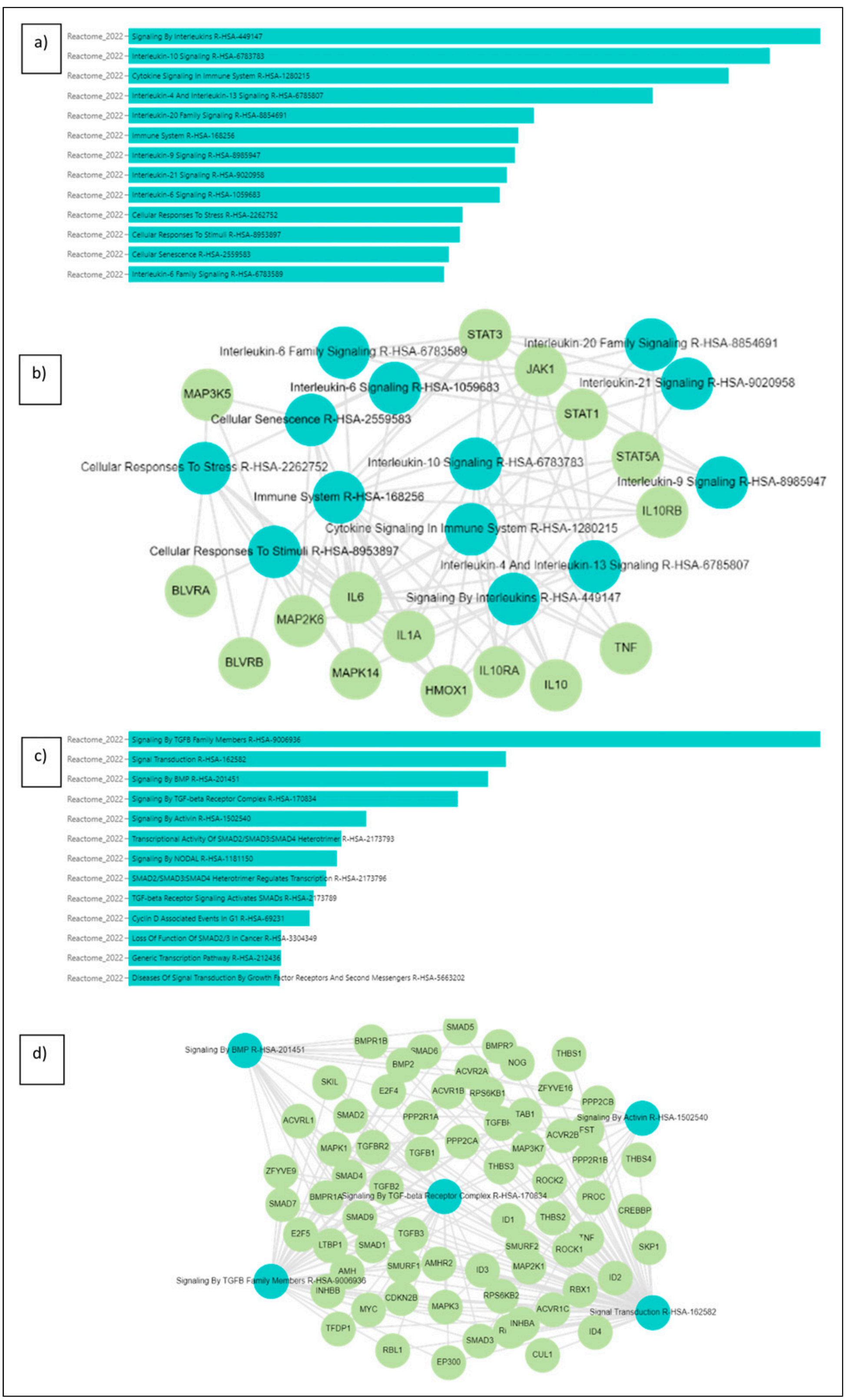

Figure 4. Enrichment analysis for DEGs between MEFs and IPSCs showing the contribution of the differentially expressed genes in the IL-10, TGF-b, and IGF-associated pathways. (a) bar view for the enrichment of the IL-10 associated pathways, (b) network view for the enrichment of the IL-10 associated pathways, (c) bar view for the enrichment of the TGF-b associated pathways, (d) network view for the enrichment of the TGF-b associated pathways, (e) bar view for the enrichment of the IGF associated pathways, (f) network view for the enrichment of the IGF associated pathways.

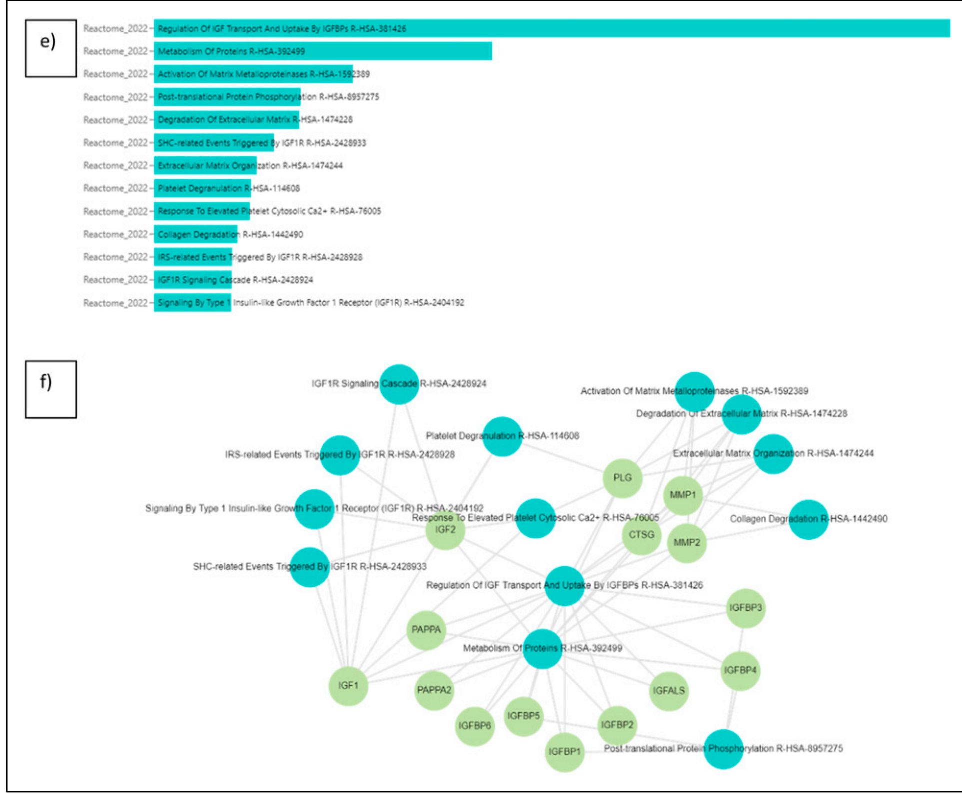

Figure 4. Continued.

immunomodulatory factors that can modulate immune reactions and support tissue regeneration.[20,21] Induced pluripotent stem cells (iPSCs) stand out among various stem cells due to their unique appeal, as they can be derived from Patient-derived somatic cells that have been transformed to become pluripotent cells by introducing a set of gene expression regulatory factors.[22] iPSCs have several advantages over other stem cells, such as avoiding the ethical issues associated with embryonic stem cells, reducing the risk of immune rejection, and having a higher proliferative and differentiation capacity.[23] However, iPSCs also have some challenges and risks, such as the possibility of tumorigenesis, genomic instability, and incomplete reprogramming.[24] Hence, it is essential to decipher the underlying molecular structures and factors that are involved in the immunomodulatory and therapeutic effects of iPSCs and to optimize their safety and efficacy. Previous studies have shown that the culture medium of mesenchymal stem cells (MSCs), which are known for their immunomodulatory properties, contains various cytokines and factors that can influence the immune response and tissue repair.[25] Some of these factors are interleukin-10 (IL-10), TGF-β, insulin-like growth factor-1 (IGF-1), insulin-like growth factor-2 (IGF-2), and insulin-like growth factor binding proteins (IGFBPs), which were shown to have immunomodulatory, anti-fibrotic, and regenerative impacts on various models of autoimmune and rheumatic diseases.[15,26,27] However, the expression and role of these factors in the culture of iPSCs have not been well studied.

Therefore, in the current study, our objective was to assess the expression of these molecules in the culture medium of iPSCs and to compare it with the expression of mouse embryonic fibroblasts (MEFs), which are the source cells for iPSC generation. We hypothesized that iPSCs would have a distinct expression profile of these molecules compared to MEFs and that this profile would reflect their immunomodulatory and therapeutic potential. To test this hypothesis, we used lentiviral vectors carrying four pluripotency genes (OCT4, SOX2, KLF4, and c-MYC) to induce iPSCs from MEFs, and after confirming the successful induction of the cells we assessed the expression of Il-10, Tgf-β, Igf-1, Igf-2, Irs1, Igfbp3 and Igfbp4, in the lysates from iPSCs and MEFs by SYBR green qPCR (Figure 5).

Our results showed that iPSCs had a significantly different expression profile of these genes compared to MEFs, which may be related to their immunomodulatory properties. Specifically, we found that IGF-1, IGFBP3, IGFBP4, and IL-10 genes were significantly overexpressed ($p \leq .01$), while IGF-2 and TGF-b genes were significantly downregulated in the lysates from iPSCs in comparison to

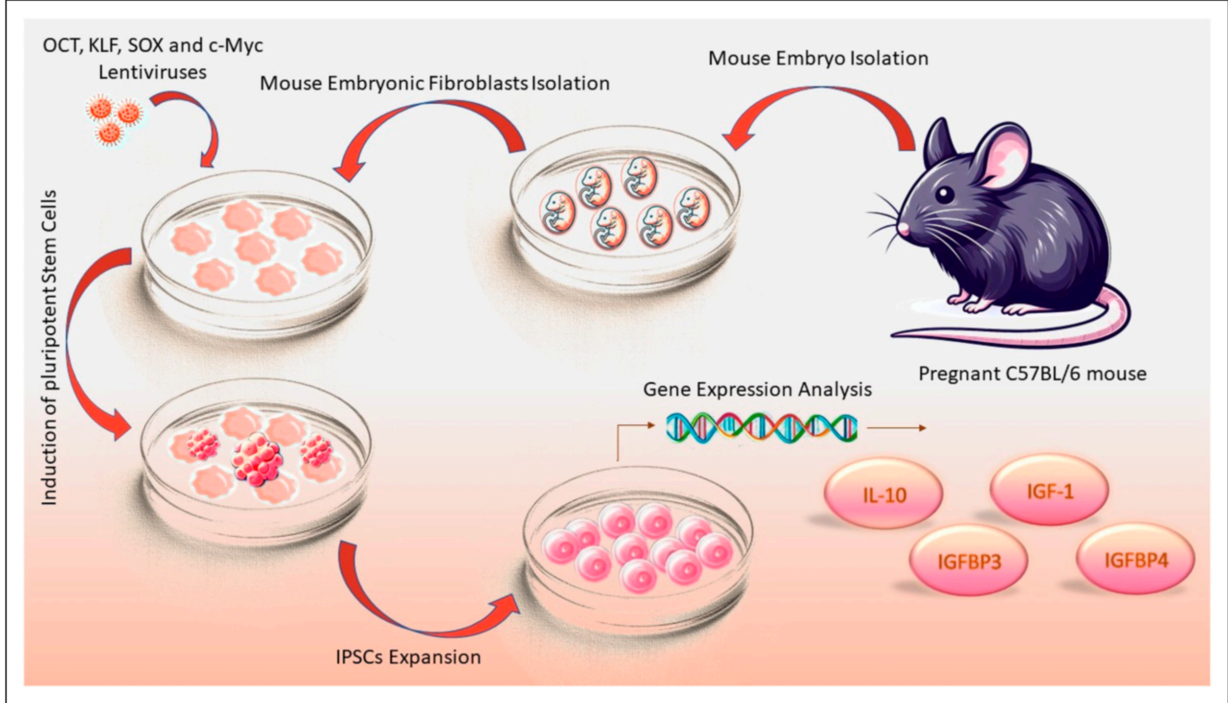

Figure 5. Schematic representation of the study flow. Mefs were isolated from a 12-day pregnant mouse and they were transduced with lentiviruses carrying a cassette of OKSM genes. iPS clones were picked, dissociated, and expanded, and then the levels of prominent IGF pathway members, along with Il-10 and Tgf-b were measured.

the MEFs. The expression of the IRS gene was not altered significantly. These results suggest that iPSCs are capable of modulating inflammatory responses through the production of various immunomodulatory factors, such as IGFBPs and IL-10. IGFBPs can bind to IGF ligands and modulate their activity and availability, while IL-10 can inhibit the expression of inflammation-related cytokines and promote the development of immune-regulating T cells.[15,28,29] Therefore, our research uncovers a novel facet of iPSCs' therapeutic effects that has the potential to be converted into further in vivo studies to explore their potential for treating inflammatory and autoimmune diseases.

There are mixed previous studies about the effects that IGF1 and IGF2 exert on the immune system and inflammation. Some studies suggest an anti-inflammatory effect of them, while some other studies support their role in eliciting inflammation.[9,30] We have observed that iPSCs overexpress the Igf-1 gene compared to MEFs which indicates the potential of the iPSCs in affecting immune responses, either by stimulating the proliferation of the immune cells or by impacting the immune cells signaling. In this regard, it has been demonstrated that IGF-1 enhances the growth and expansion of regulatory T cells, thereby alleviating autoimmune reactions.[15,31] On the other hand, it was also shown that IGF1 is capable of inducing inflammatory responses by enhancing the production of inflammation-related cytokines such as TNF-α or enhancing the polarization of Th17 cells.[32,33] Also, our data demonstrated that the Igf2 gene expression was lower in iPSCs compared to MEFs, Regarding the impact of IGF2 on inflammation the available data support an anti-inflammatory role mainly through affecting the development and expansion of Tregs[34–36]; for instance, IGF2 was shown to exert anti-inflammatory effects through the up-regulation PD-L1on macrophages which resulted in the expansion of Tregs in an in-vivo model of experimental autoimmune encephalitis.[37] However, there is evidence of its pro-inflammatory role as well. This contrast in the IGF2 effects on inflammation has been attributed to the concentration of IGF2, as in lower concentrations it can bind to IGFR2 and promote anti-inflammatory responses, while in higher concentrations it can bind to IGFR1 and act as a proinflammatory mediator.[37,38] Therefore, it could be speculated that lower expression of the Igf2 gene by iPSCs might accompany their anti-inflammatory properties. We have also shown that the expression of the Irs gene was not altered in the cultured iPSCs which might imply that the IGF signaling is not active in the iPSCs and these cells are not affected by autocrine IGFs. Furthermore, we have demonstrated that the expression of both Igfbp3 and Igfbp4 was upregulated in the iPSCs; previously IGFBP4, but not IGFBP3 was shown to be overexpressed in the culture medium of the MSCs and it was reported to be

associated with the inhibition of the IGFs anti-inflammatory effects[15]; considering our data and the previous mixed studies about both anti-inflammatory and pro-inflammatory roles for IGFs, it is difficult to conclude whether the upregulation of Igfbp genes in iPSCs favors inflammatory or anti-inflammatory consequences. One possible explanation is that the individual IGFBP family members have specific roles and interactions with IGFs and other signaling pathways. For instance, they may facilitate the binding of IGFs to their receptors which enhances their functions, while at the same time, they could impede the interaction between IGFs and IGF-Rs.[39] Considering the concentration-dependent actions of IGFs we could propose that the effects of IGFBPs could be context-dependent and anyway they could modulate the IGF's impacts. Therefore, the role of IGFBPs in iPSCs and MSCs may depend on the balance between their positive and negative effects on IGF signaling, as well as their interactions with other factors in the microenvironment. Also, it should be noted that the IGFBPs could counteract inflammation and inflammatory mediators independent of their interactions with IGFs, for instance, IGFP3 has been shown to suppress TNF-α functions in vitro.[27,40] Additional research is required to clarify the molecular processes and functional implications of IGFBP expression in iPSCs and MSCs. We have also assessed the TGF-β gene expression by iPSCs; although the anti-inflammatory effects of TGF-β are well understood, however considering our data showing reduced expression of the Tgf-b gene in iPSCs compared to the MEFs, it seems that the immunomodulatory effects of iPSCs are independent of producing or secreting TGF-β, although they were shown to indirectly contribute to the downregulation of TGF-b within the lung tissue.[41] Another potent anti-inflammatory factor with diverse suppressing effects on inflammatory responses is IL-10, which was observed to be upregulated in our study. This may suggest that the iPSCs are capable of producing IL-10 and impact inflammation and autoimmune diseases in part by this cytokine. There is no data supporting or contrasting our finding; although previous research has demonstrated that MSCs and IPSCs-derived MSCs produce and secrete large amounts of IL-10, no study has investigated the IL-10 direct production by iPSCs.[8,42] There are also some reports demonstrating increased expression of IL-10 within tissues in response to treatment with iPSCs; IL-10 could be produced by iPSCs or being upregulated through other mediators produced by these cells which indirectly promote IL-10 overexpression in their surrounding immune cells.

The in-silico analysis using GEO2R highlighted significant changes in genes related to the IGF pathway and anti-inflammatory cytokines in iPSCs; Enrichr-KG analysis also showed significant enrichment of genes in the IGF, IL-10, and TGF-β pathways, reinforcing the involvement of these pathways in the gene expression differences observed. These findings suggest that iPSCs have a distinct gene expression profile that could influence their behavior and functionality, particularly in the context of inflammation and cellular communication. The upregulation of anti-inflammatory cytokines like IL-10 in iPSCs is particularly interesting, as it may contribute to their therapeutic potential in regenerative medicine and disease modeling. The pathway enrichment analysis further supports the idea that iPSCs could be modulating inflammatory responses differently than MEFs, which is an important consideration for their use in research and therapy.

Our research has certain limitations that should be acknowledged and considered in subsequent studies. First, we only analyzed the gene expression of these molecules in the lysates from iPSCs and MEFs, but not in the culture medium itself. It is possible that the secretion and stability of these molecules in the culture medium may differ from their expression in the lysates, and that the culture medium may contain other factors that may influence the immunomodulatory and therapeutic effects of iPSCs. Therefore, it would be important to measure the protein levels and activity of these molecules in the culture medium and to identify and characterize other factors that may be present. Second, we only used one source of iPSCs (MEFs) and one method of reprogramming (lentiviral vectors). It is possible that the expression and role of these molecules may vary depending on the source and method of iPSC generation, as well as the culture conditions and differentiation status of iPSCs. Therefore, it would be interesting to compare the expression and role of these molecules in iPSCs derived from different sources (such as blood, skin, or urine) and by different methods (such as plasmids, episomes, or mRNA). Third, we only performed in vitro analysis of these molecules, but not in vivo evaluation of their effects. It is possible that the in vivo effects of these molecules may differ from their in vitro effects, and that they may interact with other factors and cells in the complex microenvironment of the inflamed tissues. Therefore, it would be essential to test the in vivo effects of these molecules in relevant animal models of autoimmune and rheumatic diseases and to assess their safety and efficacy in terms of immunomodulation and tissue repair.

Conclusion

Our study demonstrates that iPSCs have a distinct expression profile of Il-10, Tgf-β, Igf-1, Igf-2, Igfbp3, and Igfbp4 compared to MEFs and that this profile may reflect their immunomodulatory and therapeutic potential. Our research offers novel perspectives on the molecular mechanisms and factors that could be involved in the immunomodulatory and therapeutic effects of iPSCs and

suggests their possible application for the treatment of inflammatory and rheumatic disorders. This study also highlights the need for further research to unveil the effects of iPSCs on inflammation and to explore their in vivo effects in relevant animal models.

Future directions

Functional Significance

Future studies can investigate the functional significance of the identified factors by co-culturing iPSCs (or their differentiated progeny) with immune cells and measuring changes in immune cell activity upon exposure to the iPSC secretome. In vitro assays specific to immunomodulation can also be employed.

Differentiation

iPSCs could be differentiated into specific cell types relevant to immunomodulation and their secretome could be analyzed to confirm the presence and activity of the immunomodulatory factors. This will provide a more comprehensive picture of the immunomodulatory potential of iPSC-derived cells.

In vivo Studies

In vivo experiments could be designed using animal models of autoimmune or inflammatory diseases. Introducing either the differentiated iPSCs or their secretome can help assess their immunomodulatory effects and therapeutic potential through evaluation of disease progression, immune cell activity, and tissue histology.

Author contributions

PB performed all lab procedures from sample preparations to qPCR tests, also is a major contributor to statistical/bioinformatics analyses and writing of the manuscript. MT assisted with the experimental procedures. MA helped with the qPCR setup. MS assessed the histopathology results. HP edited the manuscript. NM has designed and supervised the study and provided the study materials, All authors have read and approved the final manuscript.

Declaration of conflicting interests

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Ethical statement

Ethical approval

All the animal research and experimental methods adhered to the ethical standards set by the ethics committee of the Iran University of Medical Sciences as well as the ARRIVE guidelines. Ethical approval for this study was obtained from the ETHICS COMMITTEE of the Iran University of Medical Sciences (ethics code: IR.IUMS.REC139632474).

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Data availability statement

Data will be available upon reasonable request.

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