



Mesenchymal Stem Cells for Cardiac Regeneration: from Differentiation to Cell Delivery

Santosh Gupta¹ · Akriti Sharma¹ · Archana S¹ · Rama Shanker Verma¹ 

Accepted: 12 April 2021 / Published online: 5 May 2021

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Abstract

Mesenchymal stem cells (MSCs) are so far the most widely researched stem cells in clinics and used as an experimental cellular therapy module, particularly in cardiac regeneration and repair. Ever since the discovery of cardiomyogenesis induction in MSCs, a wide variety of differentiation protocols have been extensively used in preclinical models. However, pre differentiated MSC-derived cardiomyocytes have not been used in clinical trials; highlighting discrepancies and limitations in its use as a source of derived cardiomyocytes for transplantation to improve the damaged heart function. Therefore, this review article focuses on the strategies used to derive cardiomyocytes-like cells from MSCs isolated from three widely used tissue sources and their differentiation efficiencies. We have further discussed the role of MSCs in inducing angiogenesis as a cellular precursor to endothelial cells and its secretory aspects including exosomes. We have then discussed the strategies used for delivering cells in the damaged heart and how its retention plays a critical role in the overall outcome of the therapy. We have also conversed about the scope of the local and systemic modes of delivery of MSCs and the application of biomaterials to improve the overall delivery efficacy and function. We have finally discussed the advantages and limitations of cell delivery to the heart and the future scope of MSCs in cardiac regenerative therapy.

Keywords Mesenchymal stem cells · Cardiac regenerative therapy · Differentiation · Cardiomyocytes · Cell delivery

Introduction

Mesenchymal Stem Cells (MSCs) are one of the most interesting discoveries with a high impact in the field of tissue engineering and regenerative medicine. MSCs, especially in the case of cardiac regeneration, although initially thought to be non-regenerative intrinsically, have been proven to have propensity and ability to regenerate in normal physiological condition as well as upon injury under the influence of various factors responsible for the initiation of repair and regeneration cascade mechanism [1]. Cardiovascular disease-related morbidity and mortality is expected to rise upto 23.6 million by 2030 and therefore underlines an urgent need to address the issue of repairing and regenerating the injured heart [2], [3]. MSCs have been extensively used in pre-clinical models for

regeneration of the injured heart. In initial experiments during the early years of this century, the possibilities of MSCs as a source of cardiomyocytes were being explored extensively. Its ability to differentiate into cardiomyocyte like cells (CLC) upon treatment with cardiomyogenic inducers confirmed by expression of cardiomyocyte specific genes and proteins and its ability to home to the injured site of the heart upon in vivo administration was expected to create a lot of excitement and start of an era where a stem cell with high potential, facile isolation, culture and huge number gave the field of cardiac regenerative medicine a new hope. However, the inability of MSC-derived CLC to produce beating cells upon cardiomyogenic induction created the biggest setback as cardiomyocytes must have beating property to be able to call it a derived CLC with functional beating property [4]. This was achieved by the Yamanaka group in 2006 where they introduced the concept of induced pluripotent stem cells (iPSCs) and the first cells that were made in a laboratory using these non-embryonic iPSCs were functional beating CLC. Ever since, the lack of research focusing on the derivation of functional beating cardiomyocytes like cells from MSCs and a standard differentiation strategy that gives the derived cell its

✉ Rama Shanker Verma
vermars@iitm.ac.in

¹ Stem Cell and Molecular Biology Laboratory, Bhupat and Jyoti Mehta School of Biosciences, Department of Biotechnology, Indian Institute of Technology-Madras, Chennai, Tamil Nadu 600036, India

beating property has set major limitation in its use as a cell source for deriving cardiomyocytes like cells for use in cardiac regeneration and repair [5] as compared to iPSCs [6].

Therefore, this article focuses on the use of MSCs, retrieved from three widely used sources i.e., adipose tissue, bone marrow and umbilical cord, in deriving cardiomyocytes like cells and the methodologies used along with the experimental outcomes. We have extensively discussed the ability of various tissue derived MSCs to differentiate into CLC. We have also discussed about the limitations of MSCs as a source of cardiomyocytes. We have further accentuated the angiogenic capabilities of MSCs. This is due to the ability of MSCs to differentiate into endothelial like cells and the ability of MSCs to secrete a variety of factors like growth factors and exosomes which have been proven to promote angiogenesis. We have further discussed the strategies for the delivery of MSCs to injured heart as the delivery mechanisms have also been shown to influence the therapeutic outcome of MSCs in cardiac regenerative therapy. Two majorly explored delivery methods are local injection and systemic injection of MSCs that relies upon the MSCs to home to the injured site either due to intrinsic property or external modulation. Finally, we have highlighted the advantages and limitations of using different delivery routes.

Mesenchymal Stem Cells for Cardiac Therapy

Julius Friedrich Cohnheim in 1867, discovered the existence of non-hematopoietic stem cells in the bone marrow. He suggested that this new unique population of cells from the bone marrow can travel to the injured tissue through the bloodstream and could differentiate, thereby aiding in wound healing processes in various peripheral tissue [7]. This theory was later confirmed by A.J. Friedenstein in the mid-1970s. He discovered that bone marrow consisted of a subset of fibroblast-like cells with both clonogenic potential and high replicative capacity in vitro when plated at low density in tissue culture [8]. This, therefore, indicated the existence of a minor subpopulation of bone marrow cells that are distinguishable from hematopoietic cells and can give rise to a different cell type upon being cultured in a non-native environment and condition [9]. Later, the term mesenchymal stem cell was coined by Arnold Caplan in 1991 [10]. Based on the observations of Friedenstein's work, it was therefore thought that bone marrow stromal cells might also differentiate into cardiomyocytes when exposed to standard in vitro cardiac differentiation conditions [11]. This approach could therefore overcome the limitations of conventional cardiovascular therapies.

The three minimal criteria to identify MSCs were proposed by the International Society for Cellular Therapy and it includes [1] the ability of MSCs to adhere to plastic tissue

culturing vessels [2] the positive markers for surface antigen expression including CD105, CD73, CD90 and negative markers for CD45, CD34, CD14, CD79a and HLA class II [3] its multipotent capacity to differentiate into multiple cell lineages under standard in vitro differentiating conditions [12].

The first in vitro differentiation into cardiomyocytes from bone marrow was described by Shinji et al. in 1999. The investigators exposed the marrow stromal cells with 5-Azacytidine (5-Aza), a demethylating agent. Interestingly, the cells began to beat spontaneously in vitro and exhibited cardiomyocyte like characteristics [11]. Later Toma et al., in 2002 showed the in vivo differentiation of cardiomyocytes from bone marrow mesenchymal stem cells (BMSCs) when transplanted into the adult murine heart. These transplanted cells responded to signals from the injured tissue, thereby promoting its migration, proliferation and differentiation within the infarcted area [13]. These two important discoveries guided the scientists to make advances in both clinical and basic research on MSC transplantation to treat ischemia related cardiovascular diseases. The ease of isolation, well-defined culture conditions and high number of cells made this stem cell a very interesting source of multipotent cell for treating injured myocardium. However, the major problem that significantly limits the clinical transplantation of MSCs as a source for deriving functional cardiomyocytes is its inability to produce functional beating cardiomyocytes like cell upon subjection to a variety of differentiation strategies like small molecules, growth factors, physical condition like mechanical and electrical stimulation and topological cues. Most of the strategies used have yielded cardiomyocytes like cells with increased cardiac specific gene and protein expression (Fig. 1) [14].

Since 1999, many researchers attempted but only a few have been able to show the derivation of beating cardiomyocytes like cells from MSCs. However, those selected studies have not been further explored by researchers. Especially, since the last decade, acellular based therapeutic role of MSCs has gained much interest. In particular, its role as a paracrine factory to produce a therapeutic effect as pro-angiogenic, anti-oxidative, autophagic, anti-inflammatory has created a strong surge for its use as a cell factory producing plethora of biological factors having therapeutic role. It is due to this, Arnold Caplan suggested to rename Mesenchymal stem cell as “Medicinal Signalling Cell” [15]. However, in this review article, we will focus on the strategies being used so far on the three types of most widely used MSCs to induce cardiomyogenesis and study their outcome both in vitro and in vivo.

In order to maximize the therapeutic potential of MSCs in regenerative medicine, three major parameters, [1] suitable choice of appropriate MSC cell type [2] pre-treatment of the potential cell type and [3] site of delivery of MSCs should be

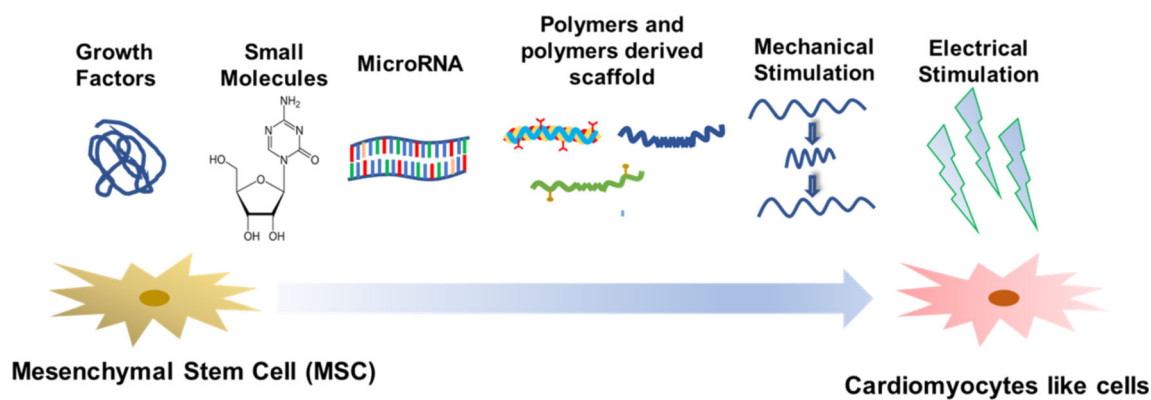


Fig. 1 Strategies used for driving cardiomyogenesis in Mesenchymal Stem cell (MSC). These include treatment with growth factors, small pharmacological molecules, micro RNAs, culture on natural and

synthetic polymers and polymers derived 3D scaffolds, sensitization and stimulation to mechanical force and electrical impulse

considered critical [16]. With more than 1050 clinical trials registered covering diseases from bone to neurological to cardiovascular diseases, it significantly substantiates the relevance of MSCs.

However, in cardiovascular regenerative therapy, the mechanistic role and the status of cardiomyocytes like cells derived from MSCs remains elusive and yet to be uncovered. Therefore, this aspect has been covered in detail in the current article as to how the differentiation strategies have taken their course over two decades and the outcome associated with it [17]. We have also provided tabular information about the relating to types of mesenchymal stem cells being used for derivation of cardiomyocytes like cells, the protocol and strategies used and whether the derived cells are beating or non-beating and finally providing information on the effect of transplanted MSCs derived cardiomyocytes like cells in vivo on the heart function, if any (Table 1).

MSCs Sources for Differentiation to Cardiomyocytes

MSCs obtained from different sources (bone marrow, adipose tissue, umbilical cord blood) have shown cardiomyogenic differentiation potential (Fig. 2). Cardiomyogenesis is an extremely complex process, regulated by various factors via different signaling pathways. The success of cell therapies mainly depends on a variety of factors which include the source of stem cells, their proliferative and differentiation status, survival capability of the newly engrafted cells and so forth.

Adipose-Derived Mesenchymal Stem Cells (ADMSC)

Adipose tissue possesses the capability of acting as a potential source of stem cells as this adult tissue is abundant and easy to sample. MSCs isolated from adipose tissue show high proliferation rates and have the capacity to differentiate into

functional CLC. Several studies have shown the differentiation of ADMSCs into cardiomyocytes using a variety of approaches. These approaches are aimed to improve differentiation efficiency and restore contractile performance and heart function. Several reports have described various exogenous stimuli such as pro-cardiogenic factors (BMPs, VEGF, Activin A, TGF- β 1 or a combination of the above factors), DNA demethylation agent such as 5- Aza, transcription factor encoding lentiviral vectors, small molecules, miRNA that were instrumental in driving myogenic differentiation in vitro and in vivo.

In one of the recent research, MSCs isolated from adipose tissue have been used to differentiate into cardiomyocyte like cells using a combination of 5-Aza and TGF- β 1 [18] (Fig. 3 A). Among the various inducers (DMSO, Angiotensin II, TGF- β , 5- Aza) known to differentiate ADMSCs into cardiomyocytes with variable efficiencies [19], 5-Aza is most widely used for cardiomyocyte differentiation to date. 5-Aza is a DNA demethylating agent. DNA methylation is a common epigenetic signaling tool that has been reported to be involved in the regulation of gene expression. The study of DNA methylation has been established in the pluripotent as well as the differentiated state. It is known from previous studies that pluripotent genome is highly methylated which is essential for the self-renewal of stem cells. As differentiation proceeds, there is a progressive loss of methylation. Thus, inhibitors of DNA methylation such as 5-Aza are a strong inducer of cardiomyocyte differentiation [20]. In this treatment regime, ADMSCs showed morphological changes like increase in size and flattening of cells, multinucleation and myotube formation similar to cardiomyocytes. Analysis of cardiac specific differentiation markers revealed an upregulated expression of Mlc-2v, SERCa2, GATA-4, cTn1 and Cx-43 in cultures treated with this protocol. However, no analysis was done to observe spontaneous beating cells after the treatment. In this study, functional analysis of newly differentiated cardiomyocytes was assessed based on calcium activities. It

Table 1 Table information relating to types of Mesenchymal stem cells being used for derivation into cardiomyocyte like cells, the protocol and strategies used and whether the derived cells are beating or non-beating and finally providing information on the effect of transplanted MSC derived cardiomyocytes like cells in vivo on the heart function. EF – Ejection Fraction, FS – Fraction Shortening, LV – Left Ventricle, LVEDP – Left Ventricular End-Diastolic Pressure, LVFS - Left Ventricular Fractional Shortening, LVEF - Left Ventricular Ejection Fraction, LVSP - Left Ventricular Systolic Pressure

MSC type	Species (Isolated from)	Species (Tested on)	Differentiation method	Days of differentiation (in vitro)	In vitro characterization	Beating/Non beating	In vivo effect	References
Adipose-derived MSC	SD rats	SD rats	Chitosan components (N-Ac-Glu/D-Glu)	14 days	<u>Morphological characteristics:</u> Elongated morphology, cross-striated myofibrils. <u>Expression of cardiac specific markers:</u> Upregulated expression of GATA-4, Nkx-2.5, α -SA, cTnI, Myl7, Myh6 at mRNA and protein level	Beating	High EF and FS, decreased LVEDP, reduction in LV infarct size and fibrosis	[124]
Adipose-derived MSC	Human	*	5- Aza and TGF- β 1	7 days - TGF- β 1 14 days- 5-Aza	<u>Morphological characteristics:</u> Flattening of cells, myotubule formation, multinucleation <u>Expression of cardiac specific markers:</u> Upregulated expression of Mlc-2 V, cTnI, Serca2, actinin at mRNA and protein level KCl- induced calcium transients were observed	Non beating	*	[18]
Adipose-derived MSC	-	*	Overexpression of GATA-4, Tbx-5 and Baf60c gene in ADMSCs using lentiviral vectors	14 days	<u>Expression of cardiac-specific markers:</u> Upregulated expression of cTnT, Myh6, Actc1 and Cx-43 at mRNA and protein level	Beating	*	[21]
Adipose-derived MSC	Human	*	Overexpression of Tbx-20 in ADMSCs using lentiviral vectors	14 days	<u>Morphological characteristics:</u> Polygonal/rod-shaped <u>Expression of cardiac-specific markers:</u> Upregulated expression of ACTN1, TNN13, ACTC1, Nkx2.5, Tbx-20 and GATA-4 at mRNA level and α -actinin, CTnI at protein level.	Functional analysis in terms of beating not shown	*	[125]
Adipose-derived MSC	Human	SD rats	DMPE-PEG mediated rTGF- β 1 modified ADSCs with exogenous TGF- β 1 treatment	14 days	<u>Morphological characteristics:</u> Cells grew in clusters, long-spindle shaped morphology, exhibited orderly actin filament arrangement in the cytoplasm <u>Expression of cardiac-specific markers:</u> Upregulated expression of α -SMA and cTnT at protein level	Functional analysis in terms of beating not shown	Immunofluorescence analysis revealed a noticeably higher expression of α -SMA and cTnT in D-P-TGF β 1R1 group when compared to D-P group. Functional evaluation was not assessed.	[126]
Adipose-derived MSC	SD rats	SD rats with MI model	Utilization of		temperature-responsive decellularized myocardial ECM hydrogel	14 days	Functional analysis in terms of beating not shown Upregulated expression of cTnT and Cx-43 at mRNA and protein level	Functional analysis in terms of beating not shown

Table 1 (continued)

MSC type	Species (Isolated from)	Species (Tested on)	Differentiation method	Days of differentiation (in vitro)	In vitro characterization	Beating/Non beating	In vivo effect	References
Increased LVEF, LVFS LV wall thickness, LV function, decreased infarct size	Adipose-derived MSC	Wistar rats	BMP4 and VEGF followed by methylcellulose based medium with cytokines (IL-6 and IL-3)	15 days	<u>Morphological characteristics:</u> Binucleate and elongated cells, dense cytoplasm with myofibril-like structures, formation of cell clusters <u>Expression of cardiac-specific markers:</u> Upregulated expression of GATA-4, cTnT, Myh, Serca2 at mRNA and protein level <u>Expression of cardiac-specific markers:</u> Upregulated expression of Nkx 2.5 in ISX1-primed ADSCs and ANF, BNP and troponin C in ISX1-primed ADSCs co-cultured with NRVMs.	Non beating	Better performance of spontaneous calcium transients was observed	[25]
Adipose-derived MSC	Mice	Mice with MI model	ISX1-primed ADSCs and co-culture with neonatal rat cardiomyocytes	21 days	<u>Expression of cardiac-specific markers:</u> Upregulated expression of Nkx 2.5 in ISX1-primed ADSCs and ANF, BNP and troponin C in ISX1-primed ADSCs co-cultured with NRVMs.	Functional analysis in terms of beating not shown	Increase in FS, protection of systolic function, decrease in LVESD	[22]
Bone marrow-derived MSC	Human	*	Utilization of electrospun Type 1 collagen nanofibre mats and also treatment with 5-Aza	28 days	<u>Expression of cardiac-specific markers:</u> → Upregulated expression of GATA-4, cTnI, MHC independent of 5-Aza treatment and Cx-43, α-SMA and cTnT with 5-Aza treatment Downregulation of CD90 Improvement in cell orientation and cell alignment	Functional analysis in terms of beating not shown	*	[48]
Bone-marrow derived MSC	Rats	*	5-Aza, Salvianolic acid and utilization of cardiomyocyte lysis medium	14 days	<u>Expression of cardiac-specific markers:</u> Upregulated expression of cTnT, α-cardiac actin, mef-2c, Cx43, GSK-3β at mRNA and protein level Downregulation of β-catenin	Non beating	*	[32]
Bone-marrow derived MSC	Wistar rats	*	Platelet lysate and 5-Aza	14 days	<u>Morphological characteristics:</u> Rod-like cells, cell aggregates were detectable <u>Expression of cardiac-specific markers:</u> Increased expression of CAA and GATA-4 at mRNA level and α-sarcomeric actin at protein level	Beating	*	[34]
Bone-marrow derived MSC	SD rats	*	Cyclic biaxial tensile strain (CBTS)	–	<u>Expression of cardiac-specific markers:</u>	Beating	*	[40]

Table 1 (continued)

MSC type	Species (Isolated from)	Species (Tested on)	Differentiation method	Days of differentiation (in vitro)	In vitro characterization	Beating/Non beating	In vivo effect	References
Bone-marrow derived MSC	SD rats	SD rats with MI model	Induction of c-kit+ BMSCs with BMP-2	28 days	Upregulated expression of GATA-4, TNNT2, Mef2c, Cx-43 and SCF at mRNA and protein level Downregulation of miR-27a <u>Morphological characteristics:</u> Columnar shape, parallel arrangement, myotube like structures <u>Expression of cardiac-specific markers:</u> Upregulated expression of cTnT, Cx43, Myom1 and α -actinin at mRNA level and cTnT, Cx-43 at protein level AP plateau similar to ventricular cardiomyocytes of adult rat <u>Morphological characteristics:</u> Fusiform shape, parallel orientation, formation of myotube like structures <u>Expression of cardiac-specific markers:</u> Upregulated expression of GATA-4, Nkx2.5, α -MHC at mRNA level and α -sarcomeric actin and cTnT at protein level	Functional analysis in terms of beating not shown	Increased EF and FS, improved thickness in left ventricular wall, presence of more myocardial tissue at the infarct regions, reduction in scar size.	[127]
Bone-marrow derived MSCs	SD rats	SD rats with MI model	TGF- β 1	28 days	Upregulated expression of GATA-4, Nkx2.5, α -MHC at mRNA level and α -sarcomeric actin and cTnT at protein level Gap junctions were observed with TEM <u>Morphological characteristics:</u> Flattened morphology, spindle-like structure <u>Expression of cardiac-specific markers:</u> Upregulated expression of GATA-4, TNNT2, ACTN2 and Mef-2c at mRNA level and Cx-43 and ACTN2 at protein level	Functional analysis in terms of beating not shown	Decreased LVEDP, Increased LVSP, maximal positive pressure development rate, reduction in scar area.	[128]
Bone-marrow derived MSC	Rats	*	Synergistic effect of electrical stimulation and TGF- β 1	10 days	Upregulated expression of GATA-4, TNNT2, ACTN2 and Mef-2c at mRNA level and Cx-43 and ACTN2 at protein level <u>Expression of cardiac-specific markers:</u> Upregulated expression of cTnT, α -MHC, cTnI, Cx-43, β -adrenoreceptor, GATA-4, Nkx2.5 at protein level	Non beating	*	[43]
Bone-marrow derived MSC	SD rats	*	Aspirin	28 days	Upregulated expression of cTnT, α -MHC, cTnI, Cx-43, β -adrenoreceptor, GATA-4, Nkx2.5 at protein level Calcium transients observed in some cells No action potential recorded	Non beating	*	[51]
Umbilical cord blood derived MSCs	Human	*	Combination of 5-Aza and mouse fetal heart extract	36 days	<u>Morphological characteristics:</u> Tube-like structure, cells tended to gather together, multinucleation <u>Expression of cardiac-specific markers:</u> Upregulated expression of GATA-4, α -Ca, Nkx2.5, β -MHC, cTnT, Des at mRNA level and sarcomeric alpha actin at protein level	Non beating	*	[60]
Umbilical cord derived MSC	Human	*	p53 inhibitor PFT- α	28 days	<u>Expression of cardiac-specific markers:</u> Upregulated		*	[129]

Table 1 (continued)

MSC type	Species (Isolated from)	Species (Tested on)	Differentiation method	Days of differentiation (in vitro)	In vitro characterization	Beating/Non beating	In vivo effect	References
Umbilical cord derived MSC	Human	Wistar rats with MI model	2-deoxycytidine	14 days	expression of cTnI, Desmin and Nkx2.5 at mRNA and protein level Downregulation of p53 and p21 <u>Expression of cardiac-specific markers:</u> Upregulated expression of GATA-4, cTnI, cTnC, α -MHC, α -actinin, Wnt-2, calcium and sodium channel at mRNA level and Nkx2.5, GATA-4, Cx-43, cardiac alpha actinin and MHC at protein level	Functional analysis in terms of beating not shown Functional analysis in terms of beating not shown	Increase in LV systolic and diastolic dimension, end systolic volume, end diastolic volume and stroke volume Increase in EF and FS and LV wall thickness Reduced fibrosis	[130]
Umbilical cord blood derived MSCs	Human	SD rats with I/R model cardiomyocytes	Oxytocin and OT-MSR cocultured with rat cardiomyocytes	7 days	<u>Expression of cardiac-specific markers:</u> Upregulated expression of cTnI, α -SA and eNOS at mRNA level and Cx-43, cTnI and α -SA at protein level Calcein AM and CM-DiI staining revealed formation of functioning gap junction <u>Morphological characteristics:</u> Spindle-shaped, multinuclear cells <u>Expression of cardiac-specific markers:</u> Upregulated expression of α -actinin and cTnT at protein level	Functional analysis in terms of beating not shown	Increase in EF. The data also correlated with the representative M-mode echograms Reduced fibrosis	[65]
Umbilical cord blood derived MSC	Human	Mice	5-Aza	14 days	Exhibited cardiomyogenic morphology with the formation of myotubules	Functional analysis in terms of beating not shown	Reduced heart tissue damages, reduction in fibrosis, increase in LV wall thickness, no sign of tumorigenesis post transplantation	[131]
Whartons jelly of umbilical cord derived MSC	Human	*	Utilization of collagenase enzyme 1S to isolate MSC and treatment with 5-Aza	21 days	Upregulated expression of α -actinin and cTnT at protein level	Slight spontaneous beating	*	[132]
Umbilical cord derived MSC	Human	*	Overexpression of Nkx2.5	28 days	<u>Expression of cardiac-specific markers:</u> Upregulated expression of cTnI, Desmin, Nkx2.5 and GATA-4 at mRNA and protein level <u>Morphological characteristics:</u> Clear striated pattern similar to cardiomyocytes <u>Expression of cardiac-specific markers:</u> Increased expression of Csx/Nkx-2.5, GATA-4, hANP, hBNP, α -actin, MHC, Mlc-2v, cTnT, cTnI and HCN2 at mRNA level Cell fusion independent cardiomyocyte differentiation were observed Action potential / recorded	Functional analysis in terms of beating not shown Beating	* *	[133]
UCBMSC/UC-BMSC-TERT	Human	*	Co-culturing with murine fetal cardiomyocytes	7 days				[61]

* Information not provided

** In vivo studies not performed

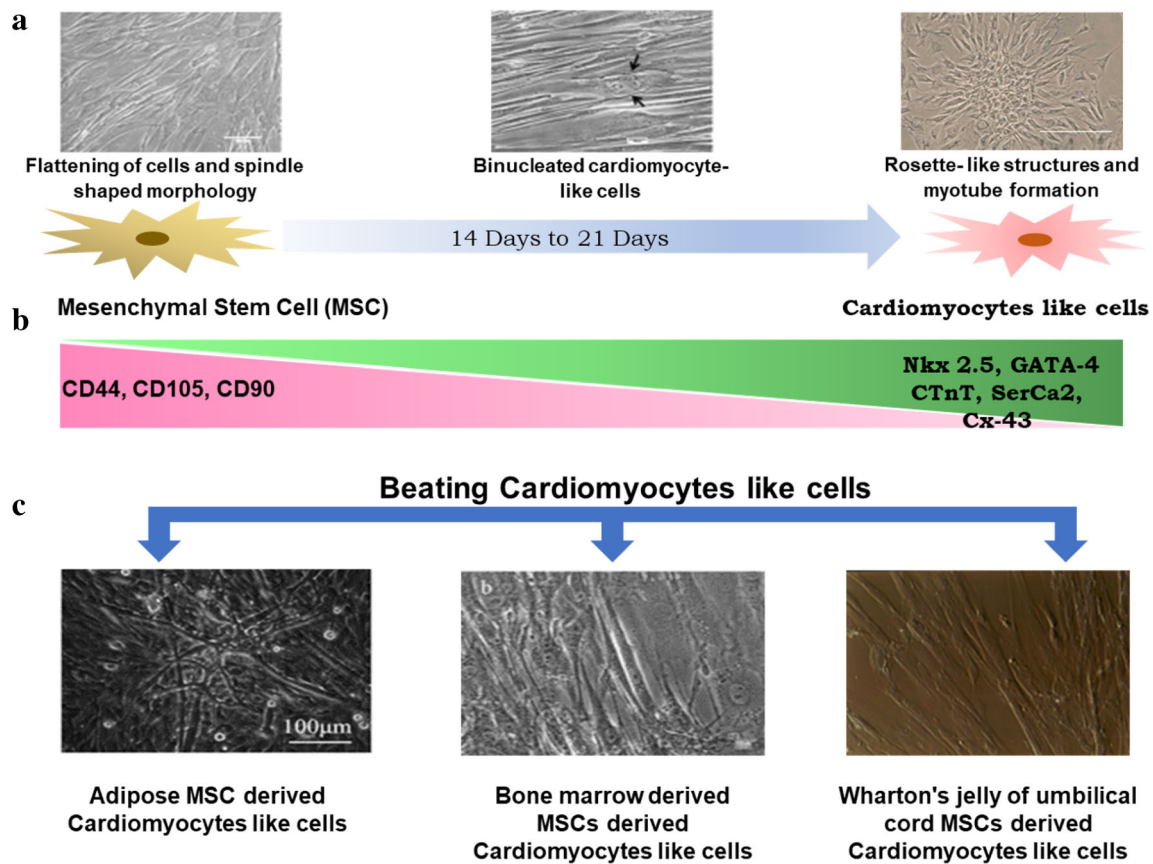


Fig. 2 Schematic representation of differentiation strategy employed to derive functional beating CLCs from different source MSCs. (A) Shows change in morphology of the initial MSCs as the differentiation progresses. (B) Variation in expression of cardiomyocyte related genes

reported in driving cardiomyogenesis using MSCs. (C). MSCs from different sources showing derived cardiomyocytes morphology. All the image represented is of spontaneously beating cardiomyocytes like cells derived from MSCs. (reproduced with permission from Ref [59, 98, 106])

was reasoned that the immediate rise in calcium flux could be due to ryanodine receptors which helped in the release of calcium whereas plateau attained could be due to the SERCa2 mediated reuptake of calcium.

Another research study conducted by Li et al. in 2015, showed that overexpressing GATA-4, Tbx5 and Baf60c in MSCs successfully differentiated ADMSCs into cardiomyocytes. Post 14 days treatment, there was a significant increase in Nkx2.5, cTnT, Myh6, ACTC1 and Cx-43. Flow cytometric analysis revealed a 5.71% cTnT expression rate when compared to the control. Importantly, this treatment protocol resulted in beating cell clusters indicating an improved differentiation efficiency (Fig. 3 B) [21]. This approach is similar to the strategy used in deriving iPSC using forced expression of Yamanaka factors. Therefore, proving a continuous supply of cardiomyogenic transcription factors could be a vital strategy to induce beating functionality in CLC derived from MSC.

A novel strategy has been established by Burchfield et al. that focuses on the synergy between pharmacology and cell-based therapy. This study utilized a small molecule, cyclopropyl-amide analog called Isoxaxoles (Isx1) which

can induce activation of cardiac gene program by priming ADMSC and promoting cardiomyocyte differentiation. The remarkable characteristic of ISX1 is its ability to promote only cardiomyogenic differentiation while limiting differentiation into other cellular lineages, suggesting that re-programming with ISX1 is cardiomyocyte specific [22]. Treatment of ADMSCs with ISX1 triggered a robust, time and dose-dependent increase in Nkx2.5, an early marker of cardiomyogenic lineage. Coculturing of NRVMs with ISX1-treated ADMSCs significantly enhanced the expression of early and late cardiac differentiation markers (ANF, BNP and Troponin C), thereby suggesting the ability of ISX1 to promote cardiomyogenic differentiation. However, no analysis was performed to detect the beating cells. For the in vivo effect, transplantation of ISX1-primed ADMSCs improved cardiac functional recovery post-MI, as evidenced by an increase in fractional shortening and a decrease in left ventricular end systolic dimension (LVESD). Administration of ISX-1 treated ADMSCs to the infarcted myocardium was associated with enhanced neovascularization which has been linked to paracrine factors secreted from ADMSCs.

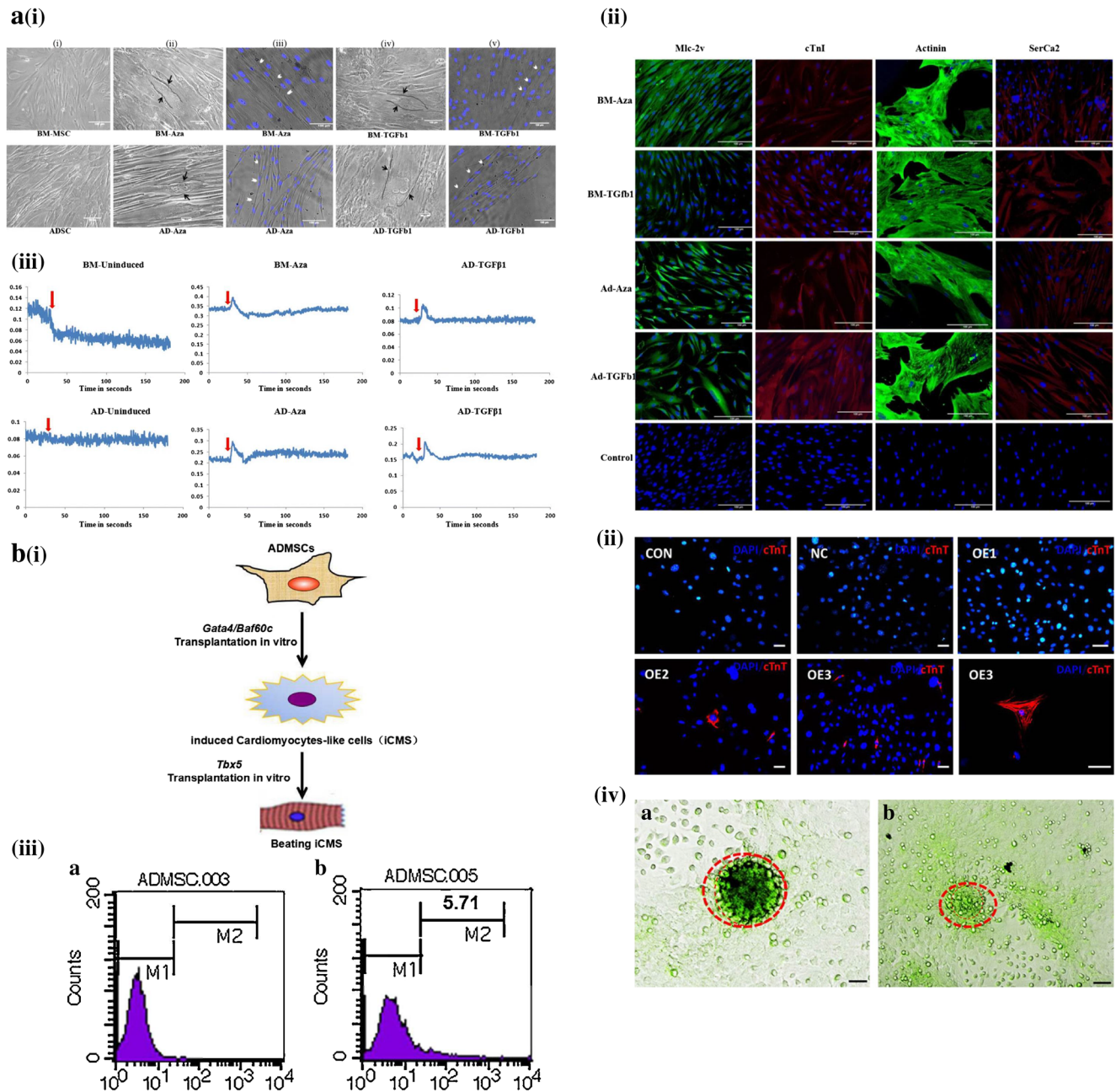


Fig. 3 (A) Differentiation of ADMSCs into CM-like cells using 5-Aza and TGF- β 1. A(i) Morphology of ADMSCs after induction exhibited CM-like features (Binucleation and myotube formation). A(ii) Immunofluorescence staining of CM-specific markers (Mlc-2v, cTn1, Actinin and SERCa2) in different treated groups. A(iii) Line graphs representing changes in calcium transients upon KCl stimulation in cells of all study groups. B- Effect of GATA-4, Tbx-5 and Baf60c in CM differentiation of ADMSCs (**reproduced with permission from Ref** [18]). (B) Effect of GATA-4, Tbx-5 and Baf60c in CM differentiation of

ADMSCs. B(ii) Schematic representation showing the effect of GATA-4, Tbx-5 and Baf60c in CM differentiation of ADMSCs. B(ii) Immunofluorescence staining of cTnT in all different treated groups [C, NC, OE-1-Tbx5-GFP-LV, OE-2-Gata4-LV and Baf60cLV, OE-3-Tbx5-GFP-LV, Gata4-LV and Baf60c-LV] with increased expression in OE-3 group. B(iii) Flow cytometric analysis of cTnT expression in OE-3 group. B(iv) Presence of beating cells (dashed red lines) in OE-3 group (**reproduced with permission from Ref** [21])

Many research studies have shown that the microenvironment plays a critical role in cell proliferation and differentiation. A study emphasized the role of soluble signaling molecules and direct cell to cell contact which can control the

differentiation into cardiomyocytes [23]. In vitro studies have also shown that ADMSCs when co-cultured with cardiomyocytes, appeared to adopt a more cardiomyocyte-like phenotype which had elongated morphology, some even

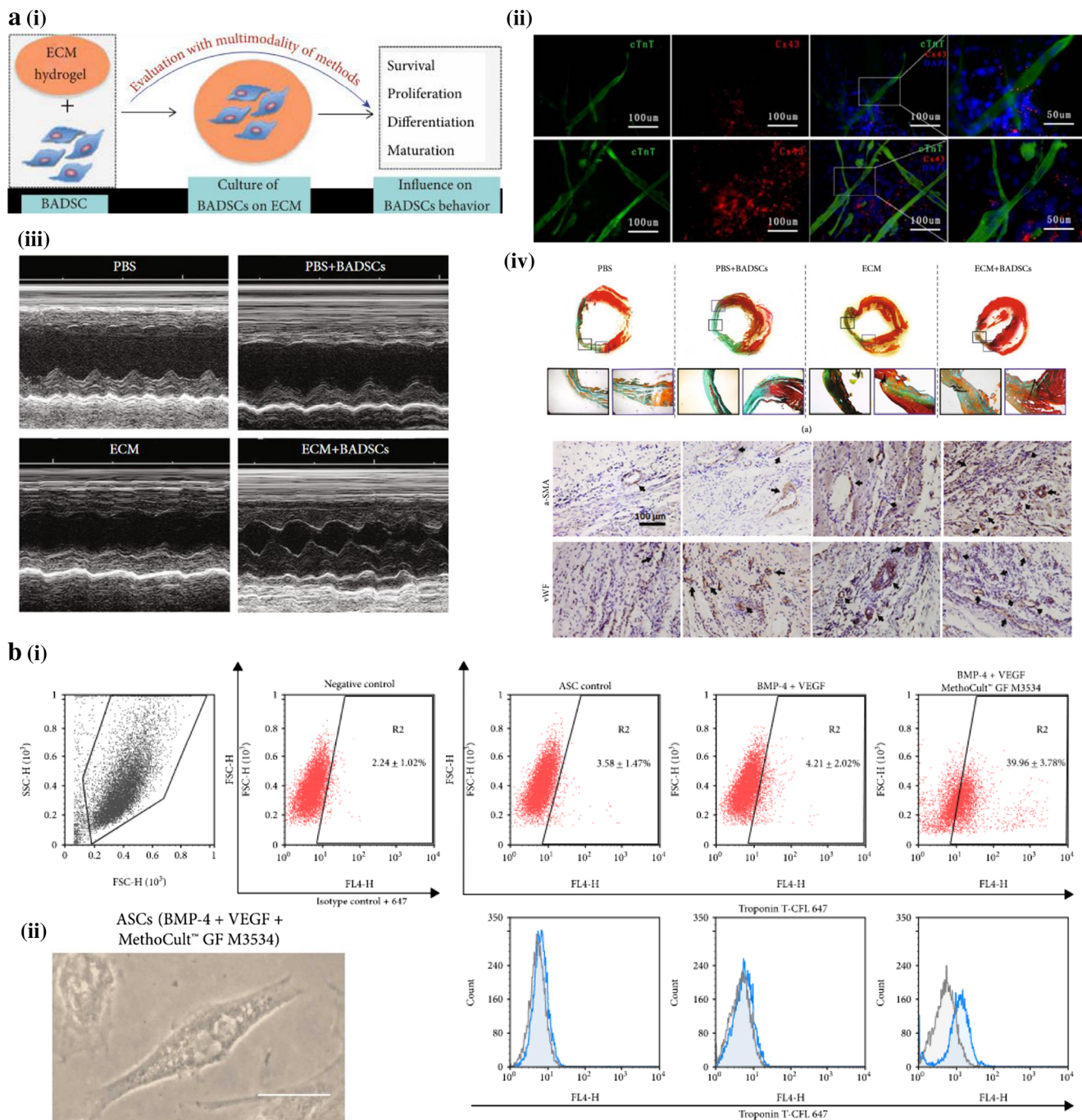


Fig. 4 (A) Effect of ECM hydrogels on CM differentiation of ADMSCs. A(i) Schematic representation of evaluating ECM hydrogels on ADMSC behaviour. A(ii) Immunofluorescent staining showing a higher expression of cTnT and Cx-43 in ADMSCs growing on ECM substrates than on Col1. A(iii) Functional evaluation by echocardiography resulted in attenuating myocardial injury post MI in ECM + ADMSC group. A(iv) MT staining (top panel) showing a decreased infarct size and increase in wall thickness and immunostaining (bottom panel) detected a higher level

of neovascularization in ECM + ADMSC group (**reproduced with permission from Ref**) [24]. (B) Effect of BMP-4 + VEGF+MethoCult™ GF M3534 in ADMSCs differentiation into CM. B(i) Flow cytometric analysis of cTnT expression at day 15 in all different treated groups (NC, ASC Control, BMP-4 + VEGF and BMP-4 + VEGF+MethoCult™ GF M3534) with increased expression observed in BMP-4 + VEGF+MethoCult™ GF M3534 group B(ii) Binucleated CM-like cells at day 10 after induction. (**reproduced with permission from Ref**) [25]

formed cardiac-like myofilaments and sarcomeres. Interestingly, synchronous contractions of cardiomyocyte like clusters were observed in this direct co-culture system.

Bai et al. in 2019, demonstrated the potential of combining extracellular matrix (ECM) hydrogel and ADMSCs for myocardial repair both in vitro and in vivo (Fig. 4 A) [24]. Study

by Bai and his colleagues explored the effects of temperature-sensitive ECM hydrogel (prepared from decellularized heart matrix) on the proliferation, cardiac differentiation and maturation of ADMSCs in vitro and its therapeutic effect in in vivo Myocardium Infarction (MI) model. ECM substrate promoted the differentiation of ADMSCs into cardiomyocyte-like cells as evidenced by a significant upregulation in the expression of cTnT and Cx-43 at both mRNA and protein levels when compared to ADMSCs grown on Col-1 coated substrate. However, functional analysis in terms of beating was not evaluated. In vivo transplantation resulted in significant improvement in heart function (evaluated by echocardiographic studies) in the ECM-ADMSCs group than in other groups (PBS, ADMSC and ECM group). Analysis of heart showed a reduction in infarct size, increase in wall thickness and neovascularization in ECM-ADMSC group when compared to other groups, thus proving the therapeutic efficacy of this combination to repair MI injury.

Another recent study has established a novel directed cardiomyocyte differentiation protocol that has shown high efficiency and reproducibility (Fig. 4 B). The protocol combined the use of BMP4 and VEGF to commit undifferentiated ADMSCs into cardiac progenitor cells, followed by using semi-solid commercial methylcellulose medium with cytokines (IL-3 and IL-6) for further cardiomyocyte differentiation [25]. BMP-4 belongs to the TGF- β family and is known to initiate the initial cardiac gene program. BMP signaling led to upregulation of Nkx2.5 and GATA-4 via Smad-mediated and MAP3K7 signaling pathway [26, 27]. VEGF upregulated KDR and PDGFR α by activating Flk-1 via the ERK pathway. The upregulated PDGFR α is instrumental in directing differentiation of specialized MSCs into cardiomyocytes. [28]. IL-6 is known to have anti-apoptotic effects on cardiomyocytes and IL-6 signaling leads to STAT3 activation which results in transcription of target genes (β -myosin heavy chain). IL-3 is also known to enhance the differentiation process. Evidence suggests that PI3K, PKCs are also involved in cardiomyocyte differentiation [29]. The efficacy of ADMSC differentiation into cardiomyocytes by this differentiation protocol was confirmed by the flow cytometric data which showed a higher percentage of cTnT positive cells ($39.96\% \pm 3.78$) in comparison to ADMSC control ($3.58 \pm 1.47\%$) and BMP4 + VEGF ($4.21 \pm 2.02\%$) groups. Although the cells exhibited cardiomyocyte like morphology and expressed cardiac specific markers, spontaneous beating cells were not observed in the culture. The author reasoned that automatism or spontaneous beating is a characteristic in vivo feature of early fetal cardiomyocytes and as the cells mature, they lose their automatism. Despite this study did not observe spontaneous beating cells, the positive expression of SERCa2, which function in calcium transportation in cardiomyocyte ER showed a further mature stage, explaining the lack of automatism in some way in adipose-derived CLCs.

Previous studies have shown that miRNAs play important roles in regulating gene expression, development, proliferation, differentiation and apoptosis. Cai et al. showed in a recent study that ADMSCs could promote cardiomyocyte differentiation in the myocardial microenvironment by transducing it with a lentiviral vector encoding miR-1 [30]. miR-1 is suggested to play a key role in heart development and can promote differentiation of cardiac progenitor cells to CLCs. Moreover, the expression levels of miR-1 are crucial for treating ischemic heart diseases [31]. The relationship between miRNA and Notch signaling pathway in cardiomyogenesis has been extensively studied. Notch-1 is considered to be a novel regulator of cell fate decision, which is involved in regulating multiple functions in heart development. The study shows that miR-1 activates Notch pathway thereby promoting differentiation of ADMSCs into cardiomyocyte-like cells. Gene and protein expression levels of Notch-1, cTn1 and GATA-4 were significantly enhanced in miR-1 expressing cells upon co-culture with cardiomyocytes. The obtained results, therefore, proved that miR-1 could promote differentiation of ADMSCs into CLCs. However, no analysis were performed to detect spontaneous beating cells in culture [30].

Bone-Marrow Derived Mesenchymal Stem Cells (BMSCs)

Bone marrow is another potential source of MSCs which can differentiate into functional phenotype of myocardial cells both in vitro and in vivo. As previously described that ADMSCs can be differentiated into functional cardiomyocytes in different approaches, similarly, several methods such as co-culture of stem cells, treatment with cardiac tissue extracts, or culturing with pro-cardiogenic factors have also been studied in the differentiation of BMSCs into functional cardiomyocytes.

In one of the studies, a cocktail method was designed for promoting cardiomyogenic differentiation from bone marrow derived MSCs. Qing Gao et al. showed that a cocktail consisting of 5-Aza, Salvianolic acid B (SalB) and cardiomyocyte lysis medium (CLM) can induce BMSCs to acquire phenotypic characteristics of cardiomyocytes [32]. Cho et al. showed that 5-Aza can increase the expression of GSK-3 β which is known to induce cardiomyocyte differentiation by downregulating β -catenin expression. GSK-3 β has shown to upregulate cardiomyocyte markers and at the same time downregulate the non-cardiogenic markers [33]. A significant increase in the expression of cardiac markers such as cTnT, alpha-cardiac actin, Cx43 GSK-3 β and decreased expression of β -catenin was observed. However, the CLCs did not show any beating property. The investigators hypothesized that soluble factors alone were not sufficient and that physical contact between cardiomyocytes and MSCs was a requisite. Thus,

further investigation is needed to determine whether induction requirements such as pharmaceutical treatment, cell to cell contact and activation of non-canonical Wnt signaling can induce differentiation into beating cardiomyocytes [32].

Another recent study showed a more powerful induction factor, platelet lysate, in inducing BMSCs into beating cardiomyocytes [34]. The unique feature of platelet lysate is that it has a storage vehicle of growth factors as well as differentiation factors [35, 36]. Among these factors, FGF is considered to be a proliferative factor. FGF acts through FGF receptors (FGFR1/FGFR2) and is involved in maintaining cell cycle progression of cardiomyocyte population [37]. Similarly, PDGF, TGF- β and IGF-1 are considered to be differentiation induction factors. Signaling mediated by PDGF/PDFGR α interaction trigger major signaling pathways including MAP kinase, PLC, and PI3K pathways which are involved in cardiomyogenic differentiation [38]. IGF1/IGF1R interaction potentiates Cx-43 expression via activation of PI3K-Akt pathways [39]. The result of this study showed that platelet lysate was able to induce differentiation of BMSCs into myocardial cells at lower concentrations (5%). The study showed increased expression of cardiogenic markers CAA and GATA-4 when treating BMSCs with platelet lysate. Most importantly this paper showed the presence of beating cells thereby indicating the reliability of RT-qPCR and staining assays [34].

Many of the protocols rely on biological or chemical cues to study cardiomyogenic differentiation. Very few studies rely on understanding the mechanical influences on the differentiation of MSCs into cardiomyocytes. One such recent study involved a mechanical stimulus called, cyclic biaxial tensile strength (CBTS) in promoting differentiation of BMSCs into cardiomyocytes. The work revealed that miR-27a which function as a mechano-sensitive miRNA is a key player in this differentiation process [40]. Previous studies reported that miRNAs could regulate mechanical stimulation triggered MSC differentiation [41]. When BMSCs were stimulated with CBTS, a strain of 10% at a frequency 1 Hz lasting for 12 h, the expression of cardiac specific markers such as GATA-4, TNNT2, MEF-2C and Cx-43 significantly increased as observed by RT-qPCR and western blotting data, meantime the expression of miR-27a was decreased and stem cell factor (SCF) was increased [42]. The challenging factor in this study was that they observed a heart rate of 300–400 beats/min which is 5–6.7 Hz but the optimal frequency was 1 Hz. They reasoned that under in vivo conditions heart beat is generally controlled by atrionector and in in vitro conditions cardiomyocytes show their own autorhythmicity without atrionector. More studies need to be performed to answer why 1 Hz was optimal in that study. They also suggested that other factors such as the percentage of longitudinal strain and time of strain could also affect the differentiation process [40].

The electrical microenvironment is also one of the key factors regulating in cardiomyocyte behaviour in vivo. Previous reports have proven that electrical stimulation can trigger mRNA expression of cardiac lineage markers in rat BMSCs, but the mechanism of electrical stimulation (ES) promoting differentiation of BMSCs remains elusive. In a study by He et al., it was demonstrated that electrical stimulation-induced cardiomyogenic differentiation of BMSCs via TGF- β 1 (Fig. 5 A). A huge body of literature has described the important roles of TGF- β 1 in growth, survival and differentiation of various cell types. mRNA and protein expression levels of cardiac markers were significantly higher in the group treated with ES and TGF- β 1 when compared to treatment with ES alone. Although BMSCs showed more flattened morphology, disruption of the organized pattern and spindle-like shape, beating cells were not observed in the groups treated with both ES and ES + TGF- β 1. Therefore, suggesting that ES alone or along with TGF- β 1 is insufficient to induced changes at the molecular level to manifest beating functionality in CLC derived from BMSC [43].

Our lab focused on studying the effects of cardiogel, a cardiac fibroblast derived extracellular matrix, in promoting cardiomyogenic differentiation of BMSCs (Fig. 5 B). Cardiogel is a natural, heterogeneous ECM scaffold derived from in vitro cultured fibroblasts, which is composed of laminin, fibronectin, collagens, growth factors, proteoglycans and other proteins [44–46], that can influence cardiomyocyte growth and differentiation from BMSCs. BMSCs grown on cardiogel coated plates showed the presence of bi and multinucleated cells post 21 days, both with and without 5-Aza induction. ICC analysis revealed an increased expression of GATA-4 and sarcomeric actin even without 5-Aza treatment but the expression was further enhanced upon 5-Aza induction, when compared to BMSCs grown on control (gelatin-coated) plates with and without 5-Aza treatment. To further validate the changed phenotype, semi-quantitative RT-PCR was performed which showed the presence of Cx-43 and 45, Mef2c in all treated BMSC groups except in untreated BMSC grown on control plates. This was confirmed by qRT-PCR which showed noticeably higher expression of GATA-4, Mef2c, Cx-43, Cx-45, BNP and α -cardiac actin in all BMSC group than in BMSC grown on control plates without preinduction. Lastly, the above studies were proved by performing reporter gene assay with pMlc2v-EGFP. pMlc2v-EGFP is a is an expression vector which has a promoter for heart specific variant of Myosin Light Chain (Mlc2v). This vector expresses enhanced green fluorescent protein (EGFP) when cardiomyocytes specific expression of Mlc2v is activated, thereby activating the cardiomyogenic specific differentiation cascade. The data revealed that there was an increase in the number of EGFP positive cells in 5-Aza induced BMSC grown on cardiogel-coated plates when compared to BMSCs grown on control plates following induction.

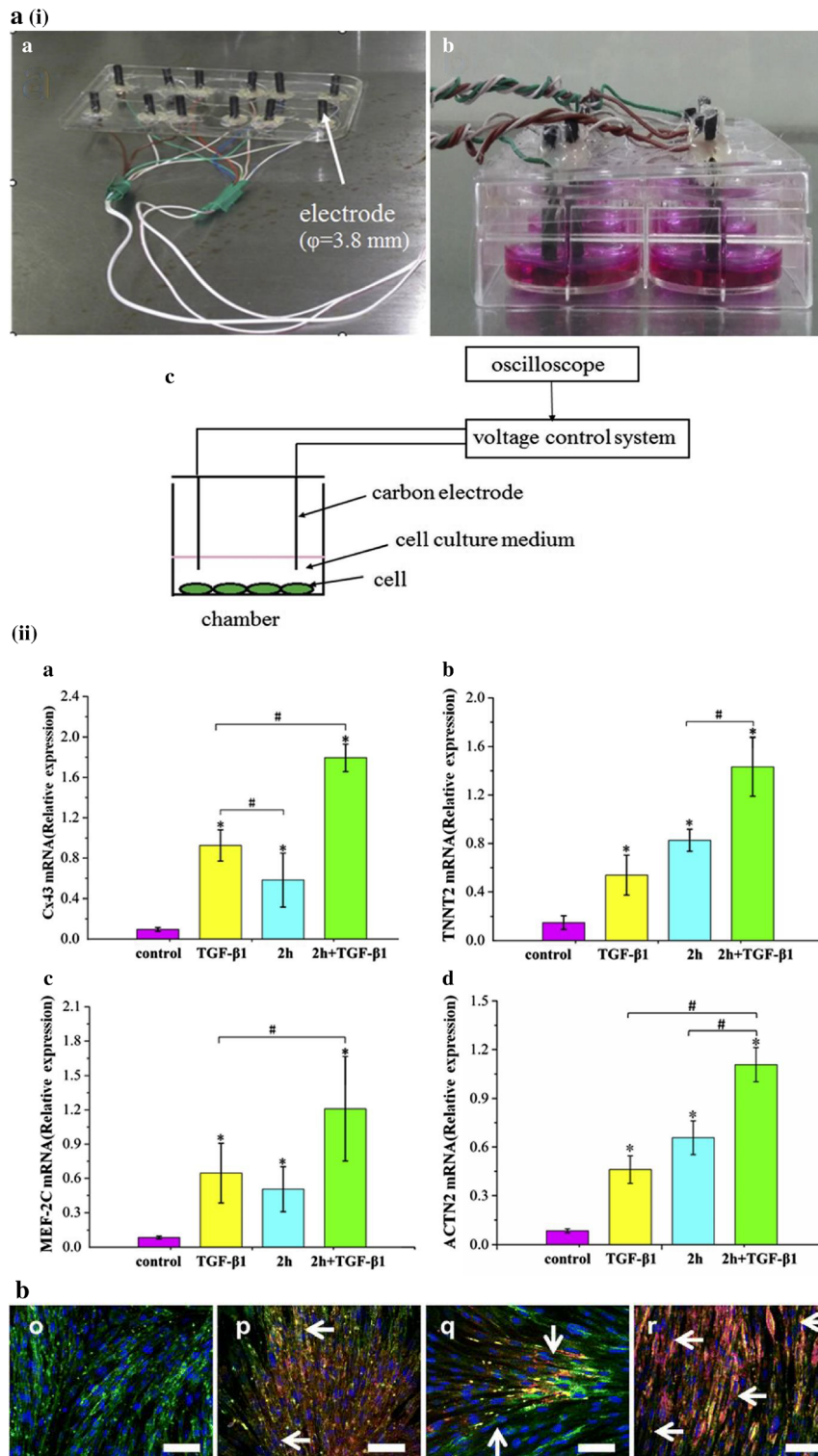


Fig. 5 (A) Effect of electrical stimulation (ES) + TGF- β 1 in CM differentiation. A(i) Biomimetic electrical stimulation device (Top panel). A(ii) Increased mRNA expression of cardiomyocyte specific markers (a-Cx-43, b-TNNT2, c-ACTN2 and d- Mef-2c) in ES + TGF- β 1 group when compared to other groups (bottom panel). This experiment confirmed that the simultaneous exposure of electrical stimulation (ES) and TGF- β to the cells caused increased expression of cardiomyocytes specific gene expression compared to condition when

the cells were exposed to ES or TGF β individually (**reproduced with permission from Ref**) [43]. (B) Cardiomyogenic differentiation of MSC on cardiac fibroblast derived extracellular matrix (cardiogel). (i) and (ii) differentiation on tissue culture plates without and with 5-Aza treatment in the absence of cardiogel respectively, (iii) and (iv) cardiomyocytes like cells derivation from MSC when cultured on cardio gel without and with 5-Aza treatment respectively (**reproduced with permission from Ref**) [47]

EGFP positive expression was also observed in BMSCs grown on cardiogel without induction by 5-Aza. However, no such expression was observed in uninduced BMSCs grown on control plates. This was the first study which showed that cardiogel was able to induce cardiomyogenic differentiation from bone marrow derived MSCs even without chemical induction [47].

One of the recent interesting studies presented a protocol wherein it utilized electrospun type 1 collagen nanofiber mats for cardiomyogenic differentiation from BMSCs spheroids in the presence of 10 μ M 5-Aza [48]. Collagen is the main structural protein constituting about 33% of the myocardium which plays important role in maintaining structural integrity, cellular orientation and providing support and strength to the tissues [49]. It was found that the architecture created a 3D microenvironment that could provide cells with better nutrient perfusion to proliferate and properly differentiate. These nanofibers generally possess a high surface to volume ratio, porosity allowing adequate cell interaction and cellular functions such as proliferation, migration and differentiation. In this study, type 1 collagen was preferred in cardiac tissue engineering as it forms 80% of the myocardial matrix and plays a significant role in inducing BMSCs into cardiomyocytes. Alongside, it also promotes electrical coupling and angiogenesis and reduces other adverse cardiac events [48]. Post 28 days treatment, exposure of BMSC with 10 μ M 5-Aza further increased the cardiac specific marker Cx-43 and sarcomeric alpha-actin. An interesting observation was that the cell orientation and cell alignment were improved over time and with 5-Aza exposure. Further investigations are needed to establish the *in vitro* and *in vivo* capability of this protocol in cardiac tissue regeneration [48].

Aspirin, also known as acetylsalicylic acid (ASA) is also used to treat and prevent cardiovascular disorders such as coronary heart disease by prevention of clotting. Much of this is believed to be inhibiting cyclooxygenase 2 and decreasing the production of prostaglandins. Previous reports have suggested that aspirin is capable of promoting differentiation of many cell types both *in vitro* and in mice models [50]. The study revealed that proliferation inhibition and apoptosis induction of BMSCs were observed in a concentration dependent manner, thus indicating its adverse effects on the proliferation of BMSCs. DNA replication was seen to be inhibited at high dose of aspirin compared to low dose and untreated cells. This study presented for the first time that although aspirin affected the growth and proliferation of BMSCs, it potentiated the cardiomyocyte differentiation of BMSCs. This illustrates the capability of aspirin in inducing cardiomyogenic differentiation of BMSCs. The limitation in this study was, BMSCs when exposed to aspirin, spontaneous beating activity and action potentials were not exhibited. There are no previous reports established which involved the use of small molecules reprogramming MSCs into

cardiomyocytes. But in this study, the results indicated that aspirin had some role in promoting cardiomyogenic differentiation of BMSCs, though the signaling pathway through which it acts remains elusive. Further investigations were needed to answer why these cells were not able to exhibit functional characteristics. Another study explored the differentiation efficiency of aspirin of BMSC. Hao et al. observed that treatment of BMSC showed highest expression of cTnT and Cx43 in 2 mM group compared to other concentration from 0 mM to 5 mM. Thus, suggesting the role of concentration depended cardiomyogenic induction in BMSC [51].

Umbilical Cord Derived Mesenchymal Stem Cells

Over the years, cell therapy has become one of the approaches to treat cardiovascular diseases. In line, many research studies have been dedicated to exploring valuable sources of MSCs including umbilical cord derived MSCs. Umbilical cord derived MSCs may be considered as an alternative source to BMSCs. It has attracted much attention for a variety of reasons: they are easily attainable, non-invasive collection, ease of storage and transport, faster cell renewal, ability to differentiate into a variety of cell types and modulate immune response *i.e.*, it has low immunogenicity characteristic with significant immunosuppressive ability [52].

MSCs Isolated from Wharton's Jelly of Umbilical Cord (WJ-MSCs)

An interesting feature of umbilical cord is that MSCs can be isolated from various compartments of umbilical cord such as Wharton's jelly, umbilical cord arteries (UCA), umbilical cord vein (UCV), umbilical cord lining membrane and sub-amnion and perivascular regions [52].

Work from our laboratory focused on understanding the effect of 5-Aza in driving cardiomyocyte differentiation from Wharton's jelly derived MSCs (WJ-MSCs) by analyzing the differential DNA methylation patterns (epigenetic modifications) in undifferentiated WJ-MSC, 5-Aza-WJ-MSC and human cardiac tissue (Fig. 6 A). Post 30 days induction of WJ-MSC with 5-Aza, the cells exhibited morphology similar to cardiomyocytes. However, functional property like spontaneous beating was not observed. FACS analysis showed a significant expression in GATA-4 (99.60%), α -SMA (98.30%), and cTnT (91.10%) and a very little augmentation in Des (11.3%) and Nkx2.5 (6.1%) in 5-Aza-WJ-MSC group. Promoter methylation analysis revealed that 5-Aza treatment caused hypomethylation of the promoter region of cardiac specific genes (CSGs) as compared to native WJ-MSCs and human cardiac tissue (hCT), thereby suggesting that 5-Aza treatment induces epigenetic changes in WJ-MSCs and thus priming it towards differentiation into cardiomyocytes. Further validation of CSGs from CLCs was analyzed by

semi-quantitative RT-PCR which revealed an upregulated expression of GATA-4, Nkx2.5, Tbx-5 and SERCA in 5-Aza-WJ-MSC group when compared to native WJ-MSCs. However, functional competency was not attained, possibly due to the negligible expression of structural genes. The expression of cardiac specific proteins such as ACTA1, ACTC1, ACTA2, and Mef2c were found to be similar in both native and 5-Aza-WJ-MSCs. This, therefore, indicated that native WJ-MSC inherently expresses cardiomyocyte specific markers, thus making them an ideal candidate for CM differentiation and potential transplantation use [53].

Another work from our lab focused on studying the effects of CHIR 99021, an inhibitor of GSK-3 β , in augmenting the differentiation potential of WJ-MSCs into cardiomyocyte-like cells (Fig. 6 B). Previous studies have shown that CHIR 99021 molecule can aid in the induction of transdifferentiation of ADMSCs into hepatic lineage in vitro. Based on this knowledge, our study hypothesized that CHIR 99021 can also enhance the trans differentiation of WJ-MSCs into cardiomyocytes in a similar manner under specific conditions. RT-qPCR analysis revealed a 3.5-fold upregulation in GATA-4, Nkx2.5 and SERCa2 in CHIR 99021 pre-treated cells when compared to differentiated native MSCs. Differentiated native MSCs expressed GATA-4), Nkx2.5 and SERCa2. Post 28 days in culture, there was a 1.5-fold increase in Myh6 and Myl7 in CHIR 99021 pre-treated cells whereas differentiated native MSCs showed only 0.2-fold in Mhy6 and 0.1-fold in Myl7 respectively. GATA-4 nuclear localization was increased in CHIR 99021 pre-treated cells with prominent expression of Mylc2v which was not evident in differentiated native MSCs. Thus our study proved that CHIR 99021 can enhance CM differentiation from WJ-MSCs [54].

In recent years, cell transplantation strategies have been extensively investigated, but their efficacy remains unestablished in both animal models and humans. A recent study presented the application of PEG/Hyaluronic acid based scaffold with MSC in cardiac regeneration in mice models in vivo [55]. Upon injection of cell/scaffold into the injured myocardium, cell morphology and vessel formation data revealed a high degree of healing in the cell/scaffold group with formation of mature vessels. End systolic volume improved the contractility of the heart in the cell/scaffold group and SPECT results confirmed the presence of beating cardiomyocytes in vivo. At both transcriptional and translational levels, the number of cardiac specific markers such as α -MHC, β -MHC, cardiac troponin T (cTnT), Nkx2.5 was higher in scaffold/cell group. These results, therefore, suggested the differentiation of injected cells into beating cardiomyocytes with no direct evidence of MSC conversion into beating cardiomyocytes in vivo. In some of the previous reports, it has been shown that hyaluronic acid-based scaffolds can directly induce or promote differentiation of a variety of cell types [56]. Many studies in literature characterize poly-

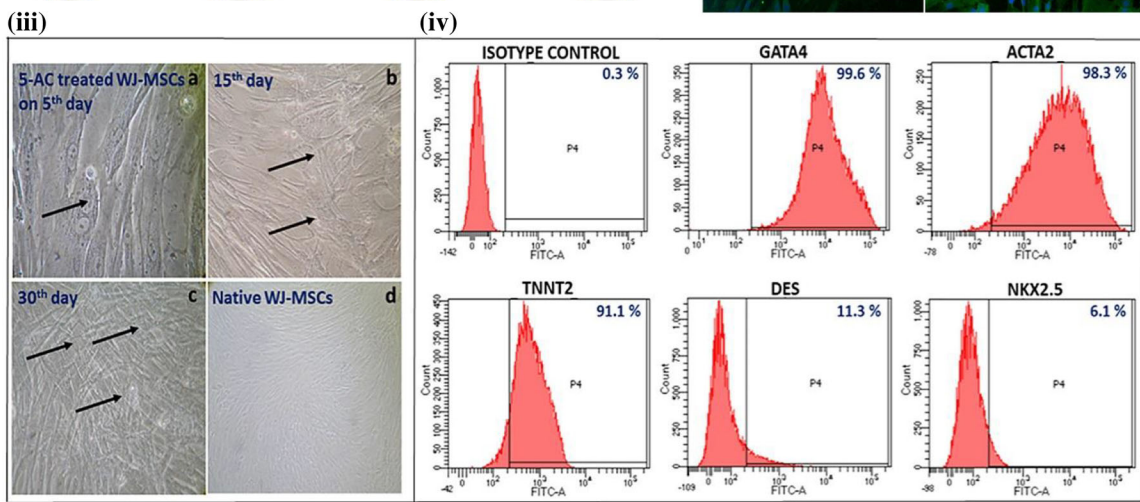
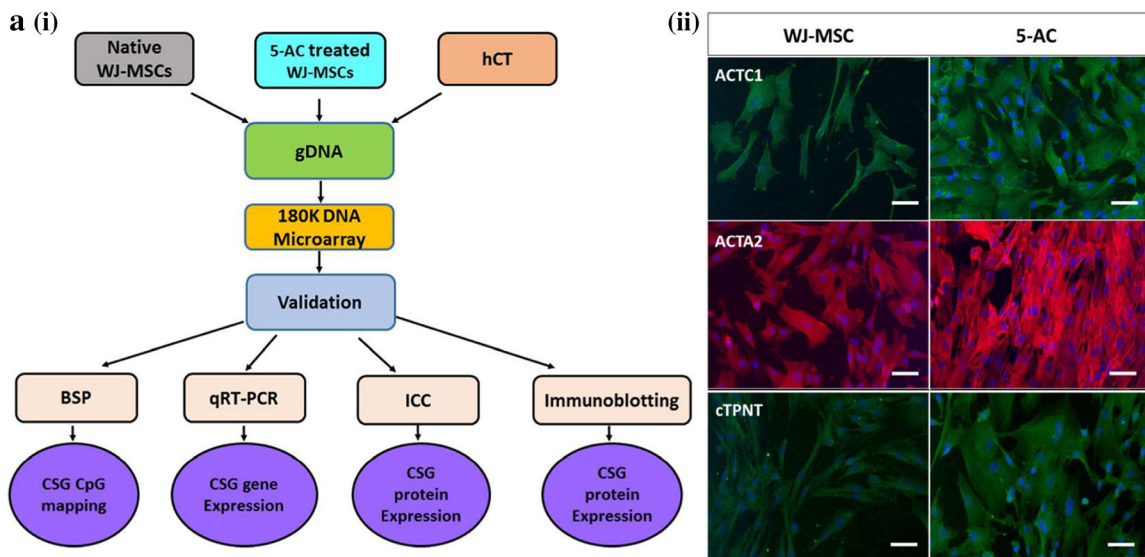
ethylene glycol (PEG) as a supporting matrix with properties to reduce inflammation after implantation [57], increase the viability of cells [58] and promote differentiation of MSCs into cardiomyocytes [59].

MSCs Derived from Umbilical Cord Blood (UCB-MSCs)

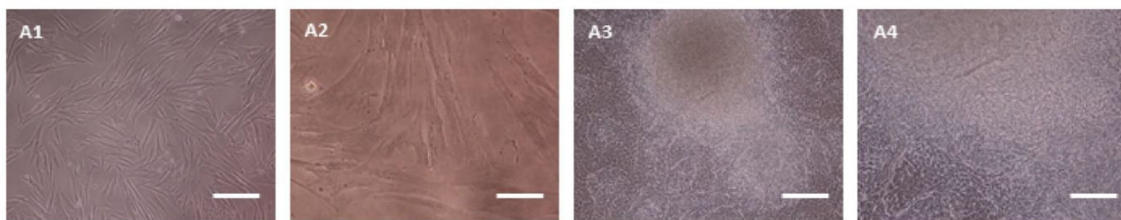
MSCs present in umbilical cord blood are sought to be at a low frequency when compared to their presence in other tissues including bone marrow, adipose tissue and umbilical cord [60]. However recent work has shown that MSCs can be expanded successfully up to 60% with low UCB units. UCB-MSCs have the distinct advantages of being young, safe and healthy, can be easily extracted and possess a lower risk of transmitting viral infections. It also has the unique potential of modulating the immune system. In context of cell-based therapies, UCB-MSCs, due to their high proliferative capacities, need lesser culture time to obtain a fixed number of expanded UCB-MSCs ex vivo [61].

Several investigations have reported the differentiation of UCB-MSCs into cardiomyocytes. a unique study aimed to increase the efficacy of differentiation of UCB-MSCs into heart muscle cells by combining 5-Aza and fetal heart extract (HE) in the induction medium [60]. The results showed that induced cells changed their morphology to a more cardiomyocyte like cell during differentiation in both kinds of induction medium: culture medium plus 5-Aza and culture medium plus 5-Aza and fetal HE. At the transcriptional level, group of cells treated with 5-Aza and HE expressed cardiac muscle specific genes much earlier when compared to cells treated with 5-Aza alone [62]. Some studies have reasoned that differentiated cells derived from MSCs have a poor sarcomeric organization and cannot, therefore, beat spontaneously [63]. Another probable reason cited by the author is that myocardial cells generally undergo three developmental stages: direction stage, precursor stage and maturation stage. Transcription factors and several structural genes are expressed in the precursor stage whereas, in mature stage, muscle fiber structure, tubular structure, ion channels are formed which will stimulate contractile activity. Therefore, the author hypothesized that their cells under study might be in progenitor stage. Few articles have even published that transplantation of beating cells into the heart might lead to arrhythmia, and therefore the investigators again hypothesized that induced cells in the progenitor stage can attain the same rhythm as normal beating cells when other factors are also considered [64].

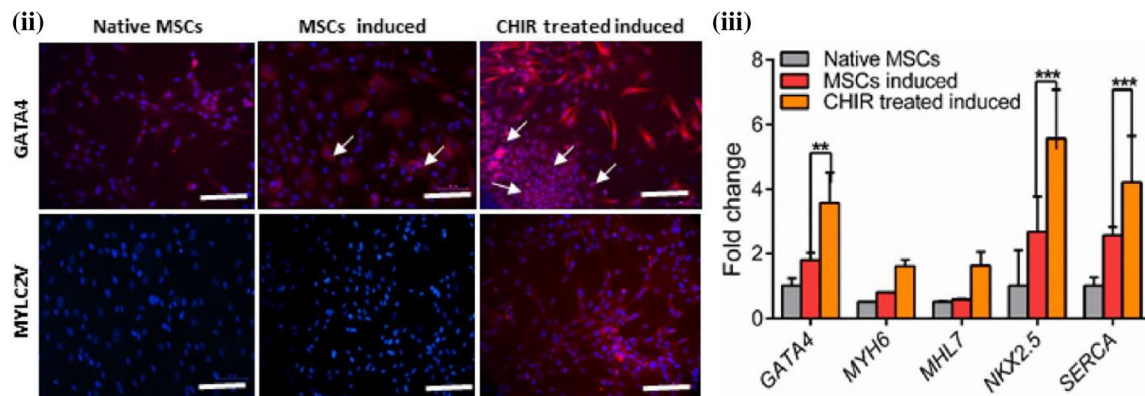
In another study, the investigators attempted to prime UCB-MSCs through supplementation with oxytocin and studied its potential to enhance the cardiomyocyte differentiation both in vitro and in vivo (Fig. 7 A) [65, 66]. Interestingly, the presence of oxytocin receptors on UCB-MSCs led to the hypothesis that UCB-MSCs supplemented with oxytocin could lead to differentiation into cardiomyocytes. UCB-MSCs were



b (i) Morphology of cardiac induced CHIR treated MSCs



Cardiomyocyte specific markers analysis in cardiac induced CHIR treated -MSCs



◀ **Fig. 6** Representative example of the driving role of epigenetic modulator, molecules encompassing growth factors and small molecules and biomaterial for cardiomyogenesis of Warton jelly derived umbilical cord MSC. (A) A study design explaining the epigenetic modulation of MSC upon treatment with 5-Aza. A(i) study plan to explore the effect of 5-Aza on the epigenetic profile of MSC and its comparison with human cardiac tissue. A(ii) MSC treated with 5-Aza showed positive staining for cardiomyocytes specific protein markers. A(iii) morphology of 5-Aza treated MSC. A(iv) Expression cardiomyocyte specific protein marker of MSC derived cardiomyocyte like cells (**reproduced with permission from Ref**) [53]. (B) Role of small molecule on improving cardiomyocyte differentiation potential of MSC. B(i) Morphology of MSC when cultured with CHIR for 30 days. B(ii) Positive staining of CHIR treated MSC-differentiated towards cardiomyocyte lineage. B(iii) Enhanced gene expression of cardiomyocyte like gene from CHIR treated UC MSC (**reproduced with permission from Ref**) [54]

treated with 100 nM oxytocin and cardiac lineage markers were measured by immunofluorescence staining. UCB-MSCs expressed cardiac proteins such as Cx-43, cTnI and α -sarcomeric actin when induced with oxytocin. A co-culture system of oxytocin-MSCs with cardiomyocytes further enhanced the expression of cardiac proteins. To study the in vivo therapeutic effect of shorter and longer supplementation (1 and 7 days) of oxytocin-MSCs, ischemia/reperfusion was induced in a rat model. The UCB-MSCs supplemented with oxytocin for 7 days, improved the cardiac function as evidenced by increase in ejection fraction (echocardiographic study) and reduction in fibrosis (MT staining) thereby ascribing UCB-MSCs to be anti-inflammatory with cardiac regeneration potential. However, no experimental studies were performed to show beating of the induced cells, although previous reports have shown that cardiac progenitor cells when induced with oxytocin for 1 week were able to differentiate into beating CLC [67].

Potential of Very Small Embryonic like Stem Cells (VSELs) as a Cardiomyogenic Source

An emerging alternative to MSCs are very small embryonic like stem cells (VSELs) as these are more primitive in nature compared to MSC. These too overcome the ethical issues related to embryonic stem cells and the tumorigenic potential of iPSCs. Though VSELs were first isolated in 2006, their existence had been a controversial topic for long as few research groups claimed them to be mere artifacts due to their small size and number. However, VSELs presence has now been established along with their mode of origin. The therapeutic effects of VSELs are now being widely studied for tissue and organ regeneration purposes.

VSELs are a small population of quiescent cells present in adult tissues and can be isolated from human umbilical cord, bone marrow and even peripheral blood. These cells are

primitive stem cells that reside in extremely small populations in adult tissues. These mostly remain quiescent under homeostatic conditions and thus serve as reservoirs only to get activated under stress and emergency conditions when body homeostasis is compromised. VSELs express markers like stage-specific antigen (SSEA), nuclear Oct-4A (octamer-binding transcription factor 4A), Nanog, and Rex1 which are also expressed by embryonic stem cells along with the expression of Stella, Prdm14, Fragilis, Blimp1, Nanos3 and Dnd1 genes, which are expressed by epiblast cells. These cells are thought to arise as a subpopulation of primordial germ cells (PGCs) that during migration to gonads, also migrate to adult tissues. The quiescent state of these cells is due to the suppression of regulatory sequences for various paternally imprinted genes due to epigenetic changes, which is also the reason for the absence of teratoma formation in these cells [68].

The quiescent stage of these cells can be reversed by different methods. Initially, the cells were expanded using C2C12 myoblast feeder layer. Lately, chemical molecules like nicotinamide, valproic acid and UM177 have been shown to expand VSELs. Sex hormones like gonadotropin and luteinizing hormone also help reverse the quiescent stage of these cells.

It has been already established the VSELs become significantly higher post myocardial infarction, suggesting the role of these cells in cardiac repair. Dwan et al. studied the therapeutic potential of VSELs by transplanting freshly isolated VSELs into mice MI model. The 6-month model study concluded that VSELs were responsible for the improvement of cardiac function in MI models through paracrine effects as well as through differentiation into myocytes and angiogenic cells. However, the number of new myocytes and capillaries formed were too less to show a pronounced effect [69]. It has also been claimed that CD34+ stem cells, which were initially thought of as transdifferentiation product of hematopoietic stem cells, are actually subpopulation derived from VSELs. CD34+ cells play a crucial role in neo-angiogenesis as well as cardiac regeneration due to their inherent high differentiation potential towards formation of cardiomyocytes and endothelial cells. This further suggests the promising outcomes of using VSELs for cardiac therapy [70].

In a recent study, VSELs were differentiated to cardiomyocytes using 5-Aza treatment. In comparison to untreated cells, VSELs treated with 5-Aza showed reduced expression of pluripotency genes like Nanog, Oct-4, and Sox2 while the cardiomyocytes marker expression (cTnT- and α -actin) was increased. The efficiency of differentiation was similar to that of MSC differentiated through similar method [71]. However, to date, there has been no study where VSELs have been shown to differentiate to beating cardiomyocytes, though their relatively higher primitive nature in comparison to MSCs put forward a greater potential of these cells to produce beating cardiomyocytes.

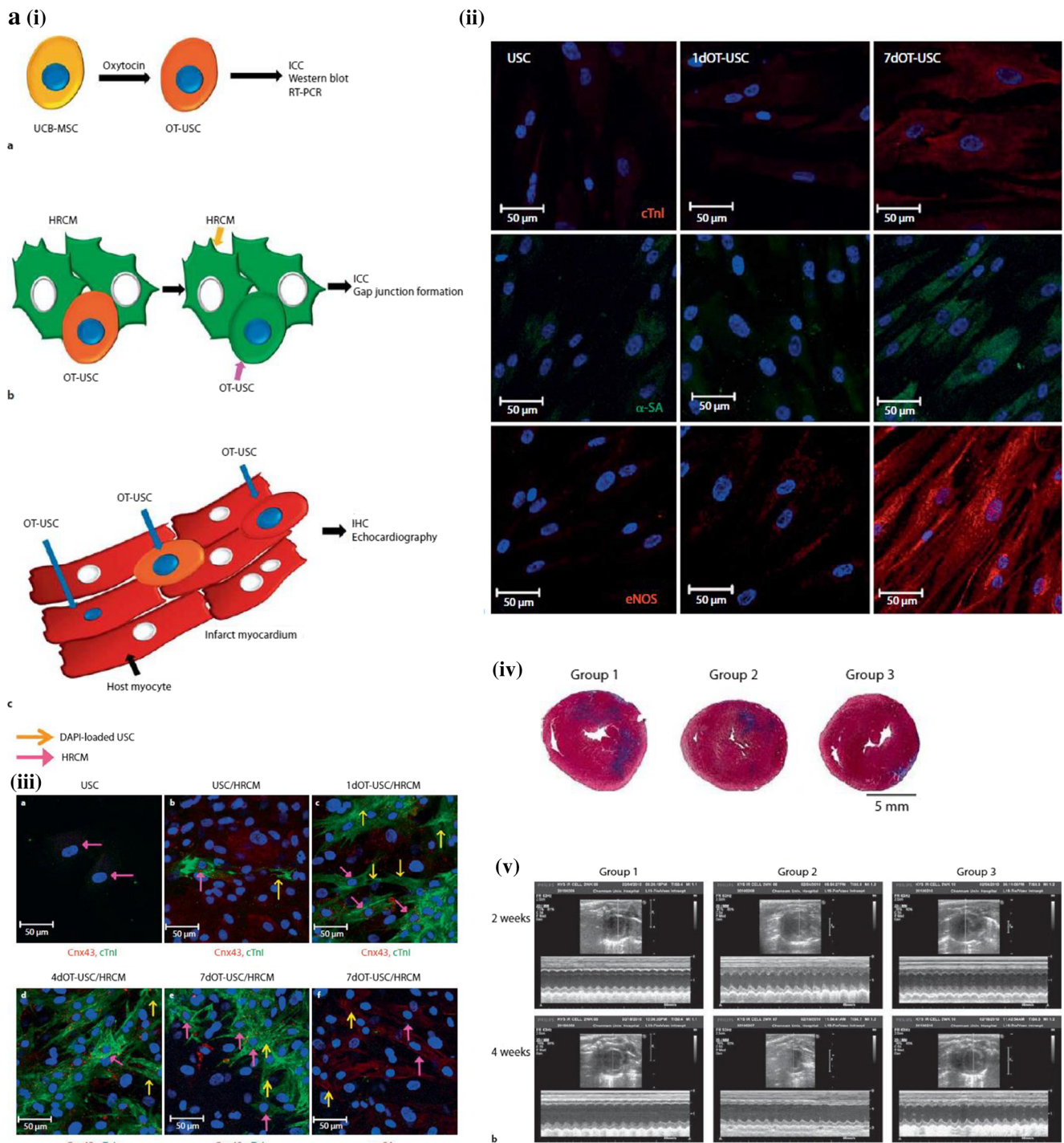


Fig. 7 (A) Effect of UC-MSC priming with oxytocin in enhancing cardiac differentiation and repair. A(i) Schematic representation of the experimental procedure followed to study UC-MSC differentiation. A(ii) IF staining showing increased expression of cTn1, α -SA and eNOS in 7 day OT-MSCs. A(iii) Double IF staining showing increased expression of

Cx-43 and cTn1 in 7d-OT-MSC when cocultured with HRCMs. A(iv) MT staining showing reduction in fibrosis in group 3 (7d-OT-MSC) [Top panel]. A(v) Echocardiographic data showing better cardiac performance in group 3 (**reproduced with permission from Ref**) [65]

Limitations and Challenges of Current Strategies of Using MSC as a Cardiomyocyte Source

MSCs in themselves possess certain limitations for their potential clinical use. Most important factors being the varied differentiation capabilities and immunomodulatory behaviour of MSC derived from adipose tissue, bone marrow and umbilical cord [72, 73, 74]. Functional beating cardiomyocytes have been successfully derived from pluripotent stem cells. The rationale behind using pluripotent state could be justified as during in utero development of heart, the progenitors which result in the mesoderm formation are pluripotent. Cardiomyocytes are derived from mesoderm. This makes the derivation of functional beating cardiomyocytes like cells from pluripotent cells more feasible as the cellular genomic architecture with respect to epigenetic landscape, signalling network is more similar to in utero development of cardiomyocytes [74]. Contrarily, MSCs are multipotent adult stem cells that have limited stemness when cultured in vitro. MSC could be considered to be more differentiated and less potency as compared to pluripotent cells like ESC or iPSC. Thus, the difference in epigenetic landscape, gene regulatory network and mechanism of pluripotency might be different [75].

Use of epigenetic modifiers induces epigenetic landscape in the MSC as observed in a study performed in our laboratory with UB-MSCs. However, the degree of difference is not estimated and requires future studies, comparing molecular differences between MSC and pluripotent cells and identify the difference in potency of MSC as compared to iPSCs. A vivid understanding of transcriptional regulatory networks and epigenetic landscape is essential to elucidate mechanisms and subsequently design strategies to induce beating functionality in cardiomyocytes derived from MSCs.

Factors like use of chemical and growth factors used for differentiation has to be standardized with rationale supporting their use either alone or in combination sequentially [76]. Another factor is optimization of time required to attain functionally mature beating cardiomyocytes as in the literature differentiation time varies anywhere between 7 days to 36 days (Table 1).

Potential of MSCs as Factory for Angiogenesis

Apart from its cardiomyogenic differentiation potential, recently role of MSC in inducing angiogenesis has been extensively studied and therefore provides an alternative regenerative strategy to induce regeneration of damaged myocardial tissues. It has been well documented that stem cell-based therapy is one of the promising strategies which could promote de novo formation of new blood vessels to the ischemic myocardial

tissue. As established by recent researches, the two major ways by which mesenchymal stem cells could potentiate angiogenesis are either by the production of paracrine bioactive signaling molecules (secretory role) or transdifferentiating into endothelial cells and pericytes (cellular role).

Secretory Role

MSCs on stimulation can secrete a wide range of signalling molecules including growth factors, chemokines and cytokines, some of which were found to play important roles in angiogenesis. Among the MSC-secretome, VEGF is the principal proangiogenic molecule that has been shown to modulate angiogenesis through its interaction with VEGFR2. This initial interaction stimulates the activation of various downstream signaling pathways in endothelial cells (ECs) relevant to angiogenesis including Ras/MAPK, PI3/Akt, FAK/paxillin, RhoA/ROCK, PLC- γ , NCK, SCK, RAK, ERK and mTOR which are involved in regulating sprout formation and vessel extension thereby resulting in formation of new capillaries and promoting vascular permeability to the ischemic myocardial tissue region [77–79]. Few reports have demonstrated that the chemokine SDF-1 plays a major role in revascularization of the ischemic tissue. This constitutive and inducible expressed chemokine interacts with its cognate receptor CXCR4 on endothelial cells (ECs), thereby, favoring revascularization, by promoting stimulation of EC proliferation and capillary tube formation [80]. The SDF-1/CXCR4 signaling pathway also modulates angiogenesis by influencing the expression of various pro-angiogenic factors including VEGF-A, IL-6 and IL-8. HGF is also capable of promoting local angiogenesis [81]. The underlying mechanism by which HGF promotes angiogenesis is dependent on their interaction with tyrosine kinase receptor c-met present on ECs and SMCs [82]. HGF/c-met signaling activates multiple downstream pathways including FAK, STAT, PI3K/Akt, and Ras/MEK pathway which directly or indirectly stimulates ECs, thereby, promoting EC proliferation, migration and vascular mural cell formation [83]. It has also been reported that MCP-1 and IL-6 from the conditioned medium of MSCs could stimulate angiogenesis both in vitro and in vivo. This effect was however inhibited by the pre-treatment of neutralizing antibodies against MCP and IL-6, thereby, emphasizing its pro-angiogenic activity [84]. The paracrine growth factor PDGF amplifies angiogenesis by its direct interaction with PDGFR- β present on ECs thereby regulating its proliferation, migration and sprout formation. PDGF can also control the angiogenic process by recruiting other cells like monocytes which can secrete angiogenic molecules (PIGF, EPO, TSP-1 and PGE2) and hence facilitating EPC migration and homing, stimulating its differentiation and promoting vessel growth to the ischemic myocardial region [85].

Cellular Role

Another way by which MSCs promote angiogenesis is through their transdifferentiation ability into either endothelial cells or pericytes [86–88]. Numerous reports have confirmed the pluripotent nature of MSCs that can potentially differentiate into endothelial lineage cells with different efficiencies. One of the recent studies reported an efficient method to induce MSC differentiation into ECs. This method incorporated an induction medium containing a cocktail of cytokines (VEGF, bFGF, EGF and IGF) to induce MSC differentiation [89]. A study implied that VEGF promotes endothelial differentiation of MSC by regulating Rho/ROCK signaling pathway [90]. EGF is known to be a potent contributor to EC migration, maturation and sprout formation. EGF promotes angiogenesis by activating PI3K and MAPK pathways. IGF, though not much potent than other growth factors, it is considered to be a main component in inducing differentiation. The potential role of IGF in angiogenesis is yet to be fully elucidated, however, is partly thought to occur via the activation of PI3K and GSK-3 β pathway [91]. Another research group has proved that nitric oxide signaling and sonic hedgehog also play an important role in inducing endothelial differentiation of MSCs [92]. Few reports have also demonstrated the usage of human platelet lysate as an alternative to serum (due to its potential pathogen transmission during culture and immunological reactions) as hPL contains various growth factors that can further enhance the differentiation potential of MSCs under cultivation [93]. New interesting research has shown that mechanical stimuli such as shear stress and cyclic strain can induce MSC differentiation into ECs. Therefore, delineating the differentiation process can offer more effective approaches for the treatment of cardiovascular repair [94].

Lastly, MSC could also be differentiated into pericytes as established by 3 separate studies. Pericytes are perivascular cells that form a close association with endothelial cells playing crucial roles in blood-vessel formation, maturation and stabilization of newly formed vessels thereby favouring angiogenesis [99–101].

MSC-Derived Exosomes

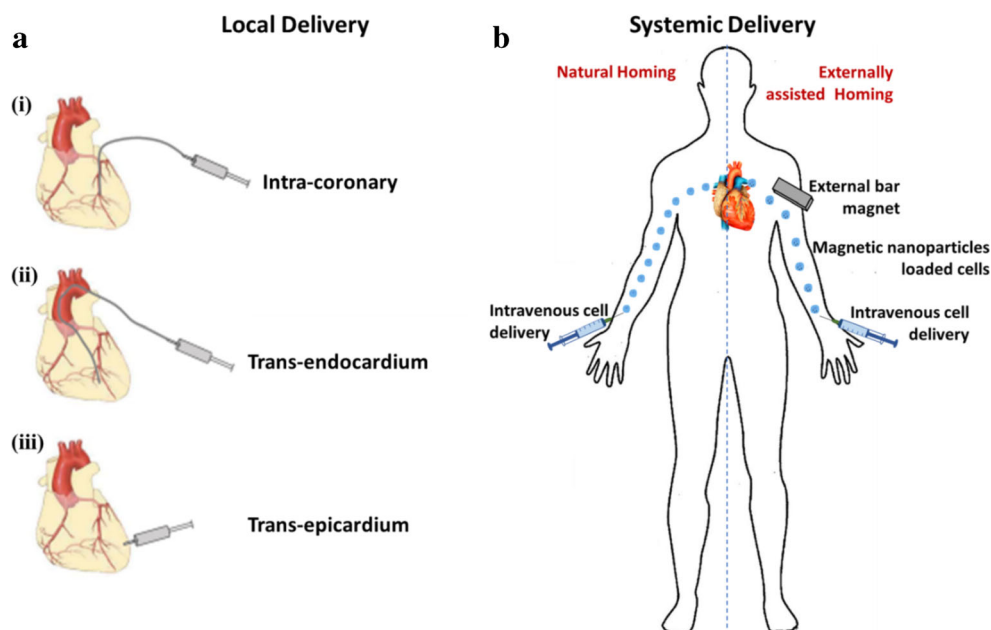
Currently, the strong paracrine capacity of MSCs has been implicated as the principal mechanism for the therapeutic modality in cardiovascular diseases, although at least in part is mediated by MSCs stimulation of resident cardiac stem cells and fusion of MSCs with cardiomyocytes (CMCs) leading to the replacement of lost cardiac cells [95]. The paracrine factors of MSCs are secreted directly or via membrane enclosed structure known as exosomes. At present, the research in MSC derived Exosomes (MSC-Exo) is gaining much interest in the field of regenerative medicine including cardiovascular repair. Exosomes are extracellular vesicles of endosomal origin that

carry a diverse array of signaling molecules to influence the recipient cells' functional activity. It appears that MSC-Exosome's membranes are rich in cholesterol, ceramide and sphingomyelin which makes them an ideal vehicle for the transfer of complex cargo molecules to the recipient cell leading to an alteration in the cells' functional activity via activation of different signaling pathways and ultimately resulting in the co-ordinative function in that cell [96]. Besides, the efficiency of exosome uptake by the recipient cell not only depends on the exosome's membrane composition but also is correlated to the recipient's intracellular pH which explains the possibility that MSC-Exo are preferentially endocytosed by ischemic cardiomyocytes which have relatively low intracellular pH. Also, exosomes contain many cell surface molecules which can engage with different cell receptors thus allowing for the exchange of cargo between the cells.

Proteomic analysis, transcriptomic analysis, miRNA array analysis or any other HTS technology can be utilized to deduce the contents in the exosomes. Over 900 species of proteins, >7540 RNA (coding, non-coding and miRNA) and >1116 lipid molecules are discovered according to the current version of ExoCarta depending upon different physiological and pathological conditions and cells types and which has proven to contribute to anti-apoptotic, immunoregulation, anti-cardiac remodeling and angiogenesis effect [97]. For example, reduced ATP production and increase in oxidative stress are the major cause of cardiomyocyte apoptosis post myocardial I/R injury. Lai et al. demonstrated that MSC-Exo contained enzymes of ATP production phase of glycolysis pathway which led to an increased ATP production in ischemic CMCs and thereby inhibiting further cardiac cell apoptosis [98]. They also showed that CD73, a surface molecule on exosomes, could activate adenosine receptors on CMCs resulting in activation of Akt/GSK-3 β survival signaling pathway [98]. Another study carried out by Yu et al. showed that miR-19a cargo present in MSC-Exo activated Akt survival pathway by inhibiting PTEN and miR-221 cargo inhibited cardiomyocyte apoptosis by downregulating PUMA [99]. The anti-inflammatory effect of MSC-Exo was investigated by Li et al., and showed that the presence of peroxiredoxins and GSTs limit the oxidative stress in CMCs and inhibit further inflammatory response [100]. Cardiac remodelling is defined as molecular and cellular changes in terms of size, mass, geometry and function post MI injury [101]. It is thought that angiogenesis is one of the major components in maintaining the functionality of the ischemic myocardium. One such study conducted by Vrijssen et al., reported that three proteins (VEGF, EMMPRIN and MMP-9) secreted from MSC-Exo, amplified angiogenesis, thereby promoting faster tissue repair [102].

To conclude, the therapeutic potential of exosomes is vast as they are non-viable and do not differentiate into an inappropriate cell type which can lead to the formation of tumors. This kind of cell-free approach is considered advantageous

Fig. 8 Schematic representation of different cell delivery routes to the infarcted heart. (A) local delivery by injecting MSC directly to the heart at through various loco-reginal route (i) Intra-Coronary delivery, (ii) Trans-Endocardial delivery, (iii) Trans-Epicardial Delivery. (B) Systemic cell delivery where MSC are injected into the systemic circulation through intravenous injection and utilizing the homing capability of MSC towards injured heart



over any other stem cell transplantation for tissue repair and regeneration.

Delivery Strategies of Cells

The numerous roles of MSC based on its paracrine activity underscore the direct role of MSC in cardiac regenerative. It also becomes essential study about the delivery strategies of MSC to heart. Therefore, delivery of cells to the injured area of the heart is critical for the successful outcome of the therapeutic intervention. The goal of an ideal delivery strategy is to deliver optimal number of cells targeted selectively to the injured area through a minimally invasive route.

Injectable Cell-Based Therapy

Even though various cell delivery routes have been explored, but there is no clinically effective delivery route to date. So far there have been two major strategies to deliver mesenchymal stem cells directly to the infarcted heart in preclinical and clinical studies i.e., intra-myocardial injection (transendocardially or transepically) or intra-coronary infusion. Apart from these two routes, other routes have also been explored [103]. It includes systemic infusion of stem cell through intravenous route and relies on the homing ability or external stimuli assisted delivery of cells to the injured heart (Fig. 8). Although systemic infusion of MSC has been explored in case of acute MI, the systemic route of delivery for stem cells to the infarcted heart is not ideally suited for chronic MI. This is due to the fact that chronically infarcted heart show downregulation of homing factors and chemoattractant which are required to recruit the cells from the systemic circulation

into the infarcted area of the myocardium. Moreover, there is more nonspecific distribution of systemically infused cells and therefore the dosage achieved at the infarcted area is not sufficient to improve the condition physiologically. Initially, intravenous infusion of the stem/progenitor cells appeared effective, lower cell retention due to the non-specific homing and redistribution has strongly challenged the successful use of this procedure which limits its use in the clinics for MI intervention [104].

Localized Cell-Based Therapy

With the recent understanding of stem cell biology and the ability to derive cardiomyocytes using various differentiation strategies, use of stem cells has been extensively explored. The exact mechanism by which the stem cells repair the injured heart remains completely unclear. However, two mechanisms have been proposed and accepted in principle for the mechanism involving heart repair. First is the direct differentiation of delivered stem cells into cardiomyocytes/endothelial cells and the second one is the stimulation of repair mechanisms like accelerated growth and proliferation of healthy functional cardiomyocytes/blood vessel endothelial cells by paracrine activity of the transplanted stem cells. Use of stem cells offers a much better prospect in repairing the injured heart. Recent reviews have elucidated very profoundly the various types of stem cells used in the repair and regeneration of damaged heart [105].

A minimal-invasive technique inspired by the trans-vascular approaches in interventional cardiac procedure is intracoronary delivery. Intracoronary delivery method delivers higher cell quantity to the injured site compared to the systemic infusion (Fig. 8 A). In this procedure, cells are administered via the lumen

of coronary artery using a block balloon technique. This technique is a brief balloon occlusion which is achieved by an over-the-wire balloon catheter. This reduces spill-back of the injected cells and, in turn, provides optimal time for the cells to make contact and extravasate in the infarcted area across the coronary vessel wall. Intracoronary delivery is only feasible in patients with patent vessels. Besides, the use of this delivery route is limited by a complex multistep process of cellular adhesion on the vessel wall, and transmigration to allow the infused stem cells to reach the infarcted area. The homing of the stem cells is also dependent on the presence of the chemoattractant which is secreted from the infarcted tissue. This is supported by the study that when EPC was infused through intracoronary route, its homing increased in patients with acute MI compared to the chronic MI [106].

Intra-myocardial or transepicardium cell injection, on the other hand, overcomes certain limitations observed in the intra-coronary cell injection. Cells can be injected into the infarcted myocardium from the trans-epimyocardial side, a procedure which can be performed in open heart surgery, or trans-endomyocardial side by using needle tipped catheter. Using this method, larger cells like MSC and SM can be delivered easily to the infarcted area. Studies have suggested that intramyocardial cell injection improves the condition at the actual site of cell delivery. Therefore, this mode of cell delivery implies that this may be effective when targeting specific regions of the myocardium, i.e., in or around the chronic scar due to MI. Preclinical studies have shown that cell retention in the infarcted myocardium is up to several folds high compared to intracoronary infusion, with no significant variation between trans-epicardial and trans-endocardial routes. Eventually this, in turn, results in less non-specific distribution of cells in other organs and gives a better therapeutic effect. The disadvantage related to this route of delivery is that the cells are delivered in clumps which limits its homogeneous distribution across the infarcted regions. Therefore, this can compromise the regeneration of total infarcted area in a given dose of cells. Such non-homogeneous distribution can also cause small disturbances to myocardial architecture or create niduses for arrhythmia [107].

Apart from non-homogeneous distribution, injection-related puncture of the viable or necrotic myocardium can cause risks, i.e., of ventricular perforation. In this scenario, an elegant and now commonly used catheter-based option is to guide the endomyocardial injection through electromechanical mapping (EMM, e.g., NOGA mapping). This technique enables to focus the delivery of cells to ischaemic but viable (hibernating) myocardium [104].

Systemic Cell-Based Therapy

Systemic delivery refers to the *in vivo* route of delivery by administration of deliverables into the bloodstream from

where the home to the affected area (Fig. 8 B). This is an excellent non-invasive mode of delivery for MSCs highly preferred by physicians as this does not cause further injury to the already afflicted heart. Another important advantage of systemic intravenous based delivery is that it allows for repeated administration of cells from time to time as per requirement contrary to invasive/ partially invasive site-specific delivery strategies that allow one-time implantation generally insufficient for efficient curing of disease [108]. Experimental and clinical trials have been widely conducted that show improved heart function as well as structural recovery post intravenous delivery of MSCs [109].

Behnaz et al. 2020, determined the effects of intramyocardial and intravenous injections of MSC on cardiac regeneration post ISO-induced heart failure in rat models. Results showed that intravenous and intramyocardial administrations of cultured MSCs had similar effect on improvement of cardiac function and prevention of fibrosis [110]. The systemic delivery method hugely relies on homing of stem cells via signals arising from area of injury. Dror et al. 2017, conducted a study to monitor the effect of intravenously administered MSCs on left ventricular function post myocardial infarction and ischemic cardiomyopathy. The intravenously administered MSCs were able to reverse the deterioration of left ventricle and remodelling of the injured tissue through MSC-induced immunomodulatory effects [111].

Dong et al. 2019, further exploring the systemic route of MSC administration, identified MSC subpopulation that had better proliferative and migratory capacity as compared to others. The study concluded that MSCs which possess CD51 marker (CD51+ MSCs) have better proliferation capacity, multi- differentiation potential, therapeutic potential and preferential migration toward infarcted heart as compared to CD51- MSCs [112].

Not only the effect of systemically administered cells but their time course of homing and distribution once injected into the body has also been studied. Ana et al. 2010, studied the kinetic and body distribution of MSC injected intravenously by labelling the cells with radioactive ^{99m}Tc-HMPAO. After 5 min of injection, cell number detected in infarcted heart model was greater as compared to sham model. The result indicated the importance local microenvironment that serves as a guiding cue for homing of cells. However, after 60 min, the MSC number in the infarcted model was similar to control. As the MSC injection showed improvement in cardiac functions of the infarcted model, one can say that MSC exerts its beneficial effects through paracrine signaling processes or by activating cardiac factors/cells in the infarcted area. It was also noticed that majority of cells were trapped in lungs, which comes across as a major disadvantage of this delivery method [113].

As described in the above study [113], one big disadvantage studied for intravenous delivery is that many

cells get trapped in lungs and other vital organs. Walazak et al., 2008, reported the occurrence of vascular occlusion through systemic delivery of cells as cells with larger size were able to escape intracerebral entrapment [114]. Therefore, intravenous MSC injections with externally guided stimuli/ have been lately explored for non-invasive systemic delivery where the movement of injected cells is manipulated using an external guiding agent to home the cells to the infarcted area. Annika et al. 2017, devised a systemic delivery system for cells through the use of a combination of magnetic nanoparticles and customized magnetic bars. The study incorporated making use of enhanced green fluorescent protein (EGFP) labelled stem cell derived cardiomyocytes which were loaded with magnetic nanoparticles composed of magnetite core and silica iron oxide shell. Optimal positioning of external bar magnet was accomplished through a series of *in vitro* simulations. A 3.4-fold increase in engraftment rate along with continuous improvement of left ventricular function was seen 8 weeks post transmission. The process was thus able to achieve improved short and long-term engraftment rates [115].

Biomaterials Used for Cell Delivery

The objective of cell delivery is successfully attained if it is loaded with a biocompatible biomaterial and is then injected into the infarcted area. Biomaterials provided structural support and other physical and biological support to the cell which is essential for proper cellular viability and delivery. Biomaterials for use in myocardial tissue regeneration are derived from either natural or synthetic sources. Natural biomaterial possesses better biocompatibility, bioactivity, biodegradability and bio-inertness. However natural biomaterials come with a limitation of its variability in batch-to-batch production and limited control over its quality control. Therefore, it becomes difficult to control its quality in mass production. This limitation is overcome in synthetic biomaterials in which the batch-to-batch variation is very less and the chemical and physical property can be fine-tuned depending on the fabrication and delivery strategy of the cells to the infarcted area. However, they don't exhibit better biocompatibility and bioactivity as compared to natural biomaterials. A list of biomaterials has been described and been extensively used in cell delivery for infarcted myocardium tissue regeneration [105, 116].

Natural Biomaterial

One of the important features that is considered while selecting biomaterial for cellular delivery is the cell-biomaterial interaction. Primary cells are adherent cells and die off if there is no interaction between the cell-

matrix. In this regard, many naturally occurring biomaterial have the motif that is essential for cellular interaction and adherence. Such biomaterials are collagen, gelatine, silk fibroin, decellularized tissue/organ ECM [117]. These materials have peptide sequences that are essential for integrin (cell surface molecules) interaction which eventually leads to cellular adherence and cell-matrix crosstalk through multimodal signaling pathways. Thus, cell-biomaterial interactions are more predominant in case of natural biomaterial, resulting in favourable cell proliferation and differentiation. Decellularized ECM-derived hydrogels are prominent natural biomaterial which shows remarkable angiogenesis and myocardial regeneration because of the intrinsic presence of growth factors, fibronectin and glycosaminoglycan (GAGs) [117]. Decellularized heart derived hydrogels have been used in rodent model which showed improved angiogenesis and myocardial function. Silk fibroin is another naturally occurring protein-based biomaterial that is derived from silkworms. Nonmulberry silkworm derived silk fibroin has intrinsic RGD peptide sequence which promotes stem cell adhesion, proliferation and differentiation [118].

Synthetic Biomaterial

Synthetic biomaterials have certain advantages over natural counterparts which include tuneable mechanical, chemical and biological properties. Certain synthetic biomaterials have been shown to improve ventricular function of infarcted heart upon injection in the infarcted region. Different forms of poly (N-isopropylacrylamide) (PNIPAm) upon injection, have shown an increase in the left ventricle end diastolic diameters (LVEDD) and decreased EF compared to sham [119]. Similarly, researchers demonstrated that injection of poly (ethylene glycol) (PEG) hydrogel decreased dilatation observed in saline-injected hearts by increase in LVEDD [120]. Conductive polymers are another set of synthetic polymers which have been shown to have significant effect and application in stem cell differentiation towards cardiomyocytes [121]. Modification of synthetic biomaterials can fine tune the desired condition anticipating better bioavailability, cellular proliferation and differentiation. Pure synthetic biomaterials do not have cell binding motif. Such adhesion properties can be easily introduced into the synthetic biomaterials by covalently grafting cell binding peptide sequences on the biomaterial. Similarly, various functional groups can be grafted covalently on the synthetic polymer which can be further utilized to link multiple biomolecules, pharmaceutical molecules on the synthetic material to promote not only cellular proliferation and differentiation but also promote angiogenesis by delivering growth factors in a spatiotemporal dependent manner [120].

Natural polymers vary in composition and lack mechanical strength. Therefore, a composite strategy has been extensively used to create composite biomaterial of natural and synthetic polymers for cell delivery. Such composite materials have better biocompatibility, mechanical properties and feasibility of grafting molecules on the hydrogels derived from the composite material [122]. For example, an injection of collagen and fibrin blended with alginate prevented expansion of an infarct [123].

Advantages and Disadvantages in Cell Delivery

Although there are many research articles in the scientific domain pertaining to cell delivery to infarcted regions, however, every strategy comes with certain advantages and disadvantages. Those have been briefly described below.

Advantage

- *Cell delivery* by injection can be minimally invasive due to the recent advances in medical technology pertaining to cardiothoracic surgery.
- *Cell Dose* – A high dose of cells can be administered to a localized region specifically on the infarcted heart.
- *Delivery Route* – Cell delivery by injection has multiple modalities of delivery which can be decided depending on the patient's condition and the urgency of delivering cells to the infarcted heart.
- *Multiple factors* – Apart from the cell delivery, other biological macromolecules like growth factors can be delivered along with the cells by encapsulating them in appropriate polymer-based matrix. This helps not only to generate cardiomyocytes but also induce angiogenesis.

Disadvantage

- *Cell Distribution* – The delivery of cells by injection is not uniform throughout the infarcted region. The administered cell forms clump and grows in a localized manner. Therefore, to administer cell in optimum dose across the infarcted area, multiple dose injection is required
- *Damage to Native Tissue* -Injection might damage the native cardiomyocytes and even lead to puncture depending on infarction size. The infarction damage reduces the mechanical stability of the ventricular region. So, differences in the injection depth may lead to perforation which might not be anticipated.

- *Delivery Route* – Trans-epicardial can be administered during open heart surgery procedure. Therefore, a more viable strategy is demanded where cells can be injected with minimal invasion
- *Non-Homogenous Cell Distribution* – Although a high dose of cells can be administered, but the distribution is not across the infarcted region. This can lead to the development of arrhythmias.
- *Biomaterial* – Although there have been reports of a variety of biomaterials both natural and synthetic and composite used for cell delivery, it is yet to be established that which is best. Such unavailability of the information can be attributed to the fact that multiple factors are varied like cell source, cell type, infarction model, patient's physiological and heart condition variability. So far, no such comparative detailed study is there which can be used on a standard basis for general or specific cell delivery to the heart.
- *Low homing efficacy* and it becomes difficult for the injected cells to survive in the hostile microenvironment because of the low vasculature, hardened tissues caused by scars, and inflammation processes. The poor survival rate has been attributed to continued ischemia within the graft and surrounding tissues.

Conclusion and Future Direction

Although the use of mesenchymal stem cells in clinical trials have been extensive for the repair and regeneration of injured heart, the mechanism behind the repair and regeneration have been owed to mostly its paracrine activity despite a good amount of evidence suggesting the ability of the MSC to differentiate into cardiomyocytes like cells. The major problem with using MSCs as a source of cell to derive functional beating cardiomyocytes is the lack of a well-defined and designed protocol and strategy and so far, there has been very limited number of papers confirming the ability of MSC to form functional beating cardiomyocytes. If at all we have to focus on the ability of MSC to provide consistency and highly efficient derivation protocol for deriving functional cardiomyocytes, we have to use a multitude approach of mimicking the developmental strategy of cardiac tissues development using MSC as the cell source. The protocol has to be expanded beyond using 5-Aza and a mixture of growth factors. However, the delivery and homing ability of MSC to the injured heart tissue can be exploited not just to repair the injured heart but can also help in delivering cargoes to the damaged heart.

Abbreviations *MSCs*, Mesenchymal stem cells; *CLC*, cardiomyocytes like cells; *CMCs*, cardiomyocytes; *iPSC*, induced pluripotent stem cells; *ESC*, Embryonic Stem Cells; *ADMSC*, Adipose-derived Mesenchymal stem cells; *CD*, Cluster of Differentiation; *BMSCs*, Bone Marrow derived Mesenchymal Stem Cells; *WJ-MSCs*, Wharton's jelly derived Mesenchymal stem cells; *UCB MSC*, Umbilical Cord Blood derived Mesenchymal stem cells; *VSEs*, Very Small Embryonic Like Stem Cells; *MI*, Myocardial Infarction; *ECM*, Extracellular Matrix; *5-Aza*, 5-Azacytidine; *DMSO*, Dimethyl Sulfoxide; *MSC-Exo*, MSC derived Exosomes

Acknowledgements SG would like to acknowledge ICMR for fellowship (3/1/3/JRF-2015/HRD-LS/90/40282/91). The authors would also like to acknowledge the support by the Department of Biotechnology (DBT), India for their funding and support (BT/PR8587/MED/31/236/2013).

Availability of Data and Materials Not applicable.

Authors Contributions SG - Conceptualization, Literature Study, Manuscript preparation; AS - Literature Study, Manuscript preparation; AJ - Literature Study, Manuscript preparation; RSV - Conceptualization, Literature Study, Manuscript preparation.

Funding Indian Council of Medical Research (ICMR) and the Department of Biotechnology (DBT), India.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Competing Interests The authors declares that they have no competing interest.

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