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## Methods to produce induced pluripotent stem cell-derived mesenchymal stem cells: Mesenchymal stem cells from induced pluripotent stem cells

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### Abstract

Mesenchymal stem cells (MSCs) have received significant attention in recent years due to their large potential for cell therapy. Indeed, they secrete a wide variety of immunomodulatory factors of interest for the treatment of immune-related disorders and inflammatory diseases. MSCs can be extracted from multiple tissues of the human body. However, several factors may restrict their use for clinical applications: the requirement of invasive procedures for their isolation, their limited numbers, and their heterogeneity according to the tissue of origin or donor. In addition, MSCs often present early signs of replicative senescence limiting their expansion *in vitro*, and their therapeutic capacity *in vivo*. Due to the clinical potential of MSCs, a considerable number of methods to differentiate induced pluripotent stem cells (iPSCs) into MSCs have emerged. iPSCs represent a new reliable, unlimited source to generate MSCs (MSCs derived from iPSC, iMSCs) from homogeneous and well-characterized cell lines, which would relieve many of the above mentioned technical and biological limitations. Additionally, the use of iPSCs prevents some of the ethical concerns surrounding the use of human embryonic stem cells. In this review, we analyze the main current protocols used to differentiate human iPSCs into MSCs, which we classify into five different categories: MSC Switch, Embryoid Body Formation, Specific Differentiation, Pathway Inhibitor, and Platelet Lysate. We also evaluate common and method-specific culture components and provide a list of positive and negative markers for MSC characterization. Further guidance on material requirements to produce iMSCs with these methods and on the phenotypic features of the iMSCs obtained is added. The information may help researchers identify protocol

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options to design and/or refine standardized procedures for large-scale production of iMSCs fitting clinical demands.

**Key Words:** Mesenchymal stem cells; Induced pluripotent stem cells; Mesenchymal stem cells derived from induced pluripotent stem cells; Differentiation methods; Culture components; Mesenchymal stem cell markers

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**Core Tip:** Heterogeneity of mesenchymal stem cell (MSC) quality might have hampered the robust success of stem cell clinical trials (CTs). The production of MSCs from a single homogeneous source (*i.e.* induced pluripotent stem cells, iPSCs) could elevate stem cell therapeutics standardization to unprecedented levels. However, a unique optimized procedure for large-scale production of MSCs, of homogenous quality, from iPSCs (iMSCs) is missing. Main methods, culture components, and common MSC markers to produce iMSCs are provided here as reference resources for the establishment of harmonized Good Manufacturing Procedures towards obtaining clinical-grade iMSCs with improved CT performance.

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## INTRODUCTION

Stem cells (SCs) are unspecialized cells capable of self-replication and of generating specific cell types through differentiation. They are necessary for the regular renewal of our tissues and organs such as the skin or gut's lining, but also for the regeneration of damaged tissues upon injury; and, thus, for human homeostasis and survival. Most of the time SCs are found in a "dormant" state and become activated by signals received from tissues needing to be repaired. SCs, however, lose their potential for repair over their lifetime[1,2]. During embryonic development, SCs are very active, they can differentiate into a wide range of cell types and can migrate easily throughout the embryonic structure, whereas in the adult they decrease in number, restrict themselves to specific tissue locations and become more specialized.

In particular, mesenchymal SCs (MSCs) are adult pluripotent SCs that can be found in various tissues at low numbers. They were initially (mid 60s) identified by Friedenstein in the bone marrow of mice[3], but later have been found in many additional human tissues[4] including adipose tissue[5], umbilical cord[6], neural crest cells[7], and dental tissues[8-11]. Basically, all vascularized human tissues seem to harbor MSCs[12]. MSCs are consensually described by fulfilling the minimal criterion established by the International Society for Cellular Therapy (ISCT) including the presence of specific cell surface markers, their capacity for tri-lineage differentiation, their fibroblast-morphology, and adherent ability[13].

MSCs can inhibit pathological immune responses and suppress inflammation, partly due to secretion of soluble factors and cell-cell contact mechanisms. Indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), transforming growth factor beta (TGF- $\beta$ ), soluble human leukocyte antigen-G5 (HLA-G5), interleukin-10 (IL-10) and IL-6 are a few of these soluble factors allowing MSCs and immune cell cross-talk. The release of cytokines by immune cells is thus regulated by MSCs, by virtue of modifying their proliferation, survival, and cytotoxicity[14].

Cultured MSCs can be safely used without major potential risks for immune rejection because of their low expression levels of major histocompatibility complex (MHC) class I molecules, and the lack of MHC class II and co-stimulatory molecules (cluster of differentiation 80 [CD80], CD86, CD40)[15].

MSC cell therapy can be applied to a wide-range of disease types such as inflammatory diseases (*i.e.* arthritis)[16], auto-immune diseases (*i.e.* lupus erythematosus)

[17], but also increases the success of organ transplants[18], improve cell engraftment [19], bone[20,21] or cartilage regeneration[22], and wound healing[23]. However, several disadvantages restrain their use in the clinic such as the requirement of invasive techniques for their isolation, the limited numbers that can be obtained from a single donor, their limited capacity to proliferate and engraft *in vivo*, the diversity of isolation and expansion protocols, and their heterogeneous quality depending on donor's features and tissue's source[24-28]. In addition, it was shown that during *in vitro* culture expansion MSC cells rapidly senesce[29-31], limiting the amounts obtained from donors. Culture conditions might impact their epigenetic profiles or induce genomic changes[32]. Still, their use for the treatment of inflammatory and autoimmune diseases is becoming recognized[33,34]. Readers are referred to recent systematic reviews summarizing the results of MSC clinical trials (CTs) for the treatment of inflammatory disorders and for regenerative therapies[35,36].

Induced pluripotent stem cells (iPSCs) represent an inexhaustible source for MSC production because they can be grown indefinitely in pluripotent state without signs of replicative senescence. Importantly, their phenotype and functions are better defined, and contrary to ESCs, they do not require destruction of embryos[37,38]. The differentiation of iPSCs into MSCs to produce iMSCs of homogeneous quality, therefore, holds great promise to overcome the actual limitations that adult MSC present for clinical applications.

Various methods to produce iMSCs with potential for industrial scale-up have already emerged[39]. They are particularly mediated *via* various culture components and growth factor complementation, such as the use of coating materials and the addition of pathway inhibitors. Ectopic expression of MSC-related genes may also be used to further refine these protocols[40]. As iPSCs can spontaneously differentiate, a need to evaluate iMSC (MSCs from iPSCs) appearance in large-scale production protocols to support reproducible iMSC quality production results obvious.

This review gathers information on the main current non-commercial protocols used to differentiate iPSC into iMSC, describes cell sources of the iPSCs assayed, and lists the characterization procedures applied to the obtained iMSCs based on cell surface markers. This information can guide future scale-up protocols, and required refinements, towards optimization of iMSC production of homogeneous quality fitting therapeutic demands.

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## IPSC CULTURE CONDITIONS TOWARDS IMSC PRODUCTION

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### **Main approaches to generate iMSCs**

We have reviewed 32 selected studies describing original methods to generate iMSCs, and have classified the identified procedures according to the following five main strategies: MSC Switch (15/32), Embryoid Bodies (EBs) (13/32), Specific Differentiation (5/32), Pathway Inhibitor (5/32), and Platelet Lysate (3/32). They will be presented by order of their frequency of use in the selected literature (Table 1-5). Some of the studies used more than one of these methods, as detailed in Supplementary Table 1. To define the method variants used by different groups, an "iPSC to iMSC Protocol" column has been incorporated in each table (Table 1-5). Additional features incorporated in the study summary tables as potential relevant for iMSC production are: the minimal required time to obtain iMSCs from iPSCs in days, the type of adult cell from which iPSCs were obtained ("iPSC origin"), and the application explored in the mentioned study.

As shown in Table 1, the MSC Switch strategy basically consists of replacing or "switching" the iPSC culture media by MSCs to induce spontaneous MSC differentiation. Some authors describe simple details, such as the addition of certain compounds or coatings, while other apply more sophisticated methods. The latter is the case of Lian *et al*[41] who use cell sorting (flow cytometry cell sorting (FACS) to select a subpopulation of cells positive for the CD105 marker and negative for CD24 before the iMSC differentiation step[41].

Another set of procedures are grouped under the modality denominated EBs as they are present in producing clusters of cells or EBs to later be seeded into MSC-specific media to induce differentiation. Again, a considerable number of options have been described by different research groups (Table 2). It is interesting that the variant method used by Eto *et al*[55] also applies FACS to select a subpopulation prior to its differentiation. In this case, however, the markers used for cell selection were the receptors platelet derived growth factor receptor alpha (PDGFR $\alpha$ ) and vascular endothelial growth factor receptor 2 (VEGFR2), both of which are mediators of cell



**Table 1** Protocols to produce induced pluripotent stem cell-derived mesenchymal stem cells by the mesenchymal stem cell Switch methods

Ref.	iPSC origin	iPSC to iMSC protocol	Time	Application	Citations
Lian <i>et al</i> [41], 2010	Lung fibroblast	(1) iPSC cultured on a gelatinized dish + KO DMEM + 10% SRM + bFGF + PDGFAB + EGF; and (2) FACS SORTING: CD24- and CD105+ and single cell clones plating	7	Limb ischemia in mice	419
Giuliani <i>et al</i> [42], 2011	Amniocytes	iPSC cultured 4 wk in DMEM/F12 + 10% hiFBS + b-FGF + NEAA + L-Glutamine + $\beta$ -ME + p/s	28	Immunomodulatory properties of iMSC on NK cytolytic activity	110
Liu <i>et al</i> [43], 2012	Dermal fibroblast	(1) iPSC cultured on collagen-coated dishes with $\alpha$ -MEM + 10% FBS + dexamethasone + magnesium L-ascorbic acid phosphate + p/s; and (2) Cells cultured on collagen-coated dishes with $\alpha$ -MEM + 10% FBS + L-Glutamine + NEAA + p/s	10	iMSC generation w/ Fibrillar Collagen Coating	118
Zou <i>et al</i> [44], 2013	Dermal fibroblasts	iPSC medium switched for MSC medium: DMEM-low glucose + 10% FBS + L-Glutamine	14	Generation of osteogenesis 3D scaffolds	98
Hynes <i>et al</i> [45], 2014	Gingival fibroblast periodontal ligaments	(1) iPSC cultured with MSC medium: $\alpha$ -MEM + FCS + sodium pyruvate + L-ascorbate-2-phosphate + L-Glutamine + NEAA + HEPES + p/s; and (2) Cells cultured on gelatin-coated-flasks then switch to non-coated flasks	14	Generation of iMSC	102
Jeong <i>et al</i> [46], 2014 <sup>1</sup>	NA	(1) iPSCs cultured in iMSC-inducing medium: DMEM/F12 + 20% KOSR + SB431542 (TGF $\beta$ inhibitor); (2) EB grown on matrigel + DMEM/F12 + 0.5% BSA + 10% ITS + SB431542 (TGF $\beta$ inhibitor); and (3) Outgrowth grown with DMEM/F12 + 10% FBS + p/s	17	Duchene muscular dystrophy	17
Hu <i>et al</i> [47], 2015	iPS-S-01, C1P33, PCKDSF001C1	iPSC medium switched for MSC medium: DMEM-low glucose + 10% FBS + L-Glutamine, then cultured in gelatin-coated dishes	14	Limb ischemia	177
Kang <i>et al</i> [48], 2015	Dermal fibroblasts	iPSC cultured with MSC medium: DMEM low glucose + FBS 10% + L-Glutamine + p/s then cultured on gelatin-coated dishes	14	iMSC plasticity (less adipogenesis)	65
Zhang <i>et al</i> [23], 2015	PBMCs	(1) iPSC medium switched for MSC medium: DMEM-low glucose + 10% FBS + L-Glutamine + NEAA + p/s; and (2) Cells cultured on gelatin-coated dishes	17	Cutaneous wound healing	262
Lian <i>et al</i> [49], 2016	NA	(1) iPSC cultured on gelatin-coated plates with MSC differentiation medium: KO DMEM + KOSR + bFGF + PDGFAB + EGF; and (2) FACS: CD24- CD105+ cells cultured on gelatin-coated plates with DMEM + 10% FBS + bFGF + PDGFAB + EGF	20	Directed differentiation of iPSC to MSC	15
Gao <i>et al</i> [50], 2017	Urine cell; Amniocytes	(1) iPSC cultured with MSC-inducing culture media: $\alpha$ -MEM + SRM + sodium pyruvate + L-ascorbate-2-phosphate + L-Glutamine + NEAA + p/s on gelatin-coated plates; and (2) Cells cultured with MSC maintenance medium = high-glucose DMEM + 10% FBS + bFGF + EGF	17	iMSC effect on dendritic cells	27
Nachlas <i>et al</i> [51], 2018 <sup>1</sup>	NA	(1) iPSC cultured in suspension (to promote cell aggregate) with differentiation media: KO-DMEM + $\beta$ -ME+ L-Glutamine + 20% FBS + NEAA + p/s, then, cells were cultured on gelatin coated plates; and (2) Cells cultured with iMSC media: KO-DMEM + L-Glutamine + 10% FBS + NEAA + p/s	12	Generation of valve interstitial-like cells from iMSC	14
Wang <i>et al</i> [52], 2018	Amniocytes	(1) iPSC cultured with induction medium: $\alpha$ -MEM + 10% FBS + p/s + L-Glutamine + NEAA + sodium pyruvate + L-ascorbate-2-phosphate; (2) Cells plated on gelatin-	14	Effect of Dexamethasone on iMSC	3

		coated plates; and (3) Cells plated on uncoated plates with iPSC-MSC medium = High-Glucose DMEM + FBS + bFGF + EGF + p/s			
Wang <i>et al</i> [53], 2018	PBMCs	iPSC cultured with MSC medium: Low-Glucose DMEM + 10% FBS + p/s + L-glutamine	NA	Immunomodulatory properties of MSC, transcriptome analysis	8
McGrath <i>et al</i> [54], 2019 <sup>1</sup>	Dermal fibroblast	(1) iPSC-MP thawed and expanded in KO DMEM + bFGF + L-Glutamine + MEM + NEAA + 20% FBS + Antibiotic-Antimycotic + $\beta$ -ME; and (2) Cell are plated into gelatin coated-plates with KO DMEM + heparin + hPL + bFGF + L-Glutamine + MEM NEAA + Antibiotic-Antimycotic + $\beta$ -ME	NA	iMSC differentiation: GMP-compatible and xeno-free cultivation	6

iPSC origin refers to the cell type used for reprogramming; Time is indicated as the minimum number of days required to obtain iMSCs; Citations show numbers on March 2020.

<sup>1</sup>In references indicate the study includes methods in more than one protocol category. bFGF: Basic fibroblast growth factor;  $\beta$ -ME:  $\beta$ -Mercaptoethanol; BSA: Bovine serum albumin; CDM: Chemical defined medium; DMEM/F12: Dulbecco's modified Eagle's medium F12; EB: Embryoid body; EGF: Epidermal growth factor; FBS: Fetal bovine serum; FCS: Fetal calf serum; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hiFBS: Heat inactivated fetal bovine serum; hPL: human platelet lysate; IMDM: Iscove's modified Dulbecco's media; iMSCs: Induced pluripotent stem cell-derived mesenchymal stem cells; iPSC: Induced pluripotent stem cell; ITS: Insulin-transferrin-selenium; KO-DMEM: Knockout Dulbecco's modified Eagle's medium; KOSR: Knock-out serum replacement;  $\alpha$ -MEM: Minimum essential medium Eagle; NA: Not available; NEAA: Non-essential amino acid; p/s: Penicillin-streptomycin; SRM: Serum replacement medium; TGF: Transforming growth factor.

proliferation but neither are on the list of minimal MSC criteria[13].

As mentioned, three other less popular methods to induce iMSC production have been described. In the Specific Differentiation method, specific progenitors are obtained before culturing the cells in MSC media to induce differentiation to mesenchymal cells (Table 3). For example, iPSCs are pre-differentiated into neural or cardiac cell progenitors (priming of iPSCs), before inducing their differentiation to MSCs. The rationale behind this procedure resides in the observation that these progenitor-like cells are more closely related to MSCs than the multipotent iPSCs. Mitsuzawa *et al*[64], Eto *et al*[55], Ouchi *et al*[65], and Fukuta *et al*[66] first generated neural progenitors as a previous step towards iMSC production. It must be noted that Fukuta *et al*[66] used SB431542 (a TGF- $\beta$  inhibitor) to support the differentiation of iPSCs, and CHIR (a glycogen synthase kinase-3 [GSK3] inhibitor, inducer of Wnt/ $\beta$ -catenin signaling pathway) to promote differentiation to neural cells. Mitsuzawa *et al* [64] modified Fukuta *et al*[66]'s protocol by using Dulbecco's modified Eagle's medium (DMEM) instead of  $\alpha$ -MEM, and added fibroblast growth factor (FGF) to the media to promote MSC differentiation. Eto *et al*[55] initially treated iPSCs with CTK (dissociation solution made of collagenase type IV, trypsin and KSR, or Knockout Serum Replacement), reducing cell oxidative stress; and the EBs with bone morphogenetic protein 4 + activin A + lithium chloride for pre-mesodermal differentiation or retinoic acid to enhance EB differentiation, plus  $\beta$ -mercaptoethanol to encourage neural differentiation before culturing the cells in MSC media. Ouchi *et al*[65] generated neural progenitors by simply culturing iPSCs with specific neural media and proved that the cells grown under these conditions had the ability to turn into MSC afterwards. On another side, Wei *et al*[59] generated EBs through a cardiac differentiation protocol "cardiomyogenic medium," promoted by the addition of SB 203580 (a p38 mitogen-activated protein kinase [MAPK] inhibitor), followed by cell plating on classic MSC media.

The pathway inhibitor method consists of the addition of chemical inhibitors of specific pathways in the culture media to induce differentiation of the iPSCs into MSCs (Table 4). Examples of these supplements are again, SB-431542, which is a TGF- $\beta$  inhibitor, the GSK-3 inhibitor CHIR, or the p38 MAPK inhibitor SB-203580. CHIR and SB-431542 had been used by Fukuta *et al*[66], as mentioned earlier, to induce neural progenitor-like cells before inducing MSC differentiation. In fact, some of the methods merge two of the five strategies here described for the production of iMSCs. Zhao *et al* [67], Jeong *et al*[46], and Chen *et al*[57] confirmed that this treatment worked with iPSCs. Indeed, SB431542 leads to the downregulation of pluripotency genes by inhibiting the TGF- $\beta$  signaling pathway, promoting differentiation into MSCs. These results are encouraging because they show that MSC differentiation methods used earlier for the differentiation of hESCs[68-70] could be applied to produce MSCs from iPSCs.

Table 2 Protocols to produce induced pluripotent stem cell-derived mesenchymal stem cells by embryoid bodies approaches

Ref.	iPSC origin	iPSC to iMSC protocol	Time	Application	Citations
Ahfeldt <i>et al</i> [56], 2012	Foreskin fibroblast	(1) iPSC cultured into low-adhesion dishes for EB formation with DMEM + 15% FBS + L-Glutamine; (2) EB plated into gelatin-coated dishes with DMEM + 15% FBS + L-Glutamine; and (3) Cells plated with Mesenchymal Progenitor Cell (MCP) medium: DMEM + 15% FBS + L-Glutamine + bFGF	12	Producing white and brown adipocytes from hPSCs	194
Chen <i>et al</i> [57], 2012 <sup>1</sup>	Lung fibroblast	SB431542 inhibitor differentiation method (feeder free); iPSC cultured in inhibitor differentiation medium: KOSR medium + SB431542 (TGFβ inhibitor) Without bFGF to enhance differentiation. Embryoid body differentiation method: (1) EB formation in KOSR medium; and (2) EB cultured with MSC medium: DMEM + 10% FCS + L-Glutamine + gentamicin + p/s	17	Generation of iMSC with TGF-beta inhibitor	136
Villa-Diaz <i>et al</i> [58], 2012	Dermal fibroblasts	(1) EB formation in suspension cultured into ultra-low-attachment plates; and (2) EB plated on gelatin-coated dishes with MSC medium: α-MEM + 10% FBS + L-Glutamine + NEAA + FGF2	21	iMSC from iPSC cultured on synthetic substrate (PMEDSAH)	262
Wei <i>et al</i> [59], 2012 <sup>1</sup>	Dermal fibroblasts	(1) EB formation through cardiac differentiation protocol involving cardiomyogenic medium CARM: High-Glucose DMEM + L-Glutamine + NEAA + Selenium Transferrin + β-ME + SB 203580 (p38-MAPK inhibitor); and (2) EB plating on gelatin-coated plates with DMEM + 2% FBS	21	Generation of iMSC	64
Shao <i>et al</i> [60], 2013	MSC	(1) iPSC cultivated in suspension in the differentiation medium: KO DMEM + 20% FBS + 1% NEAA + β-ME + L-Glutamine for EB formation; and (2) Embryoid bodies plated on gelatin-coated dishes	19	iMSC DNA methylation profiles	48
Jeong <i>et al</i> [46], 2014 <sup>1</sup>	NA	(1) iPSCs cultured in iMSC-inducing medium: DMEM/F12 + 20% KOSR + SB431542 (TGFβ inhibitor); (2) EB grown on matrigel + DMEM/F12 + 0.5% BSA + 10% ITS + SB431542 (TGFβ inhibitor); and (3) Outgrowth grown with DMEM/F12 + FBS (10%) + p/s	17	Duchene muscular dystrophy	17
Miao <i>et al</i> [61], 2014	Dermal fibroblasts	EB cultured with DMEM + 10% FBS	NA	Myocardial infarctus	38
Tang <i>et al</i> [21], 2014	bone marrow	(1) iPSC cultured in ultra-low attachment plate to form EB with differentiation medium: DMEM/F12 + 20% KSR + MEF medium + NEAA + L-Glutamine + β-ME; and (2) EB plated into gelatin-coated plates + MSC growth medium: DMEM + 10% FBS + L-Glutamine + p/s	20	iMSC and calcium phosphate scaffold for bone regeneration	88
Sheyn <i>et al</i> [20], 2016	Dermal fibroblasts	(1) iPSC plated into PCR plates to form EB with IMDM medium: MDM media + KOSR + NEAA + β-ME + PSA antifungal-antibacterial solution; (2) EB transferred to poly-HEMA-coated flasks; (3) Attached EB (aiMSCs) and Transferred EB (tiMSCs) cultured into gelatin-coated flask with medium + TGF-β1; and (4) Medium switched for DMEM + 10% FBS + L-Glutamine + p/s	10	Generation of iMSC and repair bone defect	60
Eto <i>et al</i> [55], 2018 <sup>1</sup>	Skin fibroblast	(1) iPSCs treated with CTIK (collagenase type IV + 0.25% trypsin + KSR) and transferred to petri dishes to form EB with: DMEM/F12 + 20% KOSR + glutamine + NEAA + BMP4 + p/s; (2) Specific Differentiation or Mesodermal Differentiation: EB cultured on collagen-coated plates + α MEM + 10% FBS + bFGF + BMP4 + Activin A + LiCl + p/s; or Neuroepithelial differentiation: αMEM + 10% FBS + β-ME + RA; and (3) FACS: PDGFR-α+ and VEGFR2+ cells resuspended on collagen-coated plates with αMEM + 10% FBS + 20% KOSR	10	iMSC from mesoderm or neuroepithelium differentiation	7
Nachlas <i>et al</i> [51], 2018 <sup>1</sup>	NA	(1) iPSC cultured in suspension (to promote cell aggregate) with differentiation media: KO-DMEM + β-ME + L-Glutamine + 20% FBS + NEAA + p/s, then cells were cultured on gelatin coated plates; and (2) Cells cultured with iMSC media: KO-DMEM + L-Glutamine + 10% FBS + NEAA + p/s	12	Generation of valve interstitial-like cells from iMSC	14
Karam <i>et al</i> [62], 2020	PBMC	(1) EB cultured into ultra-low attachment plates with differentiation medium: Low-Glucose DMEM + 15% FBS + p/s; (2) Later, RA is added to enhance EB formation; (3) EB plating into gelatin/matrigel coated plates + differentiation medium; and (4) Later bFGF is added	14	Generation of iMSC and adipocytes	NA

Huang <i>et al</i> [63], 2020	PBMC	(1) iPSC cultured in suspension to form EB; and (2) Cells plated into gelatin-coated plates with $\alpha$ -MEM + FGF2	NA	Repair of acute kidney injury	NA
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iPSC origin refers to the cell type used for reprogramming; Time is indicated as the minimum number of days required to obtain iMSCs; Citations show numbers on March 2020.

<sup>1</sup>In references indicate the study includes methods in more than one protocol category. bFGF: Basic fibroblast growth factor;  $\beta$ -ME:  $\beta$ -Mercaptoethanol; BMP4: Bone morphogenetic protein 4; BSA: Bovine serum albumin; CDM: Chemical defined medium; DMEM/F12: Dulbecco's modified Eagle's medium F12; EB: Embryoid body; EGF: Epidermal growth factor; FBS: Fetal bovine serum; FCS: Fetal calf serum; FGF2: Fibroblast growth factor 2; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hiFBS: Heat inactivated fetal bovine serum; hPL: human platelet lysate; IMDM: Iscove's modified Dulbecco's media; iMSCs: Induced pluripotent stem cell-derived mesenchymal stem cells; iPSC: Induced pluripotent stem cell; ITS: Insulin-transferrin-selenium; KO-DMEM: Knockout Dulbecco's modified Eagle's medium; KOSR: Knock-out serum replacement; KSR: Knockout serum replacement;  $\alpha$ -MEM: Minimum essential medium Eagle; NA: Not available; PBMC: Peripheral blood mononuclear cell; p/s: Penicillin-streptomycin; SRM: Serum replacement medium; TGF: Transforming growth factor; VEGFR: Vascular endothelial growth factor.

**Table 3 Protocols to produce induced pluripotent stem cell-derived mesenchymal stem cells by Specific Differentiation approaches**

Ref.	iPSC origin	iPSC to iMSC protocol	Time	Application	Citations
Wei <i>et al</i> [59], 2012 <sup>1</sup>	Dermal fibroblasts	(1) EB formation through cardiac differentiation protocol involving cardiomyogenic medium CARM: High-Glucose DMEM + L-Glutamine + NEAA + Selenium Transferrin + $\beta$ -ME + SB 203580 (p38-MAPK inhibitor); and (2) EB plating on gelatin-coated plates with DMEM + 2% FBS	21	Generation of iMSC	64
Fukuta <i>et al</i> [66], 2014 <sup>1</sup>	Dermal fibroblast	(1) Induction of hNCC from iPSC; (2) Cells cultured on fibronectin-coated dishes with STK2 medium + CDM (IMDM/Ham's F-12 + lipid concentrate + apo-transferrin + monothioglycerol + BSA + insulin + p/s) + SB431542 (TGF $\beta$ inhibitor) + CHIR (Wnt Agonist); and (3) Cells cultured with $\alpha$ MEM + 10% FBS	15	iMSC differentiation through neural crest lineage	80
Ouchi <i>et al</i> [65], 2016	Dermal fibroblast	(1) Generation of NCL (neural crest like-cells); and (2) NCL cultured into DMEM/F12 + Neurobasal medium + L-Glutamine + Gem21 Neuroplex + N2 Supplement + hbFGF + hEGF + insulin + p/s	10	iNCC can develop into iMSC	8
Eto <i>et al</i> [55], 2018 <sup>1</sup>	skin fibroblast	(1) iPSCs treated with CTX (collagenase type IV + 0.25% trypsin + KSR) and transferred to petri dishes to form EB with: DMEM/F12 + 20% KOSR + glutamine + NEAA + BMP4 + p/s; (2) Specific Differentiation or Mesodermal Differentiation: EB cultured on collagen-coated plates + $\alpha$ MEM + 10% FBS + bFGF + BMP4 + Activin A + LiCl + p/s or Neuroepithelial differentiation: $\alpha$ MEM + 10% FBS + $\beta$ -ME + RA; (3) FACS: PDGFR- $\alpha$ and VEGFR2+ cells resuspended on collagen-coated plates with $\alpha$ MEM + 10% FBS + 20% KOSR	NA	iMSC from mesoderm or neuroepithelium differentiation	7
Mitsuzawa <i>et al</i> [64], 2019	NA	(1) Induction of hNCC: CDM (IMDM/Ham's F-12 + lipid concentrate + apo-transferrin+ monothioglycerol + BSA + insulin + p/s) + SB431542 (TGF $\beta$ inhibitor) + CHIR (Wnt Agonist)Maintenance in DMEM + 10% FBS + FGF2; and (2) Induction of iMSC with DMEM + 10% FBS + FGF2 on fibronectin coated plates	25	Hind limb in rat allotransplantation	1

iPSC origin refers to the cell type used for reprogramming; Time is indicated as the minimum number of days required to obtain iMSCs; Citations show numbers on March 2020.

<sup>1</sup>In references indicate the study includes methods in more than one protocol category. bFGF: Basic fibroblast growth factor;  $\beta$ -ME:  $\beta$ -Mercaptoethanol; BMP4: Bone morphogenetic protein 4; BSA: Bovine serum albumin; CARM: Cardiomyogenic medium; CDM: Chemical defined medium; CHIR: Wnt agonist, potent GSK3 inhibitor; DMEM/F12: Dulbecco's modified Eagle's medium EB: Embryoid body; FBS: Fetal bovine serum; FGF2: Fibroblast growth factor 2; Ham's F12: Medium formulated for single-cell plating of near-diploid Chinese hamster ovary cells; hbFGF: Human basic fibroblast growth factor; hEGF: Human epidermal growth factor; IMDM: Iscove's modified Dulbecco's media; iMSCs: Induced pluripotent stem cell-derived mesenchymal stem cells; iPSC: Induced pluripotent stem cell; ITS: Insulin-transferrin-selenium; KOSR: Knock-out serum replacement; KSR: Knockout serum replacement; NA: Not available; NEAA: Non-essential amino acid;  $\alpha$ -MEM: Minimum essential medium Eagle; PDGFR: Platelet-derived growth factor receptor A; p/s: Penicillin-streptomycin; RA: Retinoic acid; TGF: Transforming growth factor; VEGFR: Vascular endothelial growth factor.

Only 3 of the 32 studies selected used media supplemented with human platelet lysate (hPL), in replacement of fetal bovine serum (FBS), to produce iMSCs. As the name suggests, it uses a lysate of platelets obtained from human peripheral blood to supplement iPSC's growing media, replacing the FBS component, which by its animal origin raised ethical and safety concerns. This method, therefore, could improve the safety of the final iMSCs produced.

Table 4 Protocols to produce induced pluripotent stem cell-derived mesenchymal stem cells by Pathway Inhibitor approaches

Ref.	iPSC origin	iPSC to iMSC protocol	Time	Application	Citations
Chen YS <i>et al</i> [57], 2012 <sup>1</sup>	Lung fibroblast	SB431542 Inhibitor Differentiation Method (feeder free); iPSC cultured in inhibitor differentiation medium: KOSR medium + SB431542 (TGFβ inhibitor) Without bFGF to enhance differentiation; Embryoid body differentiation method: (1) EB formation in KOSR medium; and (2) EB cultured with MSC medium: DMEM + 10% FCS + L-Glutamine + gentamicin + p/s	17	Generation of iMSC with TGF-beta inhibitor	136
Wei <i>et al</i> [59], 2012 <sup>1</sup>	Dermal fibroblasts	(1) EB formation through cardiac differentiation protocol involving cardiomyogenic medium CARM: High-Glucose DMEM + L-Glutamine + NEAA + Selenium Transferrin + β-ME + SB 203580 (p38-MAPK inhibitor); and (2) EB plating on gelatin-coated plates with DMEM + 2% FBS	21	Generation of iMSC	64
Fukuta <i>et al</i> [66], 2014 <sup>1</sup>	Dermal fibroblast	(1) Induction of hNCC from iPSC; (2) Cells cultured on fibronectin-coated dishes with STK2 medium + CDM (IMDM/Ham's F-12 + lipid concentrate + apo-transferrin+ monoethioglycerol +BSA + insulin + p/s)+ SB431542 (TGFβ inhibitor) + CHIR (Wnt Agonist); and (3) Cells cultured with αMEM + 10% FBS	15	iMSC differentiation through neural crest lineage	80
Jeong <i>et al</i> [46], 2014 <sup>1</sup>	NA	(1) iPSCs cultured in iMSC-inducing medium: DMEM/F12 + 20% KOSR + SB431542 (TGFβ inhibitor); and (2) EB grown on matrigel + DMEM/F12 + 0.5% BSA + 10% ITS + SB431542. 3. Outgrowth grown with DMEM/F12 + 10% FBS + p/s	17	Duchene muscular dystrophy	17
Zhao <i>et al</i> [67], 2015	Blood cells	(1) iPSC cultured with mTeSR1 + SB431542 (TGFβ inhibitor) on matrigel-coated plates (7.5% CO <sub>2</sub> atmosphere); and (2) Cells cultured with ESC-MSC medium: KO DMEM + KOSR + NEAA + p/s + L-Glutamine + β-ME + bFGF + EGF + SB431542	45	Tumor tropism of iMSC	79

iPSC origin refers to the cell type used for reprogramming; Time is indicated as the minimum number of days required to obtain iMSCs; Citations show numbers on March 2020.

<sup>1</sup>In references indicate the study includes methods in more than one protocol category. bFGF: Basic fibroblast growth factor; β-ME: β-Mercaptoethanol; BSA: Bovine serum albumin; CARM: Cardiomyogenic medium; CDM: Chemical defined medium; DMEM/F12: Dulbecco's modified Eagle's medium F12; CHIR: Wnt agonist, potent GSK3 inhibitor; EB: Embryoid body; FBS: Fetal bovine serum; FCS: Fetal calf serum; Ham's F12: Medium formulated for single-cell plating of near-diploid Chinese hamster ovary cells; iMSCs: Induced pluripotent stem cell-derived mesenchymal stem cells; iPSC: Induced pluripotent stem cell; ITS: Insulin-transferrin-selenium; KO DMEM: Knockout Dulbecco's modified Eagle's medium; KOSR: Knock-out serum replacement; α-MEM: Minimum essential medium Eagle; MSC: Mesenchymal stem cell; NA: Not available; NEAA: Non-essential amino acid; p/s: Penicillin-streptomycin; TGF: Transforming growth factor.

McGrath *et al*[54], Frobel *et al*[71], and Luzzani *et al*[72] decided to use this method to grant a procedure free of animal components. The differentiation protocol is similar to the already described for the MSC Switch category, with the exception that the anticoagulant heparin is used in the media to prevent clotting. It should be highlighted that Frobel *et al*[71] revealed an incomplete reacquisition of the immunomodulatory properties of iMSCs obtained with this variant of the protocol. In addition, McGrath *et al*[54] also noticed an alteration in the release of trophic factors, necessary for immunomodulatory properties being attributed to MSCs. This may impose limitations for certain therapeutic uses to the iMSCs produced with this set of protocols.

### Basic components to obtain iMSCs

As per the basic components of the culture medium used in these 32 studies, we evaluated the composition of the basal media, culture medium supplements other than chemical inhibitors, and coatings used for cell culture while producing iMSCs. All this with the intention to identify the most commonly used components within each of the five studied protocol categories. The analysis allows identification of the elements required to produce iMSCs regardless of the method of choice.

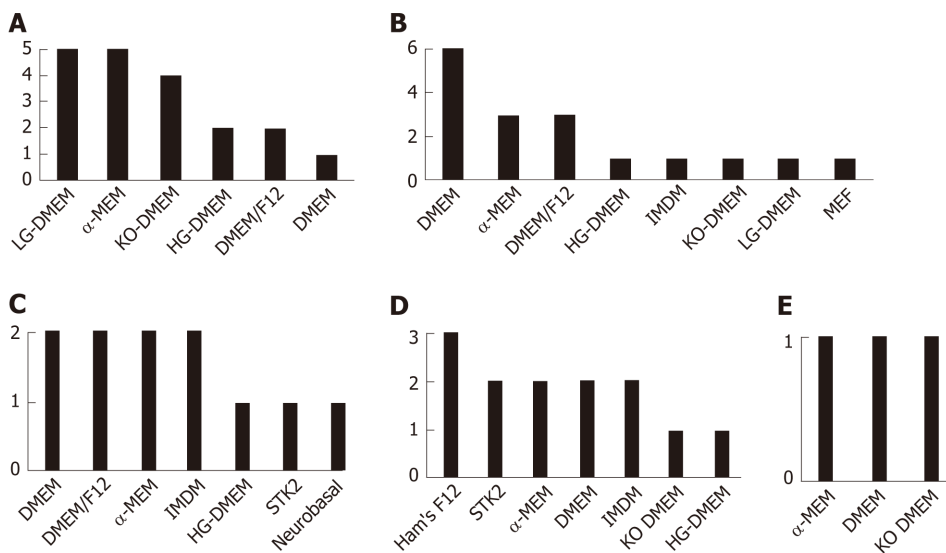
**Basal medium composition:** The main basal commercial media used to differentiate iPSC into iMSC were either DMEM, or α-MEM, or other DMEM derivatives such as DMEM knockout (KO) (a serum-free media), or DMEM F-12, which slightly vary in salt composition and glucose concentration (Figure 1). It should be noted that some studies use more than one medium type (Supplementary Table 2).

**Table 5 Protocols to produce induced pluripotent stem cell-derived mesenchymal stem cells by approaches that use Platelet Lysate**

Ref.	iPSC origin	iPSC to iMSC protocol	Time	Application	Citations
Frobel <i>et al</i> [71], 2014	BM-MSCs	(1) EB formation on ultra-low attachment plates; and (2) Cells cultured with standard medium for MSC: DMEM + L-Glutamine + p/s + hPL + heparin on matrigel-coated wells then passaged on gelatin-coated wells	35	Epigenetic study of iMSC	116
Luzzani <i>et al</i> [72], 2015	Foreskin fibroblasts	(1) iPSC cultured in matrigel/geltrex-coated dishes with $\alpha$ -MEM + 10% PL + p/s + B7 or DMEM + 10% FBS; and (2) Cells cultured with no-coated dishes	20	MSC differentiation using platelet lysate	26
McGrath <i>et al</i> [54], 2019 <sup>1</sup>	Dermal fibroblasts	(1) iPSC-MP thawed and expanded in KO DMEM + bFGF + L-Glutamine + MEM NEAA + FBS (20%) + Antibiotic-Antimycotic+ $\beta$ -ME; and (2) Cell are plated on gelatin coated-plates with DMEM KO + heparin + hPL+ bFGF + L-Glutamine + MEM NEAA + Antibiotic-Antimycotic+ $\beta$ -ME	NA	iMSC differentiation: GMP-compatible and xeno-free cultivation	6

iPSC origin refers to the cell type used for reprogramming; Time is indicated as the minimum number of days required to obtain iMSCs; Citations show numbers on March 2020.

<sup>1</sup>In references indicate the study includes methods in more than one protocol category. bFGF: Basic fibroblast growth factor; BM: Bone marrow;  $\beta$ -ME:  $\beta$ -Mercaptoethanol; EB: Embryoid body; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; GMP: Good manufacturing procedures; hPL: human platelet lysate; hEGF: Human epidermal growth factor; iMSCs: Induced pluripotent stem cell-derived mesenchymal stem cells; iPSC: Induced pluripotent stem cell; KO DMEM: Knockout Dulbecco's modified Eagle's medium;  $\alpha$ -MEM: Minimum essential medium Eagle; MSC: Mesenchymal stem cell; NA: Not available; NEAA: Non-essential amino acid; p/s: Penicillin-streptomycin.



**Figure 1 Basal commercial media used to produce induced pluripotent stem cell-derived mesenchymal stem cells.** The relative frequencies of media used by the 32 studies are displayed as number of events within each of the five categories of protocols being described: Mesenchymal stem cell Switch (A), Embryoid Bodies (B), Specific Differentiation (C), Pathway Inhibitor (D), and Platelet Lysate (E). DMEM: Dulbecco's modified Eagle's medium; DMEM/F12: Dulbecco's modified Eagle's medium F12; Ham's F12: Medium formulated for single-cell plating of near-diploid Chinese hamster ovary cells; HG-DMEM: High glucose Dulbecco's modified Eagle's medium; IMDM: Iscove's modified Dulbecco's media; KO-DMEM: Knockout Dulbecco's modified Eagle's medium; LG-DMEM: Low glucose Dulbecco's modified Eagle's medium; MEF: Mouse embryonic fibroblast media;  $\alpha$ MED/MEM: Minimum essential medium Eagle.

**Culture medium supplements:** The main supplements commonly used, as an overall, in all five described iMSC protocol categories are: FBS, L-Glutamine and antibiotics (penicillin-streptomycin or P/S), followed by non-essential amino acids, and FGF (Supplementary Table 2).

In the category of MSC Switch, an important supplement (4/15 studies) used is epidermal growth factor. The studies by Hynes *et al*[45], Gao *et al*[50], and Wang *et al* [52], commonly used l-ascorbate-2-phosphate and sodium pyruvate. KO Serum Replacement (KOSR) was used in an additional 3/15 studies.

For EB, however, a prevalent supplement is  $\beta$ -mercaptoethanol, representing 6/13 studies, followed by KOSR (3/13 studies), Insulin-Transferrin-Selenium (ITS) and SB431542 (2/13 studies). All three components were used in the study by Jeong *et al*

[46].

For Specific Differentiation methods, the most common supplement seems to be insulin (3/5 studies), followed by  $\beta$ -mercaptoethanol and SB431542, which were present in more than one study. The components being used together with apo-transferrin, bovine serum albumin (BSA), CHR, lipid concentrate, and monothioglycerol were included only in the studies by Fukuta *et al* [66] and Mitsuzawa *et al* [64].

The SB431542 supplement, which is a TGF- $\beta$  inhibitor, is used by practically all (4/5 studies) the studies reviewed fitting in the Pathway Inhibitor category. Only  $\beta$ -mercaptoethanol, BSA, and ITS were used in more than one study of this category. The low number of studies for this category ( $n = 5$ ) should be noticed.

Finally, the specific supplements of the last described category present in all three studies are hPL, per definition, and heparin (Supplementary Table 2, the latter due to the requirement to prevent media clotting, as mentioned).

**Coatings:** Coatings seem to be decisive for cell differentiation. Indeed, it has been shown that collagen type-I can activate the nuclear factor kappa B pathway and drives the epithelial to mesenchymal transition [73]. Polymer coatings such as poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] helps maintain the self-renewal and pluripotency features of SCs [58]. The most common coating for iMSC production in the protocols presented here, overall, appeared to be gelatin, with the exception of the Specific Differentiation set of protocols (Figure 2). Collagen, Matrigel, or absence of coating ( $\emptyset$ ) follow in the most commonly used coating materials list. Fibronectin is only used in Specific Differentiation and Pathway Inhibitor procedures. The most uncommon coating was found to be poly-HEMA (polymer forming hydrogel in water) [20], which was used only in 1 of the 13 EB protocols reviewed (Figure 2). It should be noted that some studies use more than one type of coating (Supplementary Table 2).

### **iPSC cell origin**

As iPSCs have epigenetic memory of their tissue of origin [74], it might be relevant to document what are the most common cell types used to produce iMSCs across the 32 original studies being reviewed here. This feature, however, could only be tracked in 25 of the studies. Among the different cell types found, dermal fibroblast represents the most frequent tissue of origin, with 12 of 25 studies using these cells, followed by peripheral blood mononuclear cells (4/25 of the studies), amniocytes and bone marrow (3/25 of the studies for each mentioned cell type), and foreskin fibroblast (2/25 of the studies). Lastly, fetal endothelial cells, lung fibroblasts, MSCs, periodontal ligaments, peripheral gingival fibroblasts, and urine-contained cells were each reported by single studies. The studies corresponding to each cell type can be consulted in Supplementary Table 3.

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## **CHARACTERIZATION OF IMSCS**

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### **Cell surface markers and cytokines**

In addition to the 32 studies selected as the main representatives for the five categories to produce iMSCs by non-commercial methods, 12 additional studies were included in this section. The reason for their inclusion was that they describe the characterization of iMSCs obtained by one of the five protocol categories studied in this review, despite not being original descriptors of the method (Supplementary Tables 1 and 4).

Top cell surface markers selected to characterize iMSCs in the reviewed literature ( $n = 44$ ) include the common markers defined for the MSC minimal criteria, as described by the ISCT: CD73, CD105, and CD90 must be expressed (positive); and cells should lack expression of CD45, CD34, CD14 or CD11b, CD79 $\alpha$  or CD19 and HLA-DR surface molecules (negative). As can be appreciated on Table 6, common markers used to characterize iMSCs additional to those defined by the ISCT for MSCs, are markers CD44, CD29, and CD166, as positive markers (they have been found on adult MSCs [75], although their significance remains unknown); and CD133 and TRA-1-81 as negative (controls to ensure absence of pluripotent SCs or cancer SCs [76]) (Table 6). Supplementary Table 4 can be consulted to identify particular studies assessing the presence or absence of these markers on iMSCs.

**Table 6** Frequency of use of iMSC surface markers

Positive CSM	%	Negative CSM	%
CD73	18.1	CD45	24.5
CD105	17.1	CD34	23.0
CD90	15.7	CD14	8.6
CD44	12.5	CD31	7.2
CD29	9.3	HLA-DR	5.8
CD166	6.9	CD11b	5.0
CD146	3.7	CD133	2.9
CD49(a)	2.8	TRA181	2.9
HLA-ABC	2.3	CD19	2.2
CD49(e)	1.9	CD24	2.2
CD106	1.4	CD3	1.4
CD271	1.4	CD40	1.4
CD49(d)	0.9	CD56	1.4
CD140alpha	0.9	CD80	1.4
Sca1	0.9	CD86	1.4
CD33	0.5	Oct3/4	1.4
CD49(f)	0.5	CD4	0.7
CD54	0.5	CD20	0.7
CD71	0.5	CD79a	0.7
CD140(b)	0.5	CD117	0.7
CD144	0.5	CD309	0.7
CD172alpha	0.5	Sox2	0.7
$\alpha$ SMA+	0.5	TRA-160	0.7
Stro1	0.5	TRA-161	0.7
		TRA180	0.7
		SSEA-4	0.7

Positive and negative surface expression markers found in mesenchymal stem cells are listed together with the frequency of findings (%) in the reviewed studies ( $n = 44$ ). CD: Cluster of differentiation; CSM: Cell surface marker; HLA: Human leukocyte antigen; SSEA: Stage specific embryonic antigen; TRA: Teratocarcinoma surface antigen.

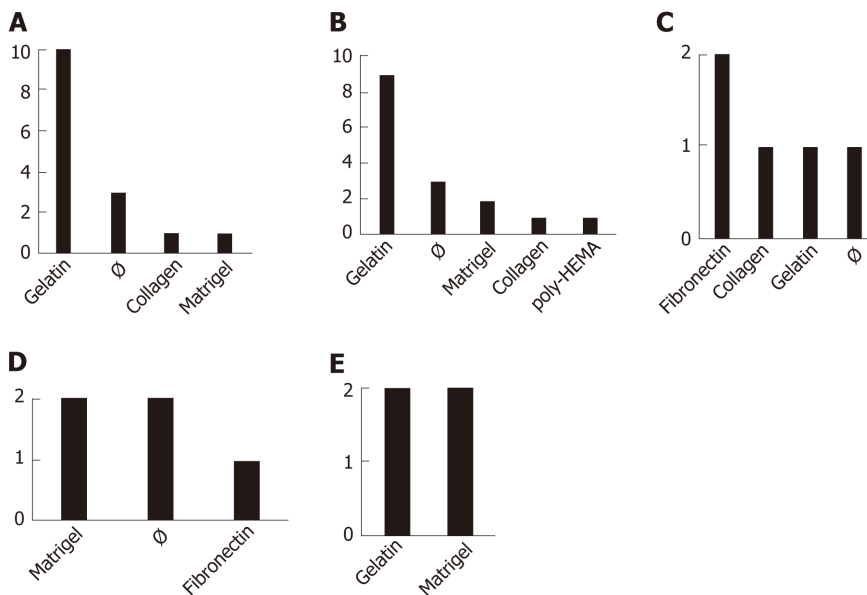
## DISCUSSION

The therapeutic potential of MSCs holds great promise for the handling of many diseases. The lack of consistency in the outcomes of adult MSC trials, sometimes leading to contradictory results and classically attributed to the heterogeneous nature of these cells, will not be overcome unless a unique standardized method of MSC production is implemented. Establishing a standard protocol for producing large-scale MSCs is therefore clearly necessary if an efficient treatment for clinical application is pursued.

Production of iMSCs seems to constitute an ideal option as these cells could be obtained in unlimited numbers from genetically homogeneous iPSC cell lines. However, the review of the literature shows that the current methods for producing iMSCs are far too different to produce homogeneous populations of iMSCs.

By reviewing 32 studies describing original methods for producing MSCs from iPSCs, we distinguished five different modalities: MSC Switch, EBs, Specific Differentiation, Pathway Inhibitor, and Platelet Lysate methods. These methods presented their own advantages, depending on the application pursued.





**Figure 2 Coatings used to produce induced pluripotent stem cell-derived mesenchymal stem cells.** The relative frequencies of coatings used by the 32 studies are displayed as number of events within each of the 5 categories of protocols being described: Mesenchymal stem cell Switch (A), Embryoid Bodies (B), Specific Differentiation (C), Pathway Inhibitor (D), and Platelet Lysate (E). Note that some studies use more than one type of coating. The symbol “Ø” indicates (absence of coating). poly HEMA: Polymer forming hydrogel in water.

MSC Switch methods appears to be the most popular method (6 method variants have been cited over 100 times) (Table 1), and seems to be the least complex of the protocols, at the expense of, perhaps, increased variability of the obtained iMSCs[24]. It presents itself as a fast (from 7 d, for the Lian *et al*[41] variant) and technically simple approach as it only requires to directly switch the growth medium. FACS sorting allows the selection of cell subpopulations possibly leading to more consistent results.

Feeder free conditions simplify the process, and the use of chemically defined media allows control over animal-derived products and reduces batch-to-batch variability.

EB methods seem to be extensively cited as well (4 variants have been cited more than 100 times), however it presents itself with some technical difficulties, such as EB average size optimization.

Specific Differentiation methods present several disadvantages: they are laborious, costly, time-consuming, and require complex media. However, they present the advantage to eliminate, to a further extent, the remaining iPSCs from the final iMSC population. By adding a pre-differentiation step before the generation of iMSC, the risk of tumorigenicity is reduced, increasing iMSC safety, an aspect of particular relevance when cells are infused into immunocompromised patients.

Pathway inhibitor protocols, focusing on controlling cellular signaling pathways to generate iMSCs, might become helpful in combination with the other described methods, perhaps allowing the development of faster or more robust protocols.

Finally, Platelet Lysate Methods are aimed at avoiding the use of FBS, to prevent transmission of animal prions and reduce the risk of rejection or undesired immune reactions upon cell transplants[77].

Despite the fact that all the studies report the production of well-characterized MSCs, according to the ISCT[13], the methods found are excessively different to produce MSCs of similar quality. To encourage the clinical use of iMSCs use, a standardized protocol should be established. We found that many of the protocols belonging to the same category show small differences in media composition for cell differentiation, suggesting that a general protocol could emerge, following empirical testing of minority factors.

Fast methods and simplicity of the technique to generate iMSC can be considered as advantages to generate important quantities. A limitation found, however, is the absolute lack of information detected with respect to yields obtained by each method. Although the most important point that should be taken into account is the safety of the therapy, the robustness of the method to generate homogeneous and reproducible cell populations is undoubtedly relevant as well.

MSCs have received significant attention due to their strong potential for cell-based and tissue regenerative therapies[20-23]. Indeed, they have been shown to secrete a

wide variety of immunomodulatory factors. A fact that has attracted interest for the treatment of immune-related disorders or diseases with an inflammatory component. According to Frobel *et al*[71] and McGrath *et al*[54], iMSCs obtained with safe platelet lysate methods, however, might limit their use as immunomodulators.

Despite the great potential for the use of iMSCs as immunomodulatory entities, the investigation of potential avenues in this direction still remains at its infancy, with few studies evaluating only some of the secreted molecules. Omic-based analysis of the obtained iMSCs should lead to a more complete phenotyping and thus a deeper understanding of iMSC potential therapeutic benefits.

As per iMSC use in the Clinic, the only CT using iMSCs we are aware of is the CT registered with the title: “Safety and Efficacy of MSC for the Treatment of Adults with Steroid-Resistant Acute Graft Versus Host Disease (GvHD)” (trial number NCT02923375). The study included 16 patients who developed refractory GvHD from Australia and the United Kingdom, who received 1-2 million cells/kg on Day 0 and Day 7 and visits on Days 0, 3, 7, 14, 21, 28, 60 and 100. Cynata Therapeutics, a company directed by Dr. Kilian Kelly, started this CT in March 1 2017. The results seem very encouraging, what should be supportive of the progress of this therapeutic use of iMSCs towards Phase 2 trials.

However, the use of iMSCs in the Clinic still presents with some handicaps to be overcome, starting by the high cost of iPSC cell culture and maintenance. Although the risk of rejection is very low, as MSC do not display MHC, autologous rather than allogeneic therapies are preferred[78,79], and iMSCs should be generated without animal components under xenofree conditions for safety compliance.

There are ethical issues concerning the use of iPSCs and iMSCs themselves that should be taken into account[80]. Importantly, iPSC donors consenting should be ensured, as well as the protection of donor data[81]. On another side, iPSCs used for iMSC generation should be generated excluding viral-based methods, even being the most efficient. Alternative methods may include micro-RNAs, plasmids or chemicals [82].

The use of iMSCs for cell therapy is not free of risks, since potential remaining iPSC or iMSC can directly generate tumor or metastasis in immunocompromised patients [83]. Indeed, the c-Myc factor used to reprogram iPSCs is an oncogenic factor in immunocompromised mice[84].

An alternative for risk reduction could be the use of cell-free iMSC derived fractions, presently being explored. For example, extracellular vesicles (EVs) might result safer while still holding healing properties of extended value[85-87].

On another end, although the differentiated cell-type used for reprogramming could translate into different iMSC products, an avenue worth of future explorations, the possibility of iMSC standardization production by far surpasses that of adult MSCs.

In summary, this review provides valuable information about the current methodological options to differentiate iPSC into iMSC to obtain MSCs of more homogeneous features than those isolated from adult tissues. It evaluates main culture media components, supplements and requirements of coatings based on a considerable number of original protocols ( $n = 32$ ). In addition, the study of main surface markers identified on iMSCs ( $n = 44$ ) show that in addition to fulfilling the ISCT minimal criteria, additional common iMSC markers are getting defined. Relationships between iMSCs surface markers and cell properties is yet another underexplored, but high priority issue towards the development of protocols meeting therapeutic demands [88]. For example, despite the availability of commercial kits to produce iMSCs already in the market, such as StemDiff Mesenchymal Progenitors kit (STEMCELL technologies, cat #05240), the specific immunomodulation properties harboring the cells differentiated and expanded under these conditions remain to be defined. Systematic combinatorial evaluations of minor components and their impact on iMSC features awaits toward settling large-manufacturing systems that give support to truly standardized MSC therapeutics.

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## CONCLUSION

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Different culture conditions have been described to produce iMSCs, which can be classified in the following five categories: MSC Switch, EBs, Specific Differentiation, Pathway Inhibitor, and Platelet Lysate methods, according to their strategy and components. Each approach presents with some advantages and limitations, as described. Some have been more widely explored than others, but there is still no

consensus Good Manufacturing Procedures improved method for a scale-up production of iMSCs. This review details these methods and lists required components and cell phenotyping markers, to serve as a reference guide for future improvements on iMSC manufacturing.

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