Regenerative Engineering and Translational Medicine (2024) 10:110–125
https://doi.org/10.1007/s40883-023-00311-3

ORIGINAL RESEARCH

Xenogeneic Stem Cell–Induced Cardiac Progenitor Cells Regenerated Infarcted Myocardium in Rat Model

Syeda Roohina Ali[1] · Waqas Ahmad[1] · Asmat Salim[1] · Marie-Christine Durrieu[2] · Irfan Khan[1]

Received: 14 February 2023 / Revised: 4 May 2023 / Accepted: 22 June 2023 / Published online: 1 August 2023
© The Author(s), under exclusive licence to The Regenerative Engineering Society 2023

Abstract

Purpose We aim to investigate the role of stem cell–based regenerative medicine to repair or regrow the infarcted myocardium and restore normal cardiac homeostasis.

Methods WIKI4-treated cells were tested for in vitro differentiation into myocytes by gene and protein expression. A myocardial infarction (MI) model was established, cells were transplanted, and cardiac function was assessed by echocardiography. Histological evaluation of the harvested heart was performed to evaluate the cardiac regeneration.

Results These pre-differentiated cardiomyocytes were assessed for the presence of cardiac markers using immunocytochemical staining for Gata-4, Alpha actinin, and Myosin heavy chain, and gene expression analysis also showed that these cells are expressing cardiomyogenic markers. Hearts transplanted with WIKI4-treated mesenchymal stem cells (MSCs) significantly (***P<0.001) improved the cardiac systolic and diastolic dimension, end-systolic, diastolic, and stroke volume, ejection fraction, and fraction shortening as compared to the MI group after two and four weeks. Fibrotic area and left ventricular wall thickness significantly (***P<0.001) improved in WIKI4-treated group as compared to the control group. Patches of normal myocytes were observed in the infarct zone showing that induced cardiac progenitor cells regenerated cardiomyocytes replacing the infarcted scar, as evident from the co-localization of fluorescently labeled cells and cardiac proteins in immunohistochemical staining.

Conclusion This study put forth a valuable approach by small molecules to induce differentiation of MSCs into cardiomyocytes, which upon in vivo transplantation regenerated the infarcted heart. These findings will lead to the development of a novel approach for modified cellular therapy and may increase the probability of better myocardial regeneration.

Lay Summary Cardiovascular diseases (CVD) are the leading causes of morbidity and mortality. The indigenous capability of the myocardium to meet the degeneration is limited. Currently, available therapeutic options for the treatment of CVD are limited and provide solutions to reduce the symptoms. To investigate the role of stem cell–based repairing or regrowing the myocardium and restoring normal cardiac homeostasis, WIKI4-treated cells were tested for in vitro differentiation into myocytes by gene and protein expression.

Treatment of human bone marrow–derived mesenchymal stem cells with WIKI4 induced differentiation of MSCs into the cardiomyogenic lineage. Xenogeneic MSCs and their derivatives in form of induced cardiac progenitors when implanted into the infarcted rat's heart showed survival, distribution, integration, and differentiation into myogenic lineage, maintained the thickness of the left ventricular wall, infarct area was reduced, and hearts performed better function.

Keywords Mesenchymal stem cell · Infarction · Differentiation · Regeneration · Ligation · Immunofluorescence

Introduction

Cardiovascular diseases (CVDs) are the leading cause of death around the globe. Among CVDs, myocardial infarction has the highest morbidity and mortality rate. According to the World Health Organization (WHO), four out of five CVD deaths are because of heart attacks and strokes. Health management officials had dedicated a huge amount of finance, 115 billion, to afford the cost of cardiovascular diseases in the year 2012 [1]. Even

✉ Irfan Khan
Khan@iccs.edu
https://orcid.org/0000-0003-1878-7836

[1] Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

[2] Univ. Bordeaux, CNRS, Bordeaux INP, CBMN, UMR 5248, F-33600 Pessac, France

though small infarcts in cardiac vasculature are managed by the internal healing system of the body with the help of resident or circulating stem cells and by re-directing the cardiomyocytes into developmental phases, but large infarcts cannot be repaired by endogenous repair mechanisms, resulting in irreversible damage and loss to cardiac muscles, smooth muscles, and endothelial cells. Besides, the endogenous repair process leads to the substitution of working cardiac muscles by fibroblast cells which do not possess the ability to contract [2]. The prevailing treatment options available for myocardial infarction are only limited to symptomatic control leaving the root cause unaddressed.

Therapeutic strategies like inhibition of enzymes that convert angiotensin-I into angiotensin-II and alternative therapy that block β-adrenergic receptors have been successful to some extent in improving the symptoms of the disease. In addition, approaches such as surgical and other interventions have failed to play any role in remodeling the left ventricle because these therapies can neither repair nor replace the injured cardiac muscles [3–5]. Though heart transplant is the only hope for heart patients, the limited number of donors and the cost of the treatment do not make it possible for everyone.

Stem cell transplant has emerged as the most prominent therapeutic strategy in regenerative medicine and has been reported to improve outcomes of cardiac failure [6]. The potential of stem cells for differentiation into desired morphological and functional cell types has opened an array of horizons in regenerative therapeutics. Out of the various types of stem cells available, mesenchymal stem cells (MSCs) have been extensively studied for their regeneration potential and biological characteristics and have also been explored in various pre-clinical and clinical studies. MSCs are categorized as multipotent stem cells and can differentiate into multiple cell types independent of the nature and type of tissue origin[7, 8]. Based on their abundant availability and easy expansion in vitro, MSCs have been observed to be a promising treatment option for cardiovascular abnormalities [9]. MSCs derived from the bone marrow have exhibited their capability to differentiate into heart muscles in both ex vivo and in vivo environments [10]. As MSCs can be obtained from heterogeneous cell types, their heterogeneity poses hurdles in achieving uniform outputs regarding their differentiation and regeneration potential. In addition, the insufficient availability of stem cells and their homing at the site of injury during stem cell therapy is the main concern. Pre-differentiation strategies aimed at differentiating MSCs into pre-cardiomyocytes before in vivo transplantion, may help this scenario. Various approaches have been described to be useful in enhancing the efficiency and rate of MSC differentiation into cardiomyocytes [11].

Among various approaches used for cellular differentiation, small molecules have exhibited promising results. They have shown interesting features in regenerative medicine because of certain advantageous features associated with them including their high purity; very minute lot-to-lot differences in their quality of preparation; chemo-stability; economic features; ease in their transport across the cell membrane and effect on the targeted signaling cascade; and ease in controlling their level at the site of action to achieve desired effects. Moreover, they also provide additional benefits for their wide usage as they are well-defined chemically. Furthermore, various small molecules have been experimentally proven and pharmacologically optimized, and are already employed clinically for various pharmacotherapeutic benefits [12]. A variety of such molecules are also under trial and being investigated for their ability to stimulate the differentiation of stem cells into cardiomyocytes. They have high induction rates of differentiation of stem cells into the cardiomyogenic lineage. Along with these molecules, the drug molecules which possess the ability to induce cardioprotection to stem cells when used concurrently are also gaining importance in the treatment of heart diseases.

Among various small molecules tested in this respect, 5-azacytidine (5-aza), which is an inhibitor of the enzyme DNA methyl transferase (DNMTi), has been reported to be the most powerful inducer of stem cell differentiation into the cardiomyogenic lineage. The initial work of Makino et al. [13] had reported differentiation of more than 30% of bone marrow–derived stromal cells into cardiomyocytes when pre-treated with 5-aza [13, 14]. Furthermore, small molecules were investigated to improve cardiac performance. Among these cardioprotective mediators, statins, anti-hypertensives, and other cardiovascular agents such as small peptides, hormones, and acids are common and can non-specifically induce cardiomyogenic differentiation. Currently, the search for new ideal compounds remains an important area of research that could induce efficient differentiation of stem cells into the cardiomyogenic lineage and are less toxic in a biological system or provide efficient cardioprotection after myocardial infarction. Therefore, in the current research study, we aim to investigate a small molecule, WIKI4, and elucidate its potential to differentiate stem cells into the cardiomyogenic lineage. WIKI4 is a novel Wnt signaling modulator, which regulates the Wnt-dependent changes in the core modulators of the Wnt/ß-catenin signaling pathway. It has been reported that it prevents ubiquitylation of AXIN and inhibits the enzymatic activity of Tankyrase-2 [15]. As Wnt pathway is known to be involved in the differentiation of stem cells and heart development [16], therefore, WIKI4 can be used as a therapeutic agent to differentiate stem cells into beating cardiomyocytes, which can be transplanted into infarcted myocardium and replenish the damaged myocardium and restore normal heart function.

Materials and Methods

Ethical Statement

The animal study was performed according with the international guidelines for the care and use of laboratory animals, and the study protocol was approved by the Institutional Animal Care and Use Committee authorization no. 2018-0020.

Cell Culture

Human bone marrow–derived mesenchymal stem cells (hMSCs) were purchased from Promo Cell (Heidelberg, Germany). Cells were then cultured in DMEM and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. All cells were used at passage 4 or 5, were subconfluently cultured, and were seeded at 5000 cells/cm^2 for the experiments. hMSCs were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) having no additional growth factors and penicillin/streptomycin (100 U), sodium pyruvate (2 mM), and L-glutamine (2 mM).

Characterization of hMSCs

hMSCs were characterized by observing cell morphology using phase contrast microscopy, and the presence of hMSC markers including CD73, CD105, CD117, and vimentin by immunocytochemistry using fluorescence imaging. In addition, the tri-lineage differentiation potential of the hMSCs was also performed.

Immunocytochemistry

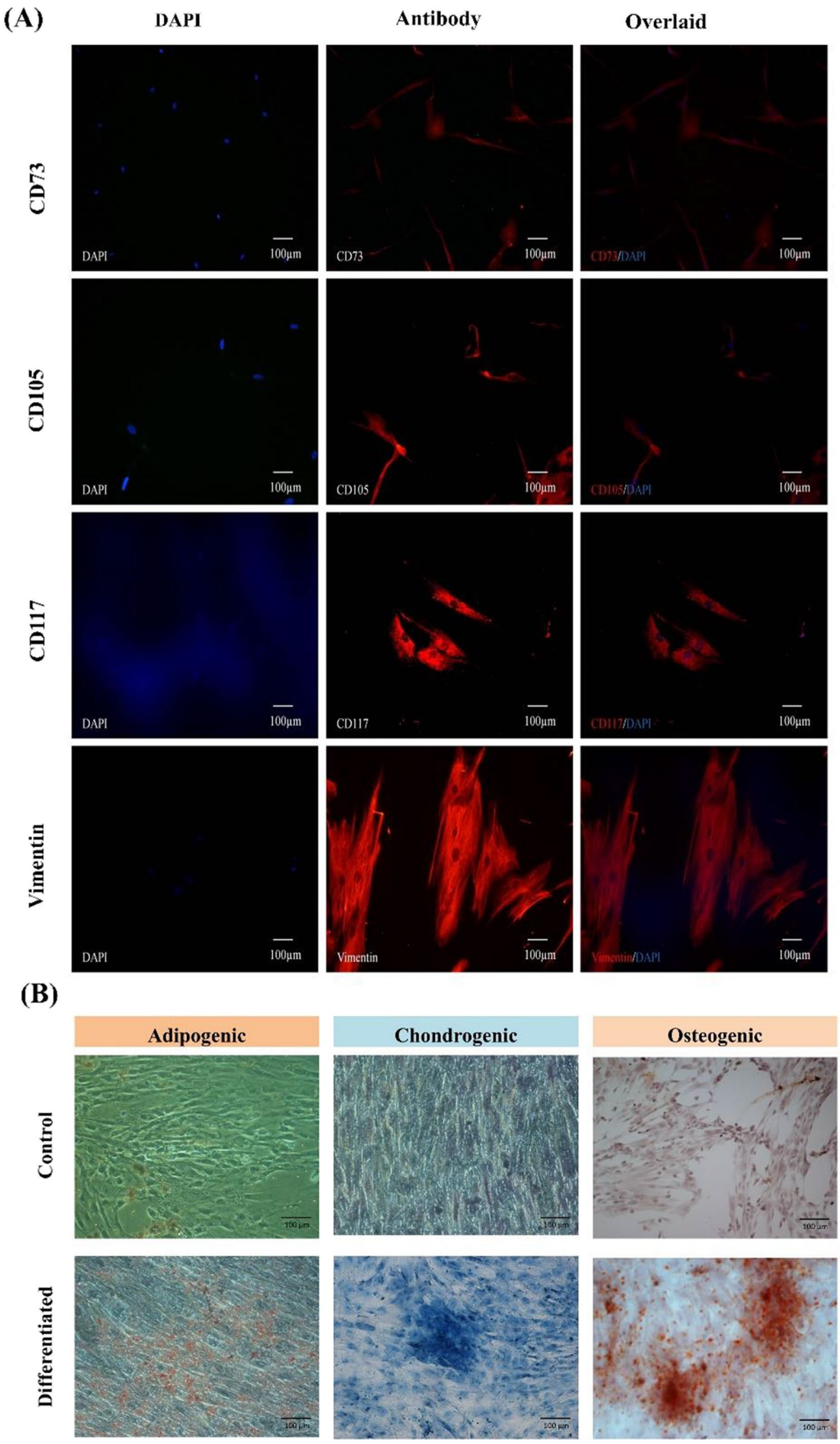
hMSCs were grown on sterile coverslips placed in a 24-well culture plate at the seeding density of 5000 cells/well. When the cells adhered to the bottom of the well, the medium was discarded, and the cells were rinsed with phosphate buffer solution (PBS) (1x) twice. Afterward, the cells were fixed with paraformaldehyde (PFA) (4%) for 10 min. After fixation, the cells were washed again with PBS (1%), and a permeabilization solution (Triton X-100, 0.1%) was added for 10 min at RT. To block the non-specific sites, cells were incubated in a blocking solution (2% BSA and 0.1% Tween 20 in PBS) for 1 h at 37 °C. The blocking solution was removed and the primary antibodies against the hMSC surface markers 5′-nucleotidase (CD73), Endoglin (CD105), c-kit (CD117), and vimentin were added as per recommended dilutions by the manufacturer and kept overnight incubation at 4 °C. The following day, the cells were rinsed with PBS (1x) 3–5 times and incubated in a secondary antibody, Alexa fluor 488 or 546 diluted (1:200) in a blocking solution. 4′,6-diamidino-2-phenylindole (DAPI, 1 µg/mL) was used to stain the nuclei. The cells were washed with PBS (1x) 3–5 times and the coverslip with cells was transfered from each well and mounted on the glass slides using a fluoromount aqueous mounting medium. The slides were observed with a fluorescence microscope and images were captured with a Nikon NiE microscope using a CCD camera.

Tri-lineage Differentiation

The cultured hMSCs were characterized for their potential of differentiation into adipogenic, osteogenic, and chondrogenic lineages. The cells were seeded in a 6-well plate and maintained till 100% confluency in the basal media, DMEM supplemented with 2mM sodium pyruvate, 2mM L-glutamine, and 10% FBS. When confluent, the cells were rinsed with PBS and allowed to grow in the respective induction media for 3 weeks followed by specific staining for each lineage. The induction media was changed every third day during the entire culture period. For osteogenic differentiation, 50 µM ascorbate phosphate, 0.1 µM dexamethasone, and 10 µM β-glycerophosphate were added to the basal media. The resulting osteocyte calcium deposits were examined using Alizarin red stain. For adipogenic differentiation, 10 µM insulin, 0.5 µM isobutyl methylxanthine, 200 µM indomethacin, and 1 µM dexamethasone were added to the basal media. After 3 weeks, the oil droplets were observed in adipocytes using an Oil Red-O stain. For chondrogenic differentiation, the basal media used was supplemented with 100 µM ascorbic acid, 20

Table 1 Primer sequence used for qPCR amplification

Gene name	Sequence	Product size
Cardiac troponin T	TTACATCCAGAAGCAGGCCC (forward) TGAAGGAGGCCAGGCTCTAT (reverse)	89bp
Cardiac troponin I	GAGCCGCACGCCAAGAA (forward) CCTTGTCCACACGGGCG (reverse)	236bp
GATA4	CTCCACAGACCAGCTCCAAG (forward) ACAGGAGAGATGCAGTGTGC (reverse)	286bp
MHC	ACCAACCTGTCCAAGTTCCG (forward) TTCAAGCCCTTCGTGCCAAT (reverse)	106bp
WNT	GTCGGGAATCTGCCTTTGTT (forward) GTTTTCCTGAAGTCGGCCAT (reverse)	376 bp
MEF2D	GGGGGCTGGAGGAGTTACC (forward) TGGGGGAACGGTGTTGTCA (reverse)	292bp
GAPDH	5'-CACCATGGGGAAGGTGAAGG -3' 5'-AGCATCGCCCCACTTGATTT -3'	274bp


(A)
DAPI
Antibody
Overlaid
CD73
CD105
CD117
Vimentin
DAPI
CD73
CD73/DAPI
CD105
CD105/DAPI
CD117
CD117/DAPI
Vimentin
Vimentin/DAPI
100μm
(B)
Adipogenic
Chondrogenic
Osteogenic
Control
Differentiated

◂**Fig. 1** Culturing and characterization of human bone marrow mesenchymal stem cell line (hMSCs): **A** characterization of hMSCs by immunostaining showing positive expression of CD73, CD105, CD117, and vimentin. **B** Tri-lineage differentiation of hMSCs into adipogenic, chondrogenic, and osteogenic lineages

ng TGFβ1, 100 nM dexamethasone, and 10 ng insulin. The differentiated cells were stained with an Alcian blue. Bright-field microscopy was used to analyze the cells and the images captured were processed with Photoshop.

Cytotoxicity Testing by Acridine Orange (AO)/ Propidium Iodide (PI) Staining

hMSCs were cultured and treated with different concentrations of WIKI4 (0, 0.1, 0.5, 1, 5, and 10 µM) for 24 h. After completion of treatment time, the cell pellet was resuspended in 0.5 mL solution of AO/PI solution (0.5–1 µg/mL) and incubated for 15 min at room temperature and analyzed by dual fluorescence for cell viability determination.

Cardiomyogenic Induction in MSCs

For inducing human stem cells to differentiate into cardiomyocytes, WIKI4 was used. The stock solution (10 mM) was prepared in distilled water. The volume of stock solution, required for the working concentrations, was added directly to DMEM. The hMSCs were cultured and treated with different concentrations of WIKI4 (0, 0.1, 0.5, 1, 5, and 10 µM) for 4 weeks, and maintained in a CO_2 incubator. The cells were treated with the compound for the entire duration (4 weeks) of incubation. However, the treatment media were replaced after every 3 days.

Gene Expression Dynamics

The cardiac-specific gene expression was examined using qRT-PCR. The expressions of GATA4, Mef2D, Wnt-2, cardiac troponin I (cTnI), cardiac troponin T (cTnT), and cardiac alpha myosin heavy chain (cMHC) were assessed in treated as well as untreated cells. Total RNA was isolated using the Trizol reagent (Invitrogen) following the manufacturer's guidelines. The total RNA isolated was reverse-transcribed to cDNA using the RevertAid™ First strand cDNA synthesis kit. Primers for gene expression analysis were designed online with primer-BLAST (www.ncbi.nlm.nih.gov/tools /prime r-blast /) and synthesized by IDT Technologies, USA (Table 1). The qPCR reaction was performed in three biological replicates of 20 µL reaction volume each, with an amplification program comprised of initial denaturation for 5 min at 96 °C, followed by 40 cycles of denaturation at 96 °C (30 s), annealing at 58–60 °C (30 s), and a final extension of 10 min at 72 °C in a 96-well optical plate. The expression of target genes was normalized according to the β-actin gene as a reference by the ΔΔCt method.

Immunofluorescence Staining

The differentiation of hMSCs into cardiomyocytes was further confirmed by the expression of cardiac markers by immunocytochemistry. The cardiac proteins GATA-4, cMHC, alpha-actinin, and stem cell stemness marker, Stro-1, were analyzed by immunofluorescence staining. Quantification of fluorescent intensity was performed with ImageJ software. Cells were selected and fluorescent intensity was calculated and plotted in MS Excel software.

Establishment of Myocardial Infarction Model

Adult Wistar male rats (250–300 g) were used for the study. The surgical procedures were in a dedicated surgical theater in animal grade biosafety cabinet. They underwent either a sham operation or left anterior descending coronary artery (LAD) ligation under anesthesia. LAD artery ligation was performed as previously described[17].

Cellular Transplantation

The animals were transplanted with normal hMSCs and WIKI4-treated hMSCs. To track the cells' migration in vivo, normal and WIKI4-treated hMSCs were labeled with a fluorescent dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Invitrogen, USA) as per manufacturer's instructions. One million cells suspended in PBS were transplanted in the left ventricular wall after LAD artery ligation.

Echocardiography Assessment

The success of the development of the MI model and potential regeneration by MSCs were evaluated by functional analysis through echocardiography as previously reported [17, 18] at the end of the 2nd and 4th week of surgery and transplantation.

Histological Evaluation of Cardiac Tissues

After the completion of 4 weeks, rats were euthanized, and their hearts were harvested using perfusion assembly. The chest cavity was dissected and a cardioplegic solution

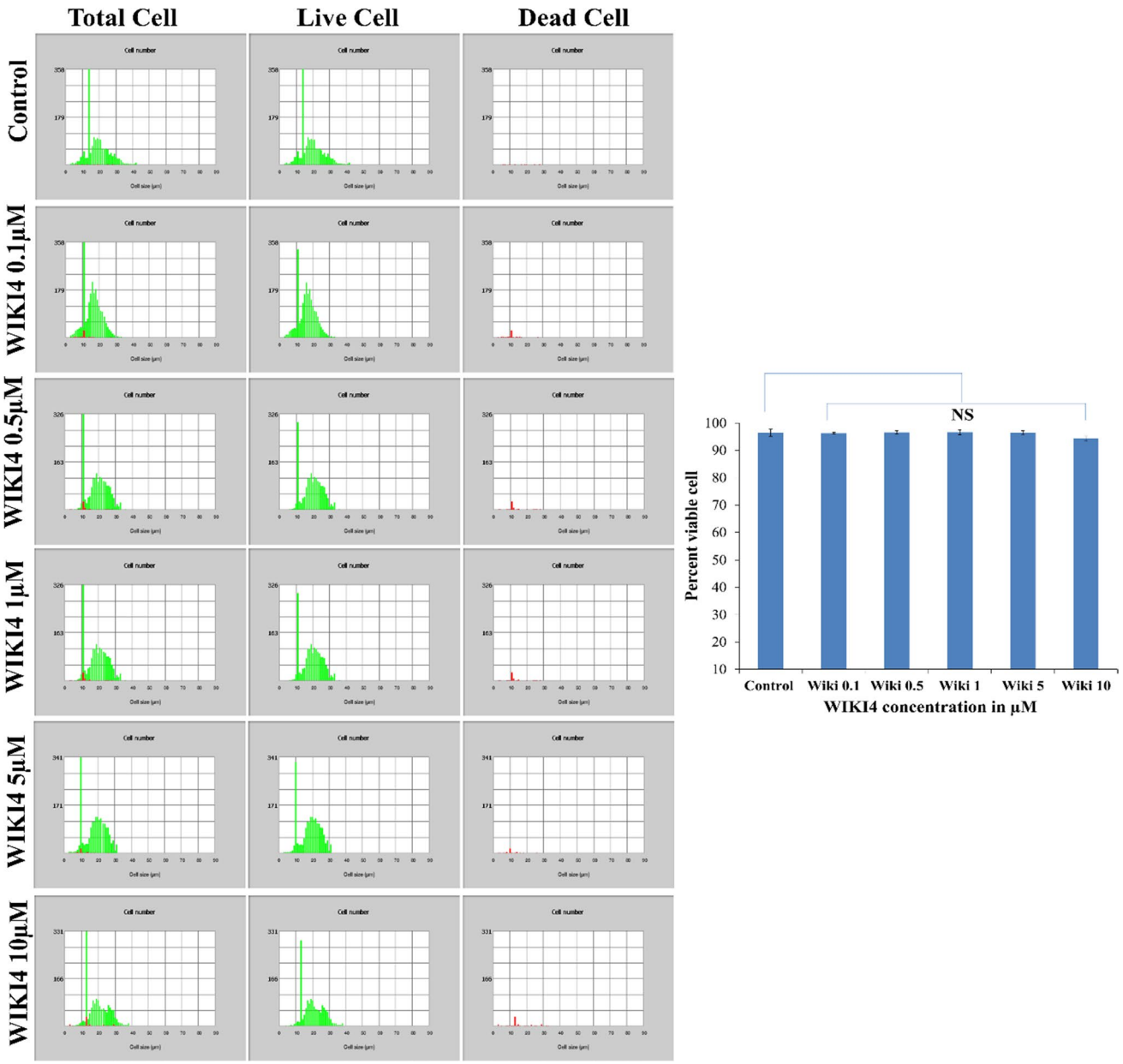


Fig. 2 Cytotoxic testing of hBM-MSCs treated with WIKI4 using Acridine orange/Propidium iodide (AO/PI) staining: the number of live and dead cells were counted at different concentrations (0, 0.1, 0.5, 1, 5, and 10 µM) of WIKI4 using a dual fluorescence cell counter. hMSCs treated with different concentrations of WIKI4 showed non-significant number of dead cells. Quantification of live hMSCs using AO/PI staining assay showed that WIKI4 did not present cytotoxicity at the tested concentrations. Values are presented as mean ± SD, $n = 5$

was infused in the heart to fully open the cardiac chambers. The right atrium was cut with a scissor to allow the flow of blood. PBS solution was flow through the heart, till blood was drained, then the fixative solution (4% PFA) was passed through the heart. The heart was removed, cut, and placed in the PFA solution for postperfusion fixation overnight at RT. The next day, the tissue was dehydrated with graded alcohol solutions and embedded in paraffin for slide preparation. The paraffin cassettes were dried and 5-µm-thick sections were sliced using a microtome. The tissue slices were allowed to spread at 42 °C in a water bath and picked on the gelatin-coated slides. The slides were dried at 42 °C on a hot plate for 8 h. The fibrosis in the harvested heart tissue was assessed by Masson's trichrome

(Carl Roth, Germany) staining following the manufacturer's instructions. Histological changes were observed using bright-field microscopy and images were captured at different magnifications. In all the groups, the MI infarct size and cardiac remodeling including chamber dilatation, fibrotic area, and ventricular wall thickness were examined and quantified by ImageJ software. The homing potential of transplanted labeled MSCs was evaluated on heart sections by processing through cryomicrotomy. Briefly, the heart sections were molded in optimal cutting medium (OCT) and frozen at −20 °C overnight. The sections (5 µm) were sliced with cryomicrotome and placed on polylysine-coated glass slides. Images were taken using a CCD camera using NIS-Elements software. The presence of cardiac markers, myosin heavy chain (cMHC), and cTnT was analyzed in cryosectioned slides of heart tissues by immunofluorescence staining. Images were taken with a fluorescent microscope.

Statistical Analysis

Data is expressed as mean values ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey's test for multiple comparisons, using GraphPad Prism version 6.07 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Differences were considered statistically significant for *P* value of at least $p<0.05$.

Results

Characterization of hMSCs

The hMSC cell line showed good proliferation and adherence ability. The cells were elongated with fibroblast-like morphology in a culture flask. The cultured hMSCs were evaluated for the presence of MSC-specific cell surface markers using immunocytochemistry. The cells were positive for MSC markers; CD73, CD105, CD117, and vimentin as shown in Fig. 1. They were also observed for tri-lineage differentiation potential by culturing in respective induction media. The results showed effective differentiation into adipogenic, chondrogenic, and osteogenic lineages after 3 weeks of culturing. Adipogenic differentiation was confirmed by visualizing lipid droplets produced within the cells using the Oil Red-O stain. The chondrogenic differentiation was detected by the synthesis of proteoglycans and glycosaminoglycans (GAG) using an Alcian blue stain. Whereas osteogenic differentiation was assured by staining mineral deposits in cells using Alizarin red stain as shown in Fig. 1.

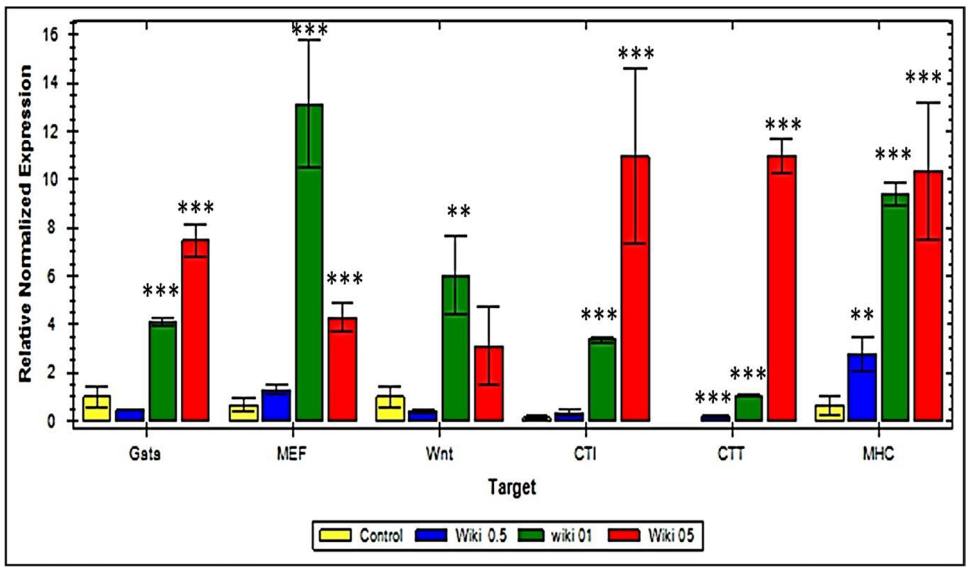


Fig. 3 Gene expression analysis in WIKI4-treated hBM-MSCs: gene expression was analyzed by real-time PCR after 4 weeks of treatment of hMSCS with WIKI4, in comparison to untreated control cells. There was significant upregulation of cardiac markers, including GATA-4, Mef, and Wnt, cardiac troponin I (cTnI), cardiac troponin T (cTnT), and myosin heavy chain (MHC). Quantitative 2-fold ($2^{\wedge-\Delta\Delta CT}$) difference of means is represented by ΔΔCt method and was subjected to ANOVA, Bonferroni's post hoc analysis. Values are shown as mean ± SD from three independent biological triplicates. The asterisk sign represents only significant ($p < 0.05$*, $p < 0.01$**, and $p < 0.001$***) fold change compared to the respective controls. The experiments were performed in triplicate

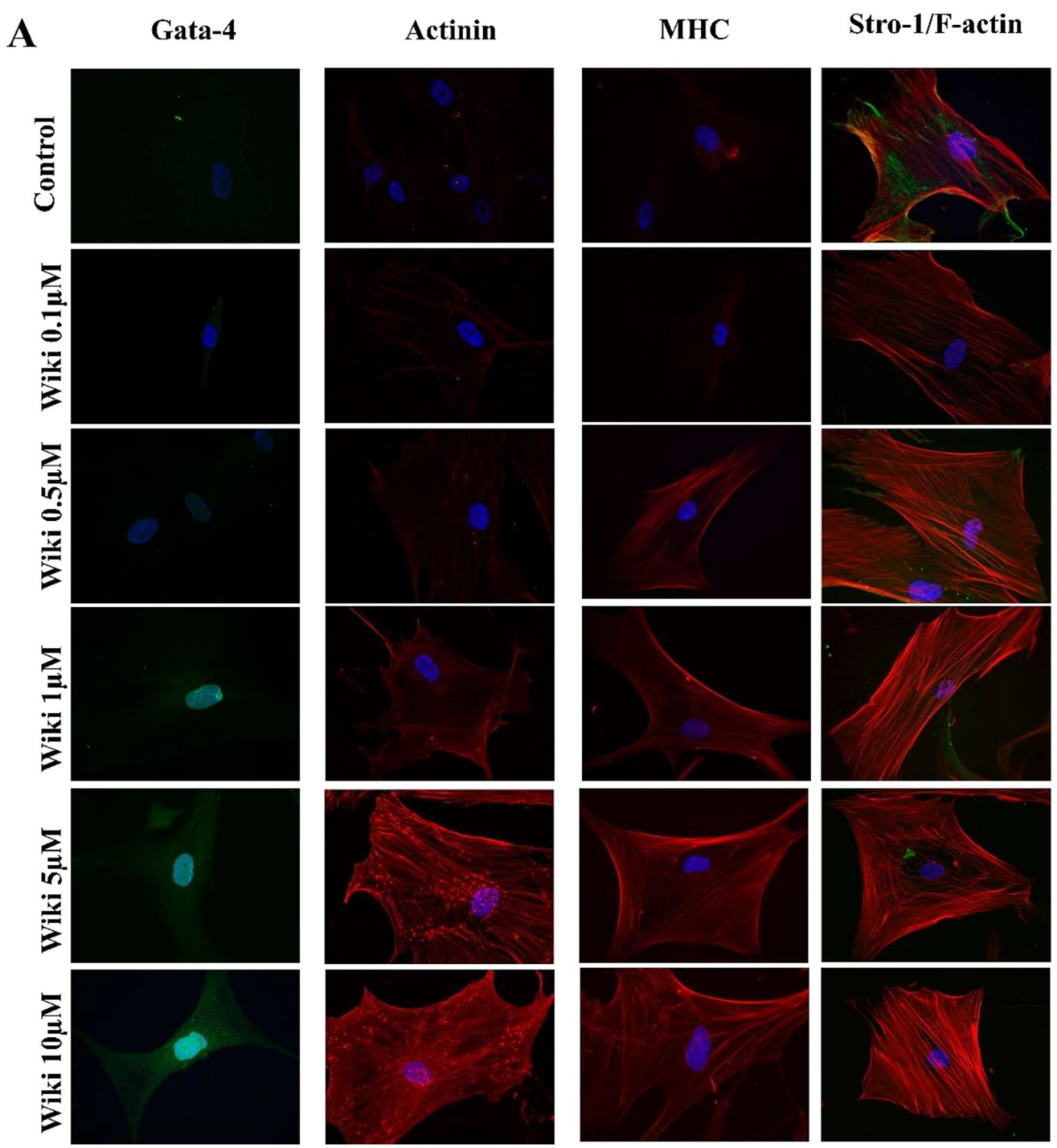


Fig. 4 Immunocytochemical analysis of WIKI4-treated hMSCs: **A** Expression of cardiomyocyte-specific proteins Gata-4, Actinin, and Myosin heavy chain after 4 weeks of treatment with different concentrations of WIKI4, and Stro-1, stemness marker for MSCs. **B** Quantification of fluorescent intensities of control and treated group. Alexa fluor 546/488 anti-mouse secondary antibody was used for detection. F-actin is stained with phalloidin in Stro-1 expression. Nuclei were stained with DAPI. Fluorescent intensity was quantified with ImageJ. Values are shown as mean ± SD (n=10). The asterisk sign represents significant ($p < 0.001$***) compared to the respective controls

Cytotoxicity of WIKI4

Cell viability assay with acridine orange and propidium iodide staining showed that there was no significant difference in the number of dead cells between control and WIKI4-treated MSCs at concentrations of 0.1, 0.5, 1, and 5 mM; however, the number of dead cells increased non-significantly at 10 mM concentrations as shown in Fig. 2.

Expression of Cardiac-Specific Genes in Treated MSCs

qPCR analysis for the expression of cardiac-specific genes showed that after 4 weeks of treatment with WIKI4, the induced cardiac progenitor cells expressed *GATA4, MEF, cardiac troponin T, cardiac troponin I, myosin heavy chain,* and *wnt* as shown in Fig. 3.

Expression of Cardiac Proteins in Treated MSCs

After separate treatments with WIKI4 at concentrations 0.1, 0.5, 1, 5, and 10 µM for 4 weeks, MSCs continued to proliferate and differentiate. After 4 weeks of cell culture, the cells showed the expression of cardiac proteins Gata-4, cardiac alpha actinin, and myosin heavy chain was significantly upregulated ($p< 0.001$) while stem cell pluripotency marker Stro-1 was downregulated ($p< 0.001$) as shown in Fig. 4 at 5 and 10 µM concentration; however, the lower concentrations 0.1, 0.5, and 1 µM did not show any significant change.

In Vivo Studies

The successful development of MI models and the effect of transplantation of hMSCs in the infarcted heart were assessed with echocardiographic measurements after 2

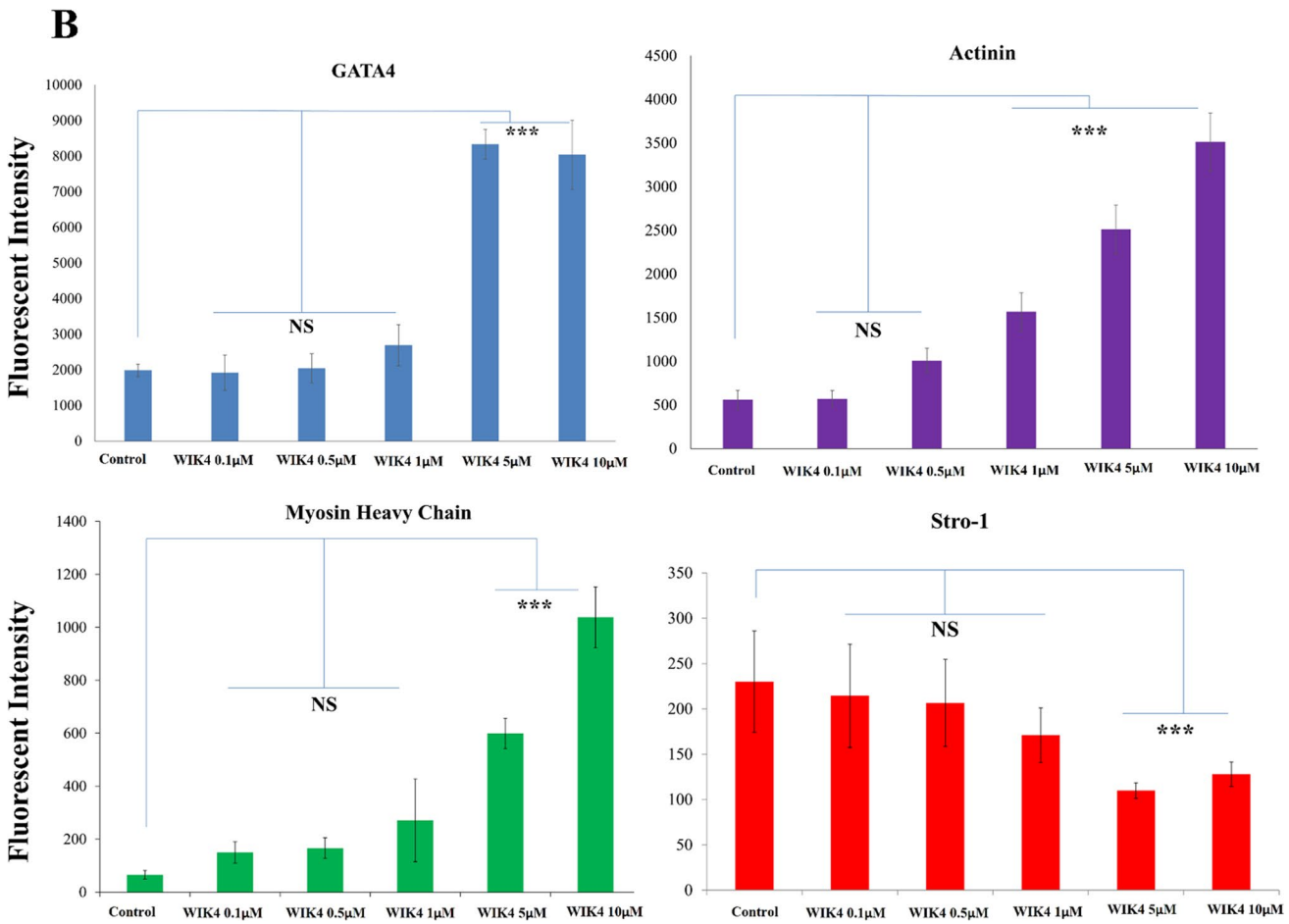


Fig. 4 (continued)

weeks and 4 weeks of development of the model and cellular transplantation. While the histological assessment was performed after 4 weeks of transplantation of hMSCs, and WIKI4-treated MSCs.

Cardiac Function Measurements by Echocardiography

M-mode echocardiographic measurements in the parasternal long axis were analyzed after two and 4 weeks of MI induction and transplantation of hMSCs and WIKI4-treated MSCs. Cardiac functional parameters were quantified and plotted and showed substantial improvement ($p<0.001$) in cardiac efficiency. There was a significant ($p<0.001$) decrease in cardiac performance in the MI model as compared to control animals. Although in the WIKI4-treated MSC group there was improved cardiac function observed, as evidenced by the decreased left ventricle dilation and improved contractility after transplantation of pre-differentiated MSCs. The treated hMSCs showed a significant ($p<0.001$) increase in ejection fraction and fraction shortening ($p<0.05$) as shown in Fig. 5.

Histological Analysis of Harvested Hearts

For histological studies, the heart tissue was cut in cross section to observe the anterior left ventricular wall. The fibrotic areas were stained by differential staining using

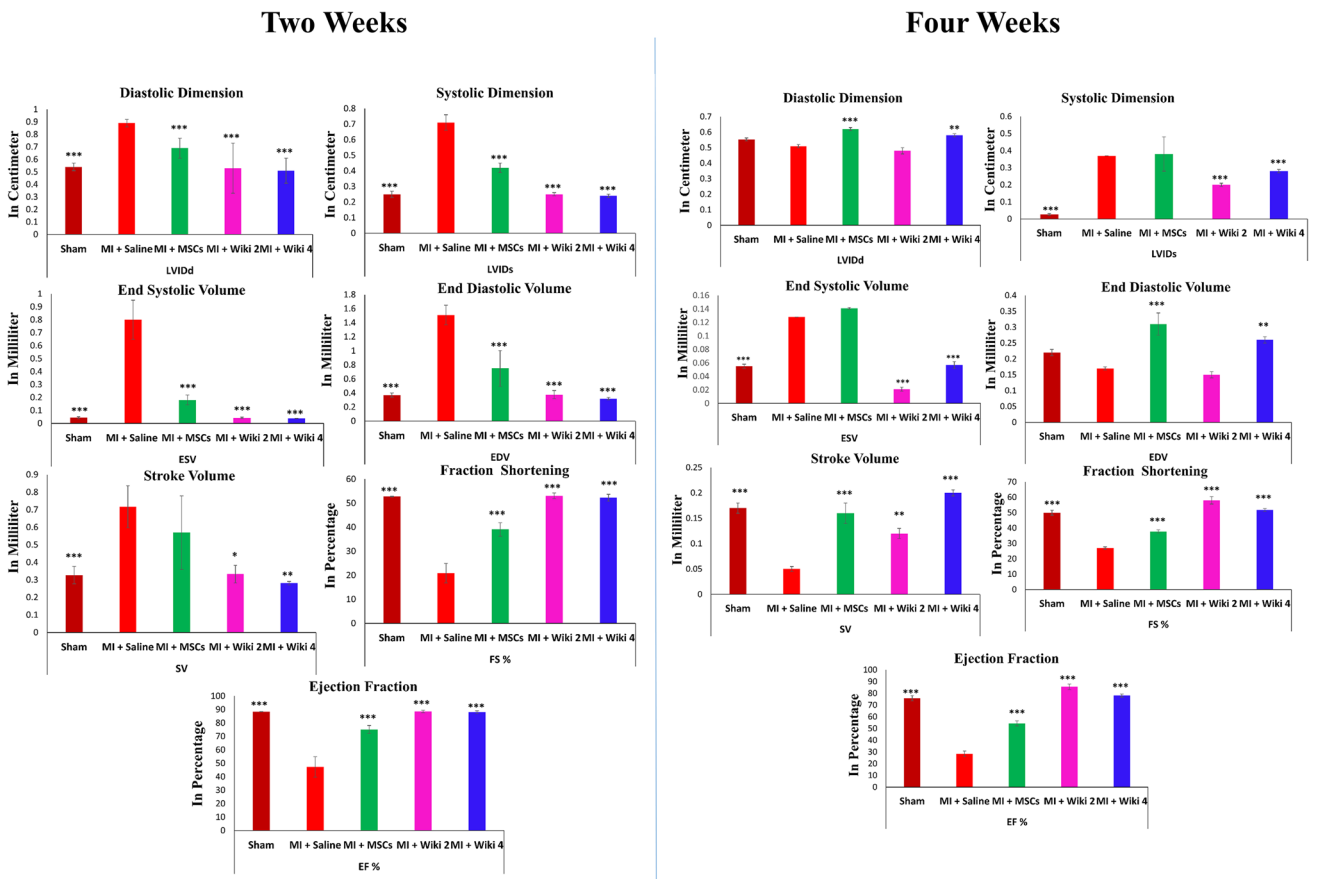


Fig. 5 Cardiac functional assessment by echocardiography: M-mode echocardiographic images in the parasternal long axis were analyzed after two and 4 weeks of MI induction and transplantation of cells. Cardiac functional parameters were quantified and plotted, and showed significant improvement (***$P<0.001$) in cardiac efficiency. There was a significant (***$p<0.001$) decrease in cardiac performance in MI model as compared to control animals. Improved cardiac function was observed as evidenced by the decreased left ventricles dilation and improved contractility after transplantation of pre-differentiated MSCs. The treated hMSCs showed a significant (***$p<0.001$) increase in ejection fraction and fraction shortening (*$p<0.05$). Values are presented as mean ± SD ($n = 5$). For statistical analysis, one-way ANOVA followed by Bonferroni's post hoc analysis was used; the level of significance is $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$)

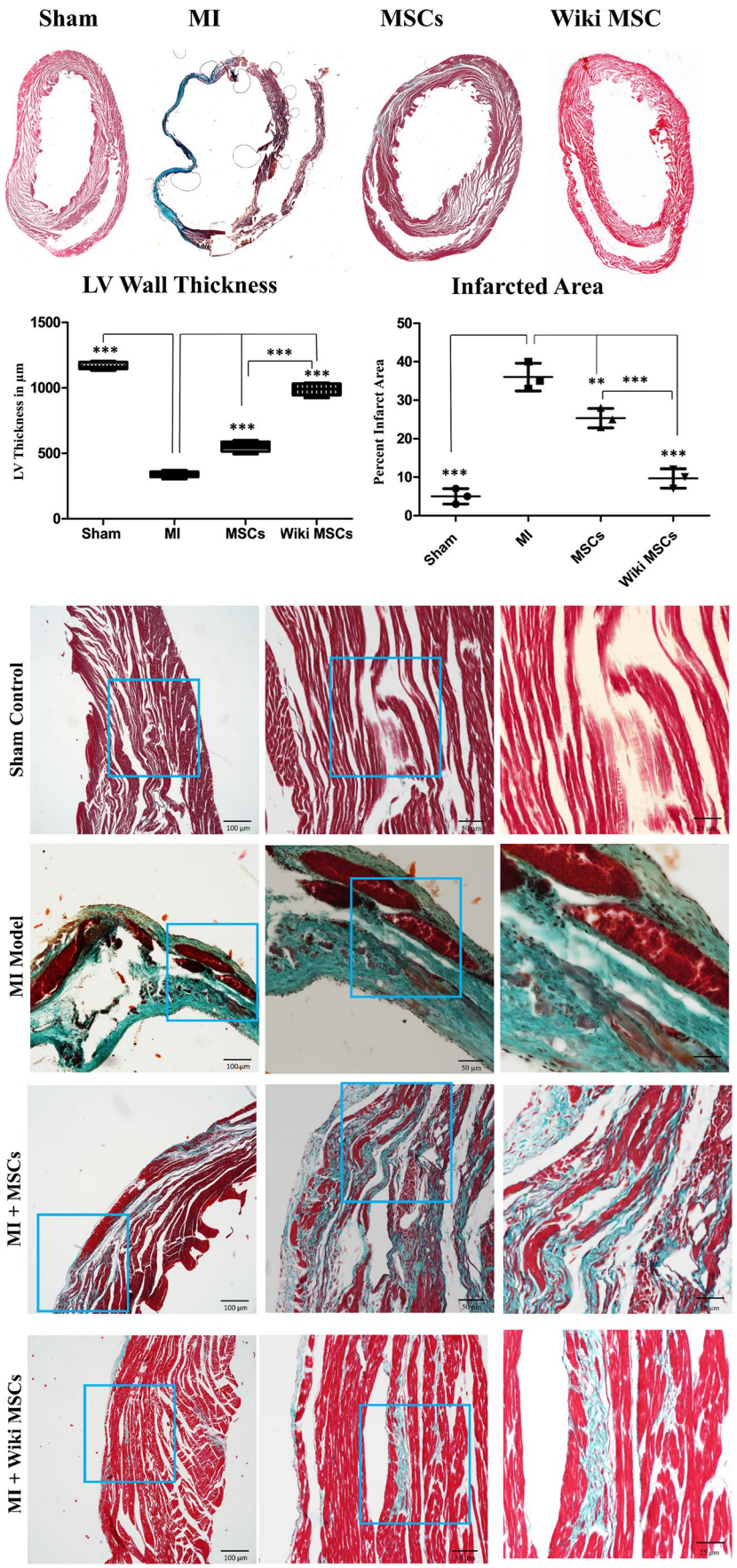
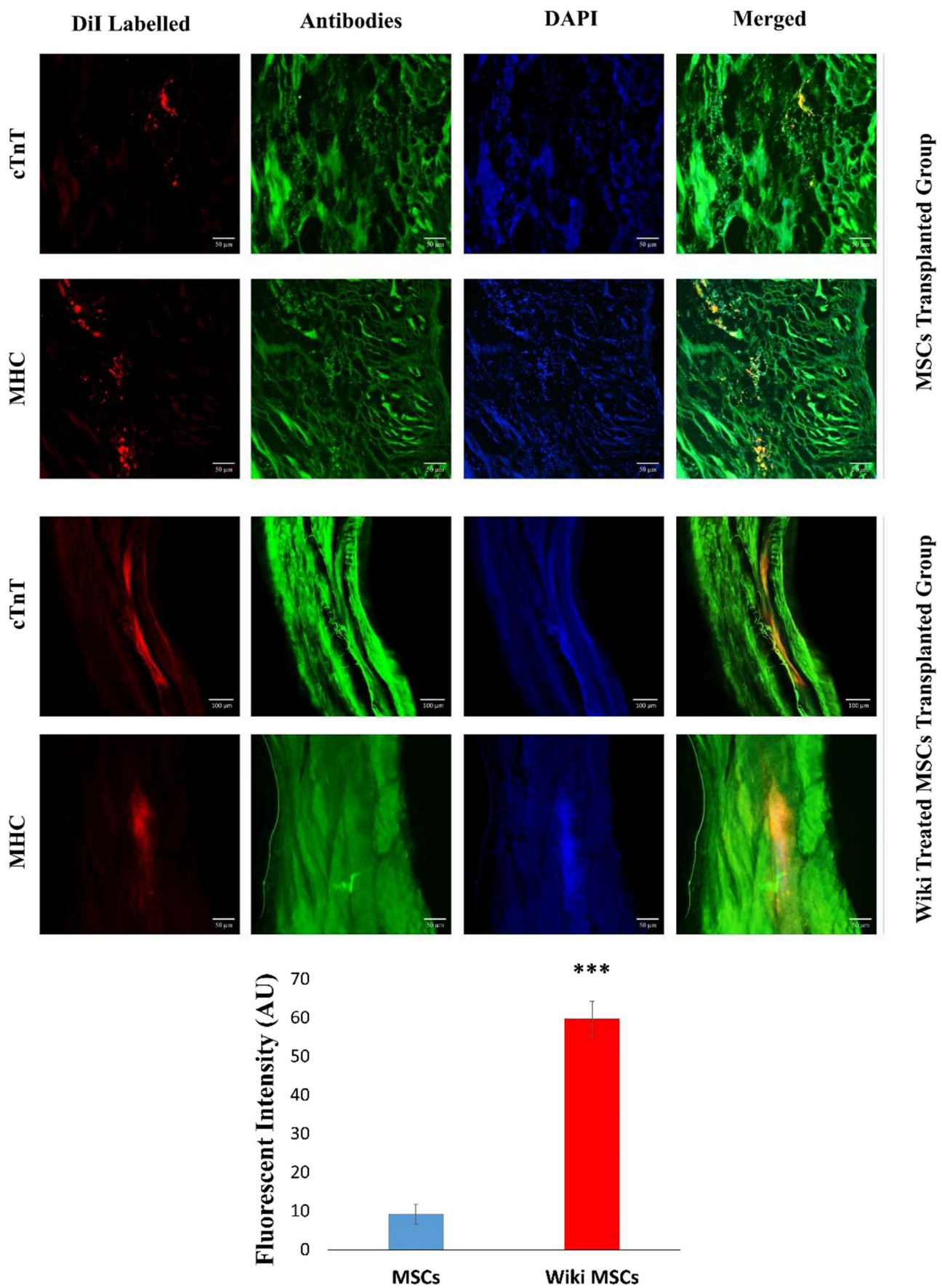


Fig. 6 Histological analysis of the infarcted myocardium and effect of cellular transplantation: heart sections of sham, MI group, and the transplanted groups, normal hMSCs, and WIKI4-treated hMSCs were visualized with Masson's trichrome staining. Fibrosis can be seen in the left ventricular myocardium in MI model group as compared to the sham group. There was a significant reduction in fibrosis in WIKI4-treated hMSCs sections with the patches of myocytes present in the infarcted and regenerating myocardium. The left ventricular wall thickness was significantly improved in the treated group as compared to MI model. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. Data are expressed as means ± SD from three independent biological triplicates; the level of significance is $P < 0.05$ ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$)


DiI Labelled
Antibodies
DAPI
Merged
cTnT
MHC
MSCs Transplanted Group
cTnT
MHC
Wiki Treated MSCs Transplanted Group
***
Fluorescent Intensity (AU)
70
60
50
40
30
20
10
0
MSCs
Wiki MSCs

◂**Fig. 7** Immunohistochemistry of the cardiac tissues: untreated and WIKI4-treated hMSCs were labeled with red fluorescent dye DiI and were transplanted in the infarcted heart. The WIKI4-treated hMSCs showed better homing in the infarcted myocardium as compared to the normal hMSC group. The expression of cardiac proteins was evaluated using human primary antibodies against cardiac myosin heavy chain (cMHC) and cardiac troponin T (cTnT). Alexa fluor 488 secondary antibodies were used for the detection of primary antibodies. The DiI-labeled cells were quantified and the fluorescence intensities were presented in bar graphs. Fluorescence intensity was significantly high (**$P < 0.001$) in WIKI4-treated MSC group as compared to normal MSCs in the infarcted myocardium

Masson's trichrome. The intact myocardium appeared red when stained with Masson's trichrome. There was significant fibrosis tracked in the MI+ve sections as compared to the sham group. The total fibrotic area was quantified, and it was evident that the group transplanted with WIKI4-treated hMSCs showed significant reduction ($p<0.001$) in the fibrotic area in comparison to the MI+ve group and untreated hMSCs group. The myocardium wall thickness was also measured and found to be better preserved in the treated hMSC transplanted group as compared to the untreated hMSC group as shown in Fig. 6. The tracking of hMSCs labeled with florescent dye (DiI) transplanted in the LV myocardial wall showed that the treated hMSCs were sequestered in the infarcted region due to the intrinsic capability of MSCs of homing in the injured area. The expression of cardiac proteins in the DiI-labeled cells was evaluated using human primary antibodies against cardiac myosin heavy chain (cMHC) and cardiac troponin T (cTnT), in the pre-treated MSCs group which confirms the myogenic differentiation of the transplanted hMSCs besides its homing in the myocardium shown in Fig. 7.

Discussion

MSCs possess immunomodulatory properties that allow allogeneic transplantation in various clinical settings [19, 20]. For use in regenerative medicine, the fundamental requisites for MSCs are their survival after transplantation, differentiation to the required cell type, and the avoidance of the formation of teratomas by the undifferentiated stem cells [21]. To overcome the undesirable effects of MSC transplantation, earlier studies evaluated the effect of various small molecules like ascorbic acid, beta-mercaptoethanol, demethylating agents (5-azacytidine, zebularine), dexamethasone, and retinoic acid on differentiation of stem cells into neuron-like, osteogenic, adipogenic, muscle-like, and cardiac-like cells [22].

Small molecules and growth factors were used to produce ventricular-like cardiomyocytes from hESC with high competence and reproducibility utilizing computational and system-biology approaches. They modulated the canonical Wnt pathway using IWR-1 which was found to have a role in cardiomyocyte subtype characterization [23] The small molecules that modulate the Wnt and BMP-4 signaling pathways in human pluripotent stem cells (hPSC) have led to efficient differentiation of stem cells to cardiomyocytes with improved electrophysiological functioning [24]. Using small molecules which modulated Wnt/b-Catenin signaling was found to enhance cardiac differentiation and more than 50–60% of cells were cardiac troponin positive [25].

In the current study, the hMSCs were cultured, expanded, and characterized according to the minimum criteria suggested by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy. They were found to be fulfilling these criteria such as hMSCs adhered to the plastic in optimum culture conditions; they expressed CD73, CD105, CD117, and vimentin. It proved that the cultured cells were MSCs. They also differentiated in vitro to adipocytes, chondroblasts, and osteoblasts which further confirmed that the isolated cells were MSCs. This can be supported by the criteria previously established [26]. The concentration of WIKI4 for the treatment of hMSCs was optimized using acridine orange/propidium iodide staining, as described earlier [27]. The lower concentrations (0.1, 0.5, 1, and 5 mM) of WIKI4 showed that there was no significant difference in the number of dead cells between control and WIKI4-treated hMSCs. However, the number of dead cells increased non-significantly at 10 mM concentrations. Pre-conditioning of hMSCs with different concentrations of WIKI4 for 14 days in culture media leads to morphological changes in hMSCs; the cells became large and flattened with the formation of myotube-like inter-cellular structures. To confirm the differentiation of MSCs to cardiac-like cells, we analyzed treated MSCs for the expression of cardiac-specific genes; these are in agreement with previous studies [21, 28, 29]. The gene analysis showed that the pre-differentiated cells express various cardiac genes including, Mef2D, GATA-4, cardiac troponin T (cTnT), cardiac troponin I (cTnI), cardiac myosin heavy chain (cMHC), and Wnt-2. It is shown that MEF2D is one of the primary MEF2 expressed in the adult heart, mice lacking Mef2d possesses an impaired response to stress signals that normally lead to cardiac hypertrophy, fibrosis, and fetal gene activation [30]. Gata4 is an important transcription factor for cardiovascular development; targeted deletion of Gata4 in mice results in early embryonic lethality and cardiac bifida [31]. Likewise, cTnI and cTnT are localized in cardiac striated muscle. Their structure is important for the function of the cardiac muscles and regulates

the contraction and relaxation of myocardial tissue. Mutations in genes encoding troponin proteins led to contractile dysfunctions and cardiomyopathies [31]. Moreover, cMHC is considered the molecular motor of the muscle and regulates contractile function [32]. Furthermore, Wnt family proteins are essential in a wide array of developmental and physiological processes [33]. The positive expression of all these cardiac markers confirmed that WIKI4 was able to differentiate hMSCs into cardiac lineage. Several studies earlier have reported that molecules such as 5-aza, zebularine, salvianolic acid B, and cardiomyocytes lysis medium induced the expression of cardiac proteins including GATA-4, cTnT, alpha-actinin, and cMHC in MSCs [22, 28].

For evaluating the effect of pre-differentiated hMSCs on cardiac regeneration in MI models, one million cells were implanted to the left ventricle immediately following ligation. It is known that the transplanted cells have challenges of survival and homing in the harsh hypoxic microenvironment of the MI [34]. MSCs were labeled with DiI (flourescent dye) to track their localization in the myocardium for tracking their homing and integration into the injured myocardium [18, 21]. Histological analysis revealed that transplanted cells successfully localized and integrated into the myocardium at the infarcted site in both the untreated hMSCs and WIKI4-treated hMSCs. The DiI-labeled section was also evaluated for the expression of cardiac proteins cMHC and cTnT, and was found positive for the expression of cardiac markers, indicating the engraftment, survival, homing, and cardiomyogenic differentiation of transplanted hMSCs. These results are in agreement with earlier studies which reported that small molecule–based differentiated stem cells are in therapeutic applications clinically [21, 29].

Heart function was evaluated by echocardiography by measuring cardiac parameters like percent ejection fraction (EF%), percent fraction shortening (FS%), and left ventricle (LV) dilatation. LV dilatation is an effective indicator for assessing left ventricular remodeling [35]. Similarly, non-invasive techniques like left ventricular end-systolic volume (LVESV) and left ventricular end-diastolic volume (LVEDV) was used to detect cardiac function [36]. Reduced EF% and FS% can signify a higher risk of heart failure [37]. All the transplanted MSCs group showed a significant increase in EF% as compared to MI +ve group was observed. Whereas a non-significant increase in FS% by the normal hMSCs while significant in WIKI4 groups as compared to MI +ve group. In addition, the LV dilatation was also decreased in the treated hMSCs group indicating the regeneration of the injured myocardium.

The xenogeneic transplantation of human MSCs into the rat MI model seems to present a significant hurdle from the host immune response. Previous investigations have shown that MSCs remain non-immunogenic in cases of allogenic transplants. MHC-I molecules are expressed on surfaces of MSCs, but not MHC-II molecules.

The ability of xenogenic MSCs to be transplanted crossways is made possible by their distinct lack of MHC-II molecules. MSCs have been linked to the release of cytokines and growth factors that modulate the host immune response [38], making them less immunogenic in pre-clinical and clinical settings [39]. They are thought to be safe for use in allogeneic and xenogeneic therapeutic applications [40–42], since they are not implicated in the immunoinflammatory response [43–45]. These cells are said to survive for 12 weeks without immunosuppressive medications while showing no signs of immunoreactivity. According to other reports, these cells survived xeno-transplantation for 4 months without displaying any signs of immunological rejection [46–48]. Additionally, it has been reported that human MSCs have been implanted in xenogeneic models for regeneration.

Conclusion

Treatment of human bone marrow–derived mesenchymal stem cells with WIKI4 induced differentiation of these MSCs into the cardiomyogenic lineage. WIKI4 treatment did not show a cytotoxic effect and was able to differentiate MSCs into cardiomyogenic lineage evident from gene and protein expression. These xenogeneic MSCs and their derivatives in form of induced cardiac progenitors when implanted into the infarcted rat's heart showed survival, distribution, integration and differentiation into the myogenic lineage. Transplanted cells maintained the thickness of the left ventricular wall, infarct area was reduced, and hearts performed better function.

Acknowledgements We acknowledge animal resource and the imaging facility of Dr. Panjwani Center for Molecular Medicine and Drug Research.

Author Contribution S. R. A. performed experiments and wrote the original manuscript. W. A. helped in experimentation and writing. A. S. evaluated and analyzed the data and reviewed the manuscript. M. C. D. evaluated and analyzed the data. I. K. conceived and designed the studies, evaluated and analyzed the data, and finalized the manuscript.

Data Availability I declare I have no other data to share. All the information's included in the manuscript.

Declarations

Ethics Approval This study was approved by the Institutional Animal Care and Use Committee authorization no. 2018-0020.

Statement of Human and Animal Rights All of the experimental procedures involving animals were conducted following the Institutional Animal Care guidelines of Dr. Panjwani Center of Molecular Medicine and Drug Research, University of Karachi, Pakistan.

Statement of Informed Consent There are no human subjects is involved and informed consent is not applicable.

Competing Interests The authors declare no competing interests.

References

1. Mozaffarian D, Benjamin EJ, Go AS, et al. Heart disease and stroke statistics—2016 Update: a report from the American Heart Association. Circ. 2016;133:e38–60. https://doi.org/10.1161/cir.0000000000000350.
2. St John Sutton MG, Sharpe N, Sutton MGSJ, Sharpe N. Clinical cardiology: new frontiers left ventricular remodeling after myocardial infarction pathophysiology and therapy. Circ. 2009;101:2981–8.
3. Bhatnagar A, Rush Z. Cardiovascular regenerative medicine: the developing heart meets adult heart repair. Circ Res. 2009;105:1041–3.
4. Hansson EM, Lindsay ME, Chien KR. Regeneration next: toward heart stem cell therapeutics. Cell Stem Cell. 2009;5:364–77.
5. Uemura R, Xu M, Ahmad N, Ashraf M. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. Circ Res. 2006;98:1414–21. https://doi.org/10.1161/01.RES.0000225952.61196.39.
6. Williams AR, Hare JM. Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. Circ Res. 2011;109:923–40.
7. Kumar BM, Maeng GH, Lee YM, et al. Neurogenic and cardiomyogenic differentiation of mesenchymal stem cells isolated from minipig bone marrow. Res Vet Sci. 2012;93:749–57. https://doi.org/10.1016/j.rvsc.2011.09.012.
8. Laflamme MA, Murry CE. Regenerating the heart. Nat Biotechnol. 2005;23:845–56. https://doi.org/10.1038/nbt1117.
9. Singh A, Singh A, Sen D. Mesenchymal stem cells in cardiac regeneration: a detailed progress report of the last 6 years (2010-2015). Stem Cell Res Ther. 2016;7:1–25.
10. Li XH, Yu XY, Lin QX, et al. Bone marrow mesenchymal stem cells differentiate into functional cardiac phenotypes by cardiac microenvironment. J Mol Cell Cardiol. 2007;42:295–303. https://doi.org/10.1016/j.yjmcc.2006.07.002.
11. Naeem N, Haneef K, Kabir N, et al. DNA methylation inhibitors, 5-azacytidine and zebularine potentiate the transdifferentiation of rat bone marrow mesenchymal stem cells into cardiomyocytes. Cardiovasc Ther. 2013;31:201–9. https://doi.org/10.1111/j.1755-5922.2012.00320.x.
12. Makino S, Fukuda K, Miyoshi S, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest. 1999;103:697–705. https://doi.org/10.1172/JCI5298.
13. Antonitsis P, Ioannidou-Papagiannaki E, Kaidoglou A, Papakonstantinou C. In vitro cardiomyogenic differentiation of adult human bone marrow mesenchymal stem cells. The role of 5-azacytidine. Interact Cardiovasc Thorac Surg. 2007;6:593–7.
14. Ye NS, Chen J, Luo GA, et al. Proteomic profiling of rat bone marrow mesenchymal stem cells induced by 5-azacytidine. Stem Cells Dev. 2006;15:665–76. https://doi.org/10.1089/scd.2006.15.665.
15. Yung T, Poon F, Liang M, et al. Sufu- and Spop-mediated downregulation of Hedgehog signaling promotes beta cell differentiation through organ-specific niche signals. Nat Commun. 2019;10:4647. https://doi.org/10.1038/s41467-019-12624-5.
16. Bhavanasi D, Klein PS. Wnt signaling in normal and malignant stem cells. Curr Stem Cell Rep. 2016;2:379–87.
17. Haneef K, Ali A, Khan I, et al. Role of interleukin-7 in fusion of rat bone marrow mesenchymal stem cells with cardiomyocytes in vitro and improvement of cardiac function in vivo. Cardiovasc Ther. 2018;36:1–11. https://doi.org/10.1111/1755-5922.12479.
18. Khan I, Ali A, Akhter MA, et al. Epac-Rap1-activated mesenchymal stem cells improve cardiac function in rat model of myocardial infarction. Cardiovasc Ther. 2017;35:e12248. https://doi.org/10.1111/1755-5922.12248.
19. Devine SM, Bartholomew AM, Mahmud N, et al. Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. Exp Hematol. 2001;29:244–55. https://doi.org/10.1016/S0301-472X(00)00635-4.
20. Devine SM, Cobbs C, Jennings M, et al. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood. 2003;101:2999–3001. https://doi.org/10.1182/blood-2002-06-1830.
21. Ali SR, Ahmad W, Naeem N, et al. Small molecule 2'-deoxycytidine differentiates human umbilical cord-derived MSCs into cardiac progenitors in vitro and their in vivo xeno-transplantation improves cardiac function. Mol Cell Biochem. 2020;470:99–113. https://doi.org/10.1007/s11010-020-03750-6.
22. Khanabdali R, Saadat A, Fazilah M, et al. Promoting effect of small molecules in cardiomyogenic and neurogenic differentiation of rat bone marrow-derived mesenchymal stem cells. Drug Des Devel Ther. 2016;10:81–91. https://doi.org/10.2147/DDDT.S89658.
23. Karakikes I, Senyei GD, Hansen J, et al. Small molecule-mediated directed differentiation of human embryonic stem cells toward ventricular cardiomyocytes. Stem Cells Transl Med. 2014;3:18–31. https://doi.org/10.5966/sctm.2013-0110.
24. Ren Y, Lee MY, Schliffke S, et al. Small molecule Wnt inhibitors enhance the efficiency of BMP-4-directed cardiac differentiation of human pluripotent stem cells. J Mol Cell Cardiol. 2011;51:280–7. https://doi.org/10.1016/j.yjmcc.2011.04.012.
25. Sharma A, Li G, Rajarajan K, et al. Derivation of highly purified cardiomyocytes from human induced pluripotent stem cells using small molecule-modulated differentiation and subsequent glucose starvation. J Vis Exp. 2015;18:e52628. https://doi.org/10.3791/52628-v.
26. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8:315–7. https://doi.org/10.1080/14653240600855905.
27. Mascotti K, McCullough J, Burger SR. HPC viability measurement: trypan blue versus acridine orange and propidium iodide. Transfus. 2000;40:693–6. https://doi.org/10.1046/j.1537-2995.2000.40060693.x.
28. Gao Q, Guo M, Jiang X, et al. A cocktail method for promoting cardiomyocyte differentiation from bone marrow-derived mesenchymal stem cells. Stem Cells Int. 2014;2014:1–11. https://doi.org/10.1155/2014/162024.
29. Schade D, Plowright AT. Medicinal chemistry approaches to heart regeneration. J Med Chem. 2015;58:9451–79. https://doi.org/10.1021/acs.jmedchem.5b00446.
30. Kim Y, Phan D, Van Rooij E, et al. The MEF2D transcription factor mediates stress-dependent cardiac remodeling in mice. J Clin Invest. 2008;118:124–32. https://doi.org/10.1172/JCI33255.
31. Chaulin A. Cardiac troponins: contemporary biological data and new methods of determination. Vasc Health Risk Manag. 2021;17:299–316. https://doi.org/10.2147/VHRM.S300002.
32. Qazi R, Naeem N, Khan I, et al. Effect of a dianthin G analogue in the differentiation of rat bone marrow mesenchymal stem cells into cardiomyocytes. Mol Cell Biochem. 2020;475:27–39. https://doi.org/10.1007/s11010-020-03855-y.
33. van de Schans VAM, Smits JFM, Blankesteijn WM. The Wnt/frizzled pathway in cardiovascular development and disease: friend or foe? Eur J Pharmacol. 2008;585:338–45.

34. Khan I, Ali A, Akhter MA, et al. Preconditioning of mesenchymal stem cells with 2,4-dinitrophenol improves cardiac function in infarcted rats. Life Sci. 2016;162:60–9. https://doi.org/10.1016/j.lfs.2016.08.014.
35. Kampaktsis PN, Ullal AV, Minutello RM, et al. Impact of paravalvular aortic insufficiency on left ventricular remodeling and mortality after transcatheter aortic valve replacement. J Heart Valve Dis. 2016;25:301–8.
36. Sekaran NK, Crowley AL, de Souza FR, et al. The Role for cardiovascular remodeling in cardiovascular outcomes. Curr Atheroscler Rep. 2017;19:1–11.
37. Marwick TH. Ejection fraction pros and cons: *JACC* State-of-the-Art Review. J Am Coll Cardiol. 2018;72:2360–79.
38. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006;98:1076–84.
39. Rocha V, Wagner JE, Sobocinski KA, et al. Graft-versus-host disease in children who have received a cord-blood or bone marrow transplant from an HLA-identical sibling. N Engl J Med. 2000;342:1846–54. https://doi.org/10.1056/nejm200006223422501.
40. Azari MF, Mathias L, Ozturk E, et al. Mesenchymal stem cells for treatment of CNS injury. Curr Neuropharmacol. 2010;8:316–23. https://doi.org/10.2174/157015910793358204.
41. Sancricca C. Mesenchymal stromal cells from human perinatal tissues: from biology to cell therapy. World J Stem Cells. 2010;2:81. https://doi.org/10.4252/wjsc.v2.i4.81.
42. Ekram S, Khalid S, Bashir I, et al. Human umbilical cord-derived mesenchymal stem cells and their chondroprogenitor derivatives reduced pain and inflammation signaling and promote regeneration in a rat intervertebral disc degeneration model. Mol Cell Biochem. 2021;476:3191–205. https://doi.org/10.1007/s11010-021-04155-9.
43. Ennis J, Götherström C, Le Blanc K, Davies JE. In vitro immunologic properties of human umbilical cord perivascular cells. Cytotherapy. 2008;10:174–81. https://doi.org/10.1080/14653240801891667.
44. Weiss ML, Anderson C, Medicetty S, et al. Immune properties of human umbilical cord Wharton's jelly-derived cells. Stem Cells. 2008;26:2865–74. https://doi.org/10.1634/stemcells.2007-1028.
45. Yoo KH, Jang IK, Lee MW, et al. Comparison of immunomodulatory properties of mesenchymal stem cells derived from adult human tissues. Cell Immunol. 2009;259:150–6. https://doi.org/10.1016/j.cellimm.2009.06.010.
46. Yang CC, Shih YH, Ko MH, et al. Transplantation of human umbilical mesenchymal stem cells from Wharton's jelly after complete transection of the rat spinal cord. PLoS One. 2008;3:e3336. https://doi.org/10.1371/journal.pone.0003336.
47. Fu Y-S, Cheng Y-C, Lin M-YA, et al. Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopaminergic neurons in vitro: potential therapeutic application for parkinsonism. Stem Cells. 2006;24:115–24. https://doi.org/10.1634/stemcells.2005-0053.
48. Xiong N, Zhang Z, Huang J, et al. VEGF-expressing human umbilical cord mesenchymal stem cells, an improved therapy strategy for Parkinson's disease. Gene Ther. 2011;18:394–402. https://doi.org/10.1038/gt.2010.152.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.