J Chin Med Assoc

ORIGINAL ARTICLE

Efficient induction of pluripotent stem cells differentiated into mesenchymal stem cell lineages

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Abstract

Background: Mesenchymal stem cells (MSCs) have garnered significant attention in the field of cell-based therapy owing to their remarkable capabilities for differentiation and self-renewal. However, primary tissue-derived MSCs are plagued by various limitations, including constrained tissue sources, arduous and invasive retrieval procedures, heterogeneous cell populations, diminished purity, cellular senescence, and a decline in self-renewal and proliferative capacities after extended expansion. Addressing these challenges, our study focuses on establishing a robust differentiation platform to generate mesenchymal stem cells derived from induced pluripotent stem cells (iMSCs).

Methods: To achieve this, we used a comprehensive methodology involving the differentiation of induced pluripotent stem cells into MSCss. The process was meticulously designed to ensure the expression of key MSC positive markers (CD73, CD90, and CD105) at elevated levels, coupled with the minimal expression of negative markers (CD34, CD45, CD11b, CD19, and HLA-DR). Moreover, the stability of these characteristics was evaluated across 10th generations.

Results: Our findings attest to the success of this endeavor. iMSCs exhibited robust expression of positive markers and limited expression of negative markers, confirming their MSC identity. Importantly, these characteristics remained stable even up to the 10th generation, signifying the potential for sustained use in therapeutic applications. Furthermore, our study demonstrated the successful differentiation of iMSCs into osteocytes, chondrocytes, and adipocytes, showcasing their multilineage potential.

Conclusion: In conclusion, the establishment of induced pluripotent stem cell-derived mesenchymal stem cells (iMSCs) presents a significant advancement in overcoming the limitations associated with primary tissue-derived MSCs. The remarkable stability and multilineage differentiation potential exhibited by iMSCs offer a strong foundation for their application in regenerative medicine and tissue engineering. This breakthrough paves the way for further research and development in harnessing the full therapeutic potential of iMSCs.

Keywords: Induced pluripotent stem cells; Mesenchymal stem cells; Regenerative medicine

1. INTRODUCTION

Mesenchymal stem cells (MSCs) have gained attention as potential therapeutic agents for both autologous and allogenic use due to their multipotency and immunoregulatory properties. However, the use of primary tissue-derived MSCs faces limitations such as finite proliferative capacity, donor variability, and pathogen exposure risks. To overcome these challenges, researchers have turned to the derivation of MSCs from pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) or induced PSCs (iPSCs). This study aims to develop a method to produce induced pluripotent stem cell-derived mesenchymal stem cells (iMSCs) that can potentially overcome the limitations of primary tissue-derived MSCs.

Notwithstanding their potential application in regenerative medicine, ESCs have long been associated with ethical concerns related to their origin, which involves the destruction of embryos.[1]

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Author contributions: Dr. Huan-Chin Tseng and Dr. Teh-Fu Hsu contributed equally to this work.

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

Journal of Chinese Medical Association. (2024) 87: 267-272.

Received May 8, 2023; accepted June 11, 2023.

doi: 10.1097/JCMA.0000000000001058

However, a breakthrough was achieved by Takahashi and Yamanaka[2] when they successfully induced the reprogramming of mouse skin fibroblasts, and later adult human fibroblasts,[3] into pluripotent cells via the simultaneous overexpression of four genes, Oct4, Sox2, Klf4, and cMyc (collectively known as OSKM) and named them iPSCs.[4] Renal tubular cells found in urine,[5] pancreatic exocrine cells, and peripheral blood cell.[6] While the derivation of iPSCs has been met with ethical concerns, the discovery of iPSCs paves the way for personalized cell-based therapies that allow the generation of patient-specific cells of tri-dermal lineage.[7–9]

The utilization of iPSCs for the generation of induced MSCs (iMSCs) brings several advantages over primary cell-derived MSCs.[10–15] iPSCs can be obtained from diverse cell sources, providing a readily available and abundant cell supply for iMSC production without the need for invasive tissue biopsies.[12,15] Additionally, iPSCs possess pluripotency, enabling controlled, and reproducible differentiation into iMSCs with consistent quality and characteristics.[14,15] This standardized and scalable manufacturing process ensures a reliable and sufficient source of iMSCs for various therapeutic applications.[15]

Furthermore, iPSCs can be genetically modified to enhance specific therapeutic properties of iMSCs, such as their immunomodulatory or regenerative capabilities.[16] This flexibility allows for tailoring iMSCs to meet specific clinical needs and optimizing their effectiveness. By overcoming limitations associated with tissue availability, inter-donor variability, and limited expansion capacity, iPSC-derived iMSCs offer a promising solution to the challenges faced by primary cell-derived MSCs.[14,15] These advancements in iMSC technology hold great potential for advancing regenerative medicine and facilitating the development of cell-based therapies.

In this study, we aim to explore the advantages and potential of iPSC-derived iMSCs as an alternative to primary cell-derived MSCs. By characterizing and validating the properties of iMSCs, we have established a foundation for their clinical application in regenerative medicine and tissue engineering. However, before iPSC-derived MSC therapy can be widely implemented in clinical settings, several challenges need to be addressed. These challenges include optimizing the differentiation protocols to ensure consistent and reproducible iMSC generation, ensuring the safety and long-term stability of iPSCs, and addressing any potential immunogenicity or tumorigenicity concerns. By overcoming these hurdles, iPSC-derived MSCs have the potential to revolutionize the field of regenerative medicine and provide new avenues for the treatment of various diseases and injuries.

2. METHODS

2.1. Establishment of human iPSCs

2.1.1. Derivation of human iPSCs

Human iPSCs were derived from peripheral blood mononuclear cells (PBMCs) using the CytoTune-iPS 2.0 Sendai Reprogramming Kit. PBMCs were cultured in StemPro-34 medium with cytokines for 4 days, infected with Sendai virus, and then cultured in PBMC complete medium for 2 days. They were then transferred to murine embryonic fibroblast (MEF) feeders and cultured in hESCs medium for 7 days. Multiple colonies of iPSCs were generated and examined.

2.1.2. iPSCs culture

The iPSCs were cultured in StemFlex™ Medium Kit (Thermo Fisher Scientific, Waltham, MA, USA A3349401) on Geltrex™-coated dishes. Geltrex™ matrix was diluted with cold D-MEM/F-12 (1X) (Thermo Fisher Scientific, Cat. no. 10565-018) and incubated in a humidified incubator at 37°C and 5% CO_2 for at least 1 hour before use. Sub-culturing of iPSCs was performed using Versene solution (Thermo Fisher Scientific, 15040066).

2.2. Establishment of induced pluripotent stem cells-derived mesenchymal stem cells

To establish iPSC-derived MSCs, we followed the protocol described by Hynes et al[17] with some modifications. Briefly, iPSCs were dissociated using Versene solution, and then reseeded onto a new Geltrex-coated T-25 flask for differentiation into MSCs in complete medium for 2 weeks, with medium replaced every 3 to 4 days. After the differentiation period, the heterogeneous cell population was passaged using TrypLE to obtain a single-cell suspension, which was then plated at a 1:3 ratio in a Geltrex-coated T-75 flask. Passaging was performed once cells reached 70% to 80% confluency, and cells were defined as passage 1 (P1) after the first passaging. From passage 2 onward, coating of the flask was not required.

2.2.1. Osteogenic differentiation

Osteogenic differentiation was induced using StemPro™ Osteogenesis Differentiation Kit (Thermo Fisher Scientific, cat. no. A1007201) according to the manufacturer's instructions. Briefly, MSCs at 70% to 80% confluency were dissociated with TrypLE and seeded at a density of 5×10^3 cells/cm^2 in 12-well plates. After 2 to 4 days of incubation in MSC growth medium, cells were re-fed with a Complete Osteogenesis Differentiation Medium. Negative controls were maintained in the MSC growth medium. The medium was changed every 3 to 4 days for >21 days. Alizarin red staining of calcium deposits within

2.2.2. Flow cytometry

MSCs were seeded in a 10-cm culture dish at a density of 2.5×10^3/cm^2. Upon reaching 70% to 80% confluency, cells were collected using TrypLE Select Enzyme (Thermo Fisher Scientific, Cat. 12563011) and incubated with conjugated antibodies including CD14, CD19, CD34, CD45, HLA-DR, TRA-1-60, CD44, CD73, CD90, and CD105 in 3% bovine serum albumin (BSA) in PBS for 35 minutes at room temperature in the dark. After washing with 10% fetal bovine serum (FBS) in phosphate-buffered saline (PBS), cells were analyzed using the FACSCanto II™ (BD, San Jose, CA) System and FlowJo™ software (BD, Ashland, OR).

3. RESULTS

3.1. Induction of mesenchymal lineage differentiation from iPSCs

In this study, we developed a method for deriving MSCs from iPSCs to establish an MSC resource that is both standardized and ethically uncontroversial (Fig. 1). By passage 2, the cells exhibited the characteristic spindle-shaped, fibroblast-like morphology of MSCs, which was consistently maintained throughout the induction process (as shown in Fig. 2).

To assess the characteristics of the derived MSC-like cells, several criteria were evaluated, including their expansion capacity, marker expression, and lineage differentiation potential. The MSCs met the first International Society of Cellular Therapies (ISCT) criteria in passage 2 when they were cultured on non-Geltrex-coated surfaces. These MSCs, derived from iPSCs, exhibited the typical fibroblast-like, spindle-shaped morphology of MSCs and showed adherence to plastic surfaces.

3.2. Established iPSCs-derived MSCs exhibited high expression of MSC markers

To confirm the identity of the derived cells as MSCs, we performed a flow cytometry analysis. The ISCT criteria require MSCs to express

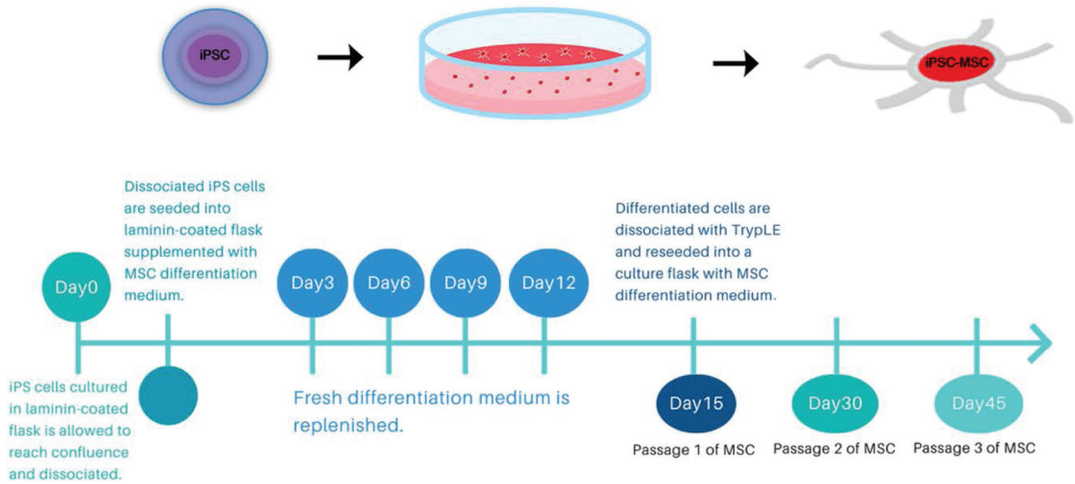

Fig. 1 Overview of the experiment to derive mesenchymal stem cells from iPSCs. The differentiation method used for generating mesenchymal stem cells from iPSCs involves a 2-wk differentiation stage, followed by multiple rounds of serial passaging to select MSCs and eliminate undifferentiated pluripotent cells. The scheme illustrates the simplified and reproducible process used for generating iPSC-MSCs. iPSCs = induced pluripotent stem cells; MSCs = mesenchymal stem cells.

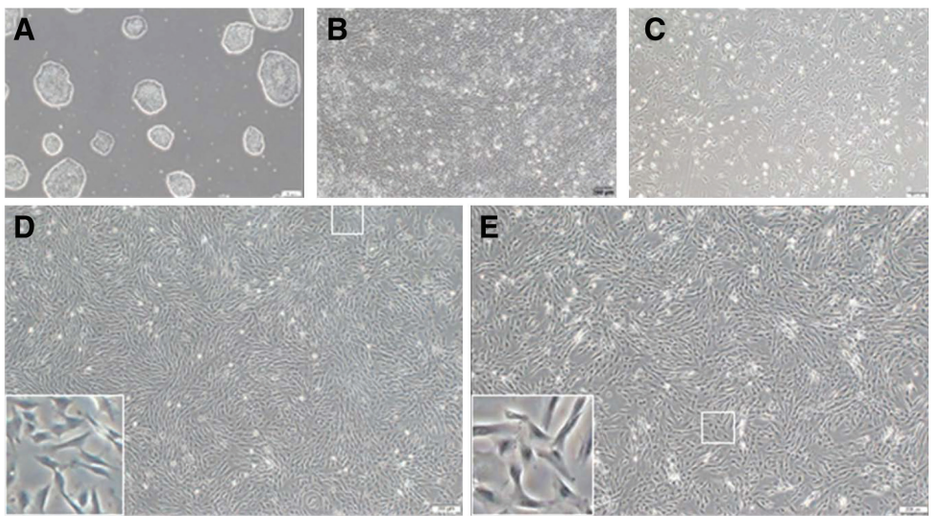

Fig. 2 Microscopy images illustrating iPSC differentiation into MSCs and corresponding changes in cell morphology. A, Undifferentiated iPSC colonies before induction. B, iPSCs cultured in MSC complete medium for 14 d. C, Differentiated iPSC cells passaged onto gelatin-coated flasks and grown in MSC complete medium for 14 d. D, Cell morphology changes at different stages of induction, including passage 2 and passage 8. E, Magnified images of the areas indicated by white boxes. Scale bar = 200 μm. iPSCs = induced pluripotent stem cells; MSCs = mesenchymal stem cells.

CD73, CD90, and CD105 markers at levels exceeding 95%, while expression of hematopoietic and endothelial markers such as CD14, CD19, CD34, CD45, and HLA-DR should be lower than 2%. The flow cytometry analysis revealed that <2% of the derived cell population expressed hematopoietic markers, while more than 95% expressed CD44, CD73, CD90, and CD105, consistent with MSC characteristics (Fig. 3). In addition, <2% of the cell population expressed major histocompatibility complex (MHC) class II DR Human Leukocyte Antigen - DR isotype (HLA-DR) and pluripotency markers, confirming the safety and immune-privileged feature of the derived MSCs.

3.3. The chondrogenic capacity of MSCs derived from iPSCs

To assess the chondrogenic differentiation potential of our iPSC-MSCs, we cultured the cells in a chondrogenic differentiation medium for 14 days. The resulting chondrocyte pellets displayed the characteristic three-dimensional (3D) round morphology of chondrocytes in culture, which is considered the gold standard for chondrocyte research (Fig. 4). Alcian blue staining of our established MSC-derived chondrocytes was positive, confirming the successful chondrogenesis of iPSC-MSCs (Fig. 4).

3.4. Adipogenic capacity of MSCs derived from iPSCs

To investigate the adipogenic differentiation potential of iPSC-MSCs, we induced differentiation using an adipogenic differentiation medium. As a result, lipid droplets were observed in the induced groups, which were positively stained with Oil Red O solution (Fig. 4). We further quantified the lipid droplets extracted by isopropanol and found that the adipocytes derived

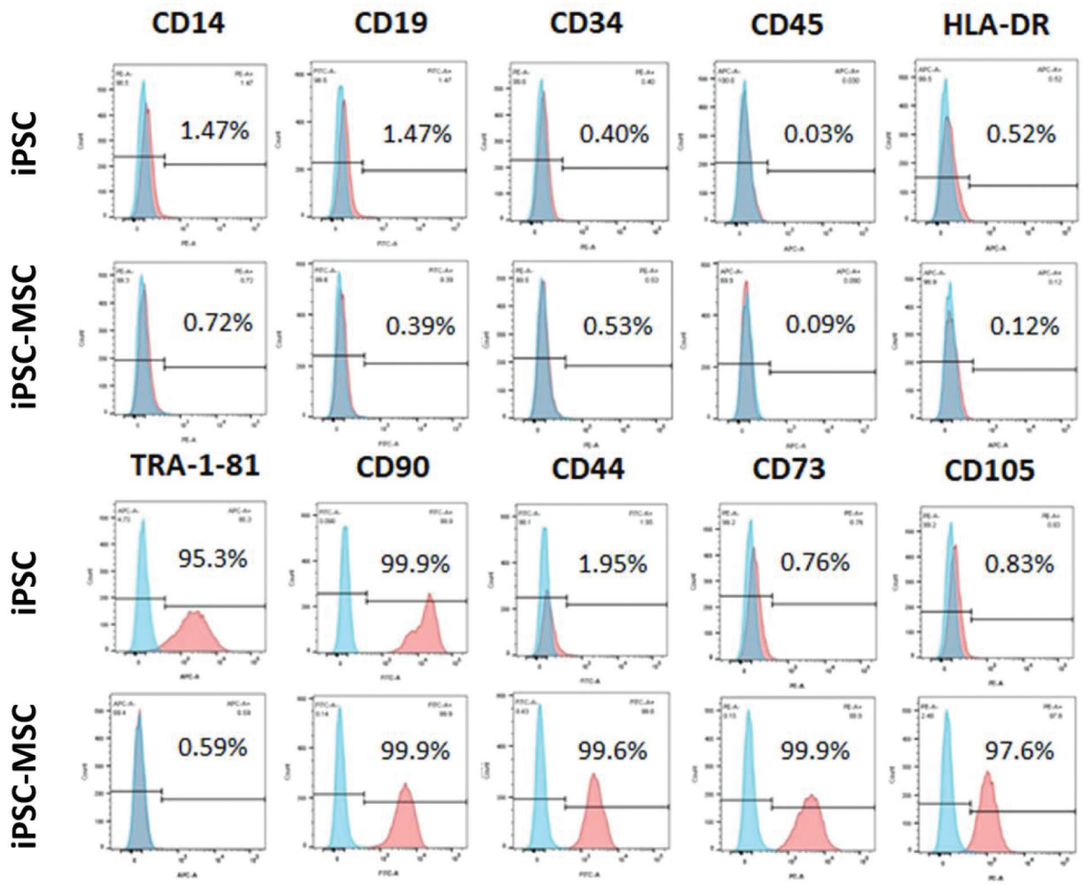

Fig. 3 Flow cytometry analysis of undifferentiated iPSCs and iPSC-MSCs. To assess the expression of cell surface markers before and after differentiation, flow cytometry analysis was conducted. The blue histograms represent the isotype controls, while the red histograms represent the expression of individual markers evaluated in both iPSCs and iPSC-MSCs. iPSCs = induced pluripotent stem cells; MSCs = mesenchymal stem cells.

from our established iPSC-MSCs had a significantly higher lipid content than the control cells.

3.5. Osteogenic ability of MSCs derived from iPSCs

To evaluate the osteogenic potential of the iPSC-derived MSCs, we cultured them in an osteogenic differentiation medium for more than 21 days. The formation of osteocytes was assessed by staining with Alizarin Red S to detect mineral deposits. In the control group cultured in standard D-MEM-low glucose supplemented with 10% FBS and 1% P/S, no calcium deposits were detected, while the induction group exhibited a bright orange-red color indicative of calcium deposits (Fig. 4).

4. DISCUSSION

The therapeutic potential of MSCs is widely recognized, attributed to their self-renewal and differentiation capabilities. However, the use of primary tissue-derived MSCs is not without challenges, such as donor variability, limited cell purity, and invasive retrieval procedures. Moreover, the iMSCs showed potential for osteogenic, chondrogenic, and adipogenic differentiation, indicating their promise for use in tissue engineering and regenerative medicine.

iMSCs derived from various sources have demonstrated potential for use in preclinical studies as therapeutic agents for the treatment of various diseases, including osteonecrosis, periodontal disease, inflammatory bowel disease, and myocardial infarction, in addition to cancer.[18–22] In clinical trials, iMSCs have been evaluated for the treatment of graft-versus-host disease (GvHD) and coronavirus disease 2019 (COVID-19).

The expression of mesenchymal genes and downregulation of pluripotent-associated genes suggest that iMSCs represent a unique cell population distinct from bone marrow-derived MSCs (BM-MSCs), although BM-MSCs have shown superior tri-lineage differentiation potential in gene expression levels.[23]

In this study, iPSCs were induced to differentiate into iMSCs, which were then characterized and evaluated for their potential therapeutic applications. The advantage of using iPSCs lies in their capacity for scalable and standardized production, enabling the generation of a consistent and homogeneous population of MSCs.[10–15] iPSC-derived iMSCs also offer the potential for personalized therapy, as they can be derived from patient-specific iPSCs.[12] Furthermore, iPSCs can be genetically modified to enhance the therapeutic properties of iMSCs or to address specific disease-related factors.[16]

However, it is important to acknowledge that iPSC-based therapies are still in the early stages of development, and several challenges need to be overcome before their clinical translation.[24] These challenges include ensuring the safety and efficacy of iPSC-derived iMSCs, optimizing differentiation protocols, addressing immunogenicity concerns, and developing strategies for large-scale production.[24,25] Further research and advancements in iPSC technology are needed to fully

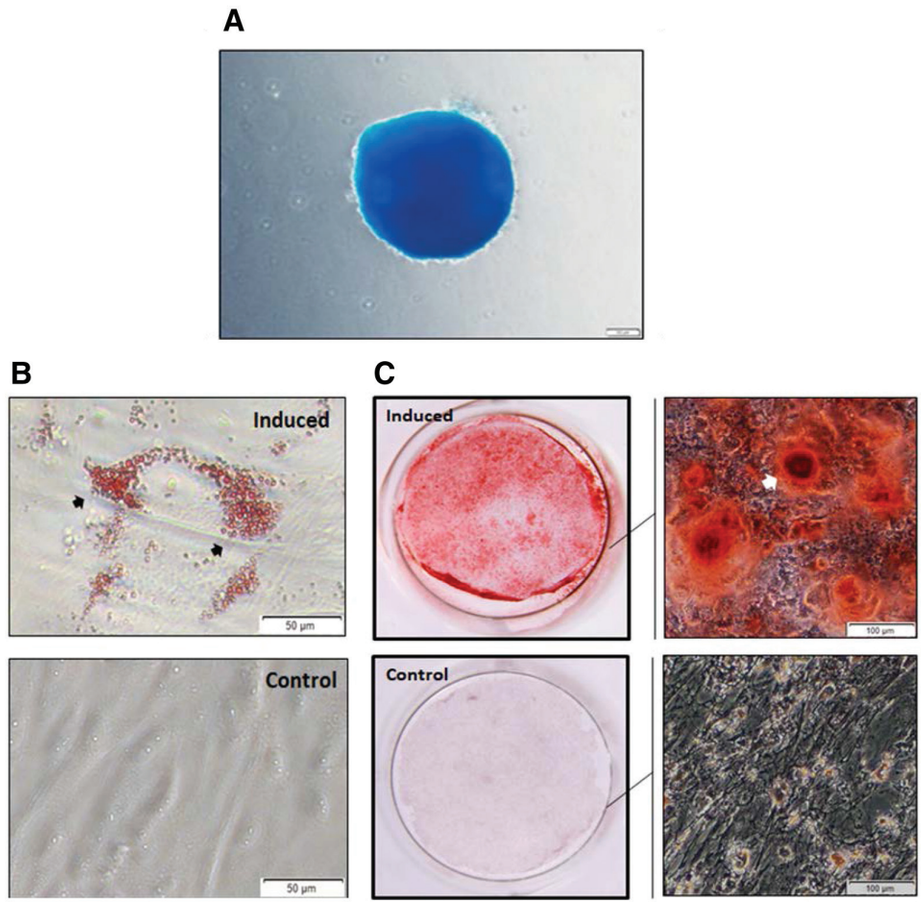

Fig. 4 Assessment of the multilineage differentiation capacity of iPSC-derived MSCs. A, Representative images of chondrogenic capacity result of iPSC-MSCs: formed chondrocyte pellets shown by staining with Alcian Blue solution. Scale bar = 100 μm. B, Representative images of adipogenesis of iPSC-MSC lines. Morphologies of formed lipid droplets are shown by staining with Oil Red O solution, indicated by black arrows. Scale bar = 50 μm. C, Representative images of osteogenic capacity results of iPSC-MSC lines. Morphologies of mineral deposits are shown by staining with Alizarin Red S solution, indicated by white arrows. Scale bar = 100 μm. iPSCs = induced pluripotent stem cells; MSCs = mesenchymal stem cells.

harness the potential of iPSC-derived iMSCs for regenerative medicine.

Therefore, in this study, iPSCs played a central role in providing a promising solution to the limitations associated with primary tissue-derived MSCs. The use of iPSCs offers advantages in terms of scalability, reproducibility, personalized therapy, and genetic modification, which contribute to the potential clinical application of iMSCs in regenerative medicine.[11] Nonetheless, continued research and refinement of iPSC-based therapies are necessary to unlock their full potential and ensure their safe and effective use in future clinical settings.

ACKNOWLEDGMENTS

This study was supported by the grants of National Science and Technology Council (NSTC 111-2321-B-A49-009 and NSTC 112-2321-B-A49-007), Ministry of Economic Affairs (112-EC-17-A-20-S6-026), and Taipei Veterans General Hospital in Taiwan (V111D61-002-MY2 and V112C-215).

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