Induced pluripotent stem cells as a new getaway for bone tissue engineering: A systematic review

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Abstract

Objectives: Mesenchymal stem cells (MSCs) are frequently used for bone regeneration, however, they are limited in quantity. Moreover, their proliferation and differentiation capabilities reduce during cell culture expansion. Potential application of induced pluripotent stem cells (iPSCs) has been reported as a promising alternative source for bone regeneration. This study aimed to systematically review the available literature on osteogenic potential of iPSCs and to discuss methods applied to enhance their osteogenic potential.

Methods and materials: A thorough search of MEDLINE database was performed from January 2006 to September 2016, limited to English-language articles. All in vitro and in vivo studies on application of iPSCs in bone regeneration were included.

Results: The current review is organized according to the PRISMA statement. Studies were categorized according to three different approaches used for osteo-induction of iPSCs. Data are summarized and reported according to the following variables: types of study, cell sources used for iPSC generation, applied reprogramming methods, applied osteo-induction methods and treatment groups.

Conclusion: According to the articles reviewed, osteo-induced iPSCs revealed osteogenic capability equal to or superior than MSCs; cell sources do not significantly affect osteogenic potential of iPSCs; addition of resveratrol to the osteogenic medium (OM) and irradiation after osteogenic induction reduce teratoma formation in animal models; transfection with lentiviral bone morphogenetic protein 2 results in higher mineralization compared to osteo-induction in OM; addition of TGF- β , IGF-1 and FGF- β to OM increases osteogenic capability of iPSCs.

1 | INTRODUCTION

The potential advantages of stem-cell-based therapies in bone tissue regeneration have been reported by several studies.^{1–5} Adult mesenchymal stem cells (MSCs) derived from autogenous sources including bone marrow,⁶ adipose tissue⁷ and dental tissue^{6,8–10} are considered promising sources of progenitors for stem-cell-based bone regeneration. However, MSCs are a highly heterogeneous population,¹¹ and their proliferation and differentiation capabilities have been shown to decrease during in vitro culture expansion.¹² Moreover, their differentiation capability is age-related.^{13,14}

Pluripotent stem cells are introduced as an attractive source of stem cells in bone regeneration because of their potential to overcome limitations associated with MSCs.^{11–14} Embryonic stem cells (ESCs) have an unlimited self-renewal capacity with the ability to differentiate into all cell types of the three germ layers.¹⁵ However, ethical/legal problems associated with the utilization of human ESCs in several countries¹⁶ and the risk of immune rejection after transplantation¹⁷ hamper their clinical application. The generation of patient-specific pluripotent stem cells, induced pluripotent stem cells (iPSCs), from the transduction of patients' own somatic cells,^{18,19} was a major breakthrough in tissue regeneration. iPSCs possess the same

characteristics as ESCs, including morphology, unlimited self-renewal capacity and gene expression profiles.²⁰ Since iPSCs can be derived from the patients' own somatic cells, the risk of immune rejection after transplantation is avoided. Given such properties, iPSCs are expected to replace ESCs in tissue regeneration.

Recently, there has been great interest in the application of iPSCs for bone regeneration. In order to limit the tumorigenicity associated with pluripotency of iPSCs, in vitro differentiation of iPSCs towards the MSCs and osteoprogenitor cells prior to transplantation is essential. To generate osteoprogenitors from iPSCs, they are normally directed to embryoid body (EB) formation as an intermediate step during osteogenic differentiation.^{21–23} Other studies have applied different strategies to skip this step in the osteogenic induction of iPSCs.^{24–26} Direct differentiation of iPSCs into the osteoblast lineage has also been reported.^{27–29}

The osteogenic medium (OM) consist of β -glycerol phosphate, ascorbic acid and dexamethasone has been commonly used for the osteo-induction of iPSCs.^{30,31} However, several protocols, including supplementing the medium with transforming growth factor- β (TGF- β), insulin growth factor-1 (IGF-1), basic fibroblast growth factor (FGF- β) or vitamin D3, have been reported to enhance the osteogenic capability of iPSCs.^{32,33}

To best of our knowledge, two reviews have been published on the application of iPSCs for bone tissue engineering.^{34,35} However, the current review aimed to review all the available literature in this context systematically and to discuss the recent methods applied to enhance their osteogenic potential. This systematic review would help to elucidate the limitations in reported studies and assist in designing future pre-clinical and clinical studies on the utilization of iPSCs for bone regeneration.

2 | METHODS AND MATERIALS

2.1 | Eligibility criteria

2.1.1 | Types of studies

All in vitro and in vivo studies that conducted an osteogenic induction of iPSCs or used iPSCs for bone regeneration were included. Included studies were limited to English-language studies from January 2006 to September 2016. Abstracts, reviews, letters and PhD theses were excluded.

2.1.2 | Types of participants

Any type of adult somatic cells (such as dermal and gingival fibroblasts), adult stem cells (such as bone marrow stem cells [BMSCs] and adipose-derived stem cells [AdSCs]) and iPSC lines (prepared iPSCs) used as a source of iPSCs were considered.

2.1.3 | Types of interventions

Studies that conducted an osteogenic induction in given cells were included. Studies that used only osteogenic induction as a part of a multilineage characterization test were excluded.

2.1.4 | Types of outcome measures

Osteogenesis was reported in in vitro studies using the alkaline phosphatase (ALP) activity test, Alizarin Red staining, osteocalcin (OCN) content kits or gene expression analysis using real-time RT-PCR. Bone regeneration in in vivo studies was reported using histological evaluation (such as haematoxylin and eosin [H & E] and Masson trichrome), histomorphometric analysis or radiographic evaluation (such as computed tomography [CT] scans and micro-CT).

2.1.5 | Information source

The MEDLINE (NCBI PubMed and PMC) database and other scientific electronic databases (acta.tums.ac.ir, hindawi.com/journals, rsc.org/journals-books-databases) were the information sources. In addition, a further handsearch was performed in the following journals: *PLoS ONE*, *Proceedings of the National Academy of Sciences of the United States of America*, *Stem Cells*, *Stem Cells and Development*, *Stem Cell Reports*, *Tissue Engineering: Part A*, *Acta Biomaterialia*, *Biomaterials* and *Journal of Dental Research*. Our search was limited to English-language studies from January 2006 to September 2016.

2.1.6 | Search strategy

An electronic search was performed to select articles relevant to osteogenesis of iPSCs using the following terms: "induced pluripotent stem cell" AND "osteogenesis" OR "osteogenic differentiation" OR "bone tissue regeneration" OR "bone formation" OR "bone reconstruction" OR "bone development" OR "bone tissue engineering." A search of the selected articles bibliographies was also performed manually.

2.1.7 | Study selection

Three independent reviewers conducted the study selection according to the defined keywords. They also performed the initial screening of titles and abstracts from the selected articles according to the pre-defined eligibility criteria. Disagreements between reviewers were resolved through discussion. Then, the reviewers performed the review on the full texts of articles which were agreed upon and selected by all the authors.

2.1.8 | Data collection process

Review and data extraction were performed according to the PRISMA flow diagram illustrated in Figure 1. As explained in the study selection segment, three independent reviewers performed the initial screening of the selected articles. Then, they reviewed the full text of the articles and tabulated all data. Disagreements between the three reviewers were resolved by consensus. In case of no agreement, a fourth reviewer decided. Fourth and fifth authors contributed in the discussion section.

FIGURE 1 Flow chart of the search strategy used in this study. *One article evaluated EBs formation approach *in vitro* and direct use of iPSCs *in vivo*

*One article evaluated EBs formation approach *in vitro* and direct use of iPSCs *in vivo*

2.1.9 | Data items

Results and data were extracted from the full text of the articles and tabulated as follows: (1) Types of included studies, including *in vitro* and *in vivo* studies (2) types of cells used as the source of iPSCs; (3) transduction methods and vectors used for reprogramming the cells; (3) any type of treatment or induction method conducted on iPSCs and their duration, including EB medium, MSC medium and OM; (4) types of scaffolds used for cell seeding; (5) types of animals and types of defects, defect location, and defect size used for *in vivo* studies; and (6) outcomes of *in vitro* and *in vivo* studies, ie, the results of the ALP activity test, Alizarin Red staining, OCN content kits and gene expression analysis using real-time RT-PCR for *in vitro* studies and the results of histological evaluation (such as H & E and Masson trichrome), histomorphometric analysis and radiographic evaluation (micro-CT and CT scans) for *in vivo* studies. Definitions for variables and data items are given in Box 1.

3 | RESULTS

3.1 | Study selection

Article review and data extraction were performed as illustrated in Figure 1, the PRISMA flow diagram. The search showed 784

results from the NCBI PMC and PubMed databases and two results from other sources and three other articles were also obtained and included by hand searching. A total of 60 articles were selected after duplicates were removed with respect to eligibility criteria. In reviewing the full text of the 60 selected articles, 10 more articles were excluded. One of the studies was excluded as it only described a step-wise procedure for the induction of mesenchymal-like progenitors from both iPSCs and ESCs and an *in vitro* bone development model by the subsequent cultivation of these progenitors on decellularized bovine bone scaffolds in perfusion bioreactors. Six of the studies were excluded because osteogenesis was only a part of the multilineage differentiation characterization tests. In three other studies, the effect of osteogenic induction was evaluated only in neurogenesis and PDL regeneration, and not for bone regeneration. Finally, a total of 50 articles were included in the systematic review.

3.2 | Cell sources used for generating iPSCs:

In the included articles, iPSCs were conventionally generated from the reprogramming of adult somatic cells including bone marrow CD34+ cells,^{23,36–38} human gingival and dermal fibroblasts,^{22,29,32,39–48} human fibroblasts (IMR90),^{26,44} human foreskin,⁴⁹ human periodontal

Box 1 Definitions of variables and data items

Induced pluripotent stem cells (iPSCs): A type of stem cells produced by converting differentiated somatic adult cells or non-pluripotent adult stem cells into pluripotent stem cells. Transcription factors such as Oct3/4, Sox2, Klf4 and c-Myc were commonly used for reprogramming the cells.

Teratoma formation: It is a golden standard evaluation to define stem cell as pluripotent stem cells. It is the capability of stem cells to generate tumoral structures containing tissues representing all the three germ layers in immune-compromised mice.

Embryoid bodies (EBs) formation: EBs are the three-dimensional aggregates formed in suspension by pluripotent stem cells including embryonic stem cells (ESCs) and iPSCs. Standard methods of achieving EBs include hanging drop technique and formation in static suspension culture condition. EB formation is important step-directed differentiation of pluripotent stem cells. However, differentiation of iPSCs can be induced with or without intervention of the EB stage

Transduction (transfection): It is a common method for reprogramming the cells into iPSCs and involves the introduction of transcription factors such as Oct3/4, Sox2, Klf4, and c-Myc using different vectors.

In vitro osteogenesis/mineralization evaluation

Alizarin Red staining: It visualized the mineralization of the extracellular matrix.

Alkaline phosphatase (ALP) activity test: It evaluates the enzymatic activity to show the rate of osteogenesis created by the cells. It can be measured by using an ALP Colorimetric Assay Kit that uses p-nitrophenyl phosphate (pNPP) as a phosphatase substrate.

Von Kossa staining: It is used to quantify mineralization in cell culture and tissue sections. The staining is based on precipitation reaction between silver ions (in staining solution) and phosphate (in mineralized culture) in the presence of acidic material.

Reverse transcription-polymerase chain reaction (RT-PCR): It includes conventional RT-PCR and real-time RT-PCR to evaluate the expression of specific genes. For osteogenic differentiation analysis, the expressions of ALP, BSP, Col1, Runx2, Spp1, Flt1, OCN, OPN, and ON genes were evaluated.

In vivo bone regeneration evaluation

Immunocytochemistry and immunohistochemistry: They are used to find a specific protein such as ALP and OCN localized in cells or biologic tissue using specific antibodies.

Histologic evaluation: They are commonly performed to study the microscopic anatomy of cells or tissues sectioned, stained and mounted on a microscope slides. The staining of haematoxylin and eosin (H&E) and Masson trichrome are useful for evaluation of bone regeneration.

Radiographical evaluation: They are commonly performed for initial evaluation of bone regeneration. Micro-CT and CT scans are common for evaluation of bone regeneration.

ligament (PDL) cells,⁴¹ mouse fibroblasts,^{21,50,51} murine fibroblasts,^{22,52} equine fibroblasts,⁵³ rhesus macaque dermal fibroblasts⁵⁴ and human lung.⁴¹ Alternatively, iPSCs were also generated by converting adult stem cells like BMSCs,^{29,46,54,55} AdSCs²⁸ and stem cells from human exfoliated dental tissue (SHED).⁵⁶

3.3 | Reprogramming methods used for the generation of iPSCs:

A combination of four transcription factors, including octamer-binding transcription factor 3/4 (Oct3/4), sex-determining region Y-box 2 (Sox2), Kruppel-like factor 4 (Klf4) and cellular Myelocytomatosis Oncogene (c-Myc), were conventionally used for pluripotency induction.^{21,22,28,29,41-43,45,46,52,53,55,57} Alternatively, combinations of given factors plus Lin28,^{23,24,36-38,58} Oct3/4, Sox2 plus Nanog^{44,49} and Lin28, Oct3/4, Sox2, and Klf4 without c-Myc^{26,50,59} were utilized as well. These genes were transferred to the cells by various vectors including retroviral vector,^{21,29,41,46,50-52,56,57} episomal plasmids,^{23,37,38,47,48,52,58} lentiviral vector,^{28,42,43,54,59} Sendai virus vector,²⁴ pMSCV vector,²¹ StemCCA lentiviral reprogramming kit,²⁹ cell-penetrating peptide³⁰ and Piggy-Bac transposon-based vector.⁵³ Three studies used the mRNA reprogramming method for transduction.^{26,44,45}

3.4 | Treatment approaches used for the osteo-induction of iPSCs:

Three main approaches were utilized in application of iPSCs for bone regeneration (Figure 2): (1) Generating MSCs from iPSCs with the EBs formation step^{21-23,30,32,33,36-39,42,43,46-52,55,57-70} (Table 1); (2) generating MSCs from iPSCs without the EB formation steps^{24-26,41,44,45,53,54,56,71} (Table 2); and (3) using iPSCs directly without generating MSCs or EBs^{27-29,40,62,72} (Table 3). One study evaluated the osteogenic potential of osteo-induced iPSCs derived with the EB formation step seeded on a nanofibre-based scaffold in vitro and investigated the ability of bone regeneration in rat critical-sized bone defects using osteo-induced iPSCs without generating MSCs or EBs.⁶²

3.5 | Scaffolds used for iPSC seeding:

Scaffolds used for bone regeneration in several studies were polyethersulfone nanofibres,^{39,62} gelfoam surgical sponges,²¹ polycaprolactone (PCL),^{27,44} hydroxyapatite/tricalcium phosphate (HA/TCP),^{22,29,30,41,54} HA/poly-lactic-co-glycolic acid (PLGA),²⁸ HA-coated PLGA/poly(L-lactic acid) (PLLA) scaffold,⁶⁹ PCL functionalized with natural polymer hyaluronan and TCP,²⁶ decellularized bone scaffold,^{24,73} calcium phosphate cement (CPC),^{36-38,58,70} apatite-coated

FIGURE 2 Three main approaches used for osteogenic induction of iPSCs

silk fibroin,⁴⁹ fibrin,³⁰ silk,⁶⁸ collagen type I,⁴⁷ HA/Collagen/Chitosan (CTS),^{59,61} nanoHA/CTS/gelatin^{42,43,59,61} and gelatin-methacrylate-co-acryloyl 6-aminocaproic acid and gelatin-methacrylate-co-poly(ethylene glycol)-diacrylate.⁷²

3.6 | Defect models used for bone regeneration:

the bone regeneration capability of osteo-induced iPSCs was evaluated by implantation subcutaneously,^{21,24,26–29,41–43,51,52,54,65,69} intramuscular injection,⁴⁷ or by transplantation in critical-sized calvarial,^{23,28,30,57–59,61,64,68} long segmental bone^{30,47} or periodontal defects.^{49,71}

4 | DISCUSSION

4.1 | Cell types of origin and osteogenic differentiation potential of iPSCs

Various differentiated adult somatic or stem cells were obtained from human and animal tissues for generating iPSCs. Somatic cells are easily accessible, and the discovery of iPSCs basically originated from the reprogramming of these types of cells.^{18,19} Moreover, it has been demonstrated that iPSCs derived from MSCs maintain their normal karyotype even up to 120 population doublings.⁷⁴ From the included studies, five compared the osteogenic potential of iPSCs originating from various cell sources.^{24,29,41,46,56} The results of three studies are in accordance with the theory that differentiation potentials of human iPSCs can be affected by the cell type of origin.⁷⁵ de Peppo et al. used human iPSCs from both dermal fibroblasts (11c and 1013A) and BMSCs (BC1). Both 1013A and BC1-derived osteogenic progenitors showed significantly higher expression of the ALP gene, a key early marker of osteogenesis, and calcium deposition, but 11c-derived osteogenic progenitors demonstrated poor osteogenic differentiation and less mesenchymal differentiation potential. In addition, after 12 weeks of subcutaneous implantation, 1013A-derived osteogenic

progenitors demonstrated bone-like tissue with embedded cells, and BC1-derived osteogenic progenitors demonstrated areas of dystrophic calcification and high mineral content, indicating that cells from different origins showed different regenerative properties.²⁴ The study by Ishiy et al. reported that iPSCs derived from both SHED and human fibroblast presented the up-regulation of ALP. However, ALP activity and calcium mineralization of SHED-derived iPSCs were more than fibroblast-derived iPSCs,⁵⁶ which may refer to the somatic epigenetic memory of the cell origin.⁷⁶ Hynes et al. demonstrated the different characteristics of iPSCs generated from three various somatic cells, ie, gingival fibroblasts (GFs), PDL and lung fibroblasts.⁴¹ They also demonstrated that the subcutaneous implantation of these cells with an osteoconductive scaffold showed greater mineralized structure in osteo-induced iPSCs generated from PDL compared with the other two cell sources. Furthermore, the MSCs derived from PDL-iPSCs have demonstrated the potential to regenerate ligamentous structures including Sharpey's fibres and mineralized tissue similar to bone and cementum in vivo; therefore, it may be hypothesized that even after the induction of iPSCs into MSC-like cells, their epigenetic memory is retained.⁴¹

In contrast, two studies raised a debate regarding the influential role of cell origin on osteogenic potential. Nasu et al. demonstrated no significant difference in osteoblast gene expression profiles comparing iPSCs originated from BMSCs and those with a dermal fibroblast (DF) origin.⁴⁶ However, in their study, Philips et al. suggested that cell origin plays a more determining role than the differentiation method in vitro,²⁹ yet both the quantity and quality of in vivo bone formation of dermal fibroblast-derived iPSCs were similar to the BMSC-derived iPSCs. It is assumed that in vivo results may not be predicted from in vitro results.

In addition to the cell type of origin, it has been demonstrated that the differentiation potential of iPSCs is not influenced by donor age,²⁹ even though sex-specific differences were observed in the study by Anguera et al.⁷⁷; female iPSCs were noted to be epigenetically less stable.

Considering the conflicting evidences and numerous variables regarding the effect of cell origin on the osteogenic differentiation potential of iPSCs, it is evident that further studies with other influential factors eliminated, such as various pluripotency factors and medium combinations, should be performed to thoroughly elucidate the role of epigenetic memory in this context.

4.2 | Reprogramming methods used for the generation of iPSCs

An ideal method should have high transfection efficiency with no genome integration, be easy to use, and be reproducible. However, highly efficient reprogramming with sustained expression of transgene usually happens when transgenes are integrated into the host genome. The studies included in this review had utilized various vectors to reprogramme of different cell sources into iPSCs. However, the reprogramming efficiency was barely reported by these studies, which makes a feasible comparison difficult.

TABLE 1 iPSC-osteoprogenitors obtained with EB formation step

Authors	Type of study	Cell Source for iPSCs	Transduction	Treatment	Scaffolds	Animal Model	Test Groups	Results
Bilousova et al. ²¹	In vitro	ICR mouse dermal fibroblasts	pMXs retroviral vector for Oct3/4, Klf4 and Sox2 MSCV-ires-GFP vector for c-Myc	EB formation: KO-DMEM, GlutaMAX-I, NEAA, β -Mercaptoethanol, 15% FBS (2 days) MSC generation: ES cell medium, 10^{-7} M ATRA (2 days) Osteogenic induction: OM (8 weeks)	Gelfoam surgical sponges	—		Alizarin Red staining, Von Kossa silver, ALP activity test showed high level of osteogenesis in osteo-induced iPSCs Runx2, Col1a1, Spp1, BSP and Flt1 were highly expressed in osteo-induced iPSCs Osteo-induced iPSCs seeded on gelfoam demonstrated expression of Runx2, Col1a1, Spp1, BSP and Flt1. No tumor formation Histological evaluation demonstrated mineralization and vascularization in osteo-induced iPSCs on gelfoam
	In vivo					Subcutaneous implantation in nude ICR mice		
Wang et al. ²³	In vitro	Human iPSC BC1 line (derived from bone marrow CD34+ cells)	—	EB formation: 80% (DMEM)/F12, 20% Knockout Serum Replacement, NEAA, L-glutamine, β -Mercaptoethanol (10 days) MSC generation: DMEM-LG, 10% FBS Osteogenic induction: OM (2 weeks)	RGD-coated macroporous CPC	—	-Osteo-induced iPSCs -Osteo-induced hUCMSCs -Osteo-induced hBMSCs	At day 7, ALP peaked, while OCN down-regulated in osteo-induced iPSCs At day 14, ALP peaked, while OCN up-regulated in osteo-induced hUCMSCs and hBMSCs. For each group, the mineral amount synthesized by cells increased dramatically from 1 to 14 days with no significant differences among them were observed
	In vivo					8 mm calvarial defect in athymic nude rats	-CPC -hiPSC-MSCs on CPC -Osteo-induced iP-SC-MSCs on CPC -BMP2-transduced iPSC-MSCs on CPC	At week 12, the new bone area was significantly higher in all groups compared with CPC control. No significant differences were detected among the three cell types

(Continous)

TABLE 1 (Continued)

Authors	Type of study	Cell Source for iPSCs	Transduction	Treatment	Scaffolds	Animal Model	Test Groups	Results
Li et al. ²²	In vitro	Murine fibroblast	pMXs retroviral vector Oct3/4, Klf4 and Sox2, c-Myc	EB formation: DMEM 15% FBS, NEAA, β -Mercaptoethanol, L-glutamine, (3 days) MSC generation: - Some EBs treated with RA (2 days) - 0.1% gelatin-coated plates and DMEM, 15% FBS, NEAA, β -Mercaptoethanol, L-glutamine, TGF- β 1 (2 days) - DMEM, 10% FBS, ascorbic acid - Osteogenic induction: OM (28 days)	—	—	-Osteo-induced iPSCs from TGF- β 1 and RA treatment -Osteo-induced iPSCs from RA treatment alone	More calcium deposition by Alizarin Red staining and Von Kossa silver was observed in osteo-induced iPSCs from TGF- β 1 and RA treatment Higher expression of Runx2 was observed in Osteo-induced iPSCs from RA treatment alone, while treatment with TGF- β 1 and RA resulted in higher expression of OCN and OSX.
Lee et al. ⁶⁰	In vitro	Prepared human iPSCs line (Kor-WT-iPSC 1)	—	EB formation: β -depleted ESC Medium (EB medium) MSC generation: EB medium mixed with hMSC medium (1:1) (v/v) (4 days) Osteogenic induction: OM (2 weeks)	—	—	-EBs -Osteo-induced iPSC-MSCs	Expression of ALP, Col1, OPN, ON and OCN in significantly enhanced in osteo-induced iPSCs Alizarin Red staining, Von Kossa silver showed the calcium deposition was higher in Osteo-induced iPSCs
Li et al. ⁵²	In vitro	Murine tail tip fibroblasts	pMXs retroviral vector Oct3/4, Klf4 and Sox2, c-Myc	EB formation: DMEM 15% FBS, NEAA, β -Mercaptoethanol, L-glutamine (3 days) MSC generation: -ES medium with RA (2 days) -Medium supplemented with either TGF- β 1, TGF- β 3, FGF- β or BMP2 (2 days) -DMEM with FBS and ascorbic acid Osteogenic induction: OM (28 days)	—	—	-iPSC-MSCs treated with RA -iPSC-MSCs treated with FGF- β -RA -iPSC-MSCs treated with TGF- β 1-RA -iPSC-MSCs treated with TGF- β 3-RA -iPSC-MSCs treated with BMP2-RA	Alizarin Red staining showed that treatment with TGF- β 1 and TGF- β 3 resulted in higher differentiation ability towards the osteoblastic lineage Treatment with RA alone and or FGF- β and BMP2 exhibited lower expression of Runx2, OCN, OSX and OPN
	In vivo				HA/TCP ceramic scaffolds	Subcutaneous implantation in thymic SCID mice	-iPSC-MSCs treated with TGF- β 1-RA on scaffold -iPSC-MSCs treated with RA on scaffold	OCN and DMP1 immunohistochemistry staining showed that iPSC-MSCs treated with TGF- β 1-RA differentiate into osteoblasts More bone with blood vessels were observed in iPSC-MSCs treated with TGF- β 1-RA

(Continues)

TABLE 1 (Continued)

Authors	Type of study	Cell Source for iPSCs	Transduction	Treatment	Scaffolds	Animal Model	Test Groups	Results
Villa-Diaz et al. ⁵⁷	In vitro	Human fibroblasts	Retroviral vector Oct3/4, Klf4 and Sox2, c-Myc	EB formation: PMEDSAH-coated plates with human cell conditioned medium supplemented with FGF- β (7 days) MSC generation: α -MEM, 10% FBS, L-glutamine and NEAA supplement and FGF- β (2 weeks) Osteogenic induction: OM (3 weeks)	Gelfoam sponge	—	-Osteo-induced iPSCs -iPSC-MSCs	Expression of BMP2 Col1a1, OCN, ALP, SPP1, OCN and DLX5 was increased in osteo-induced iPSCs Alizarin Red staining showed calcium deposition in osteo-induced iPSCs
	In vivo					5 mm calvarial defect in immunocompromised mice	-iPSC-MSCs on Gelfoam -Gelfoam	Micro-CT analysis demonstrated a 4.2-fold increase in new bone formation in treatment with iPSC-MSCs
Tang et al. ³⁷	In vitro	Human iPSC BC1 line (derived from bone marrow CD34+ cells)	Episomal vector pEB-C5 Oct3/4, Klf4 and Sox2, c-Myc and Lin28	EB formation: DMEM/F12, 20% Knockout Serum Replacement mixed with 50% MEF-conditioned medium, NEAA, L-glutamine (10 days) MSC generation: DMEM, 10% FBS, L-glutamine, Osteogenic induction: OM (3 weeks)	CPC	—	-iPSC-MSCs on CPS -Osteo-induced iPSC on CPC	Osteo-induced iPSCs represented higher expression of ALP, OCN, Col1 and Runx2 ALP activity in osteo-induced iPSCs was 10 fold higher Alizarin Red staining showed that bone minerals in osteo-induced iPSCs was 3- to 4-fold higher
TheinHan et al. ³⁸	In vitro	Human iPSC BC1 line (derived from bone marrow CD34+ cells)	—	EB formation DMEM/F12, 20% Knockout Serum Replacement mixed with 50% MEF-conditioned medium, NEAA, L-glutamine (10 days) MSC generation: DMEM, 10% FBS, L-glutamine Osteogenic induction: OM	CPC-RGD CPC-Fn CPC-FEPP CPC- Geltrex CPC- Platelets	—	Osteo-induced iPSCs seeded on five CPC scaffolds	The expression of ALP, Runx2 and Col1 was significantly higher on CPC-platelet group Alizarin Red staining showed that mineral synthesis by cell seeded on CPC-RGD, CPC-Fn and CPC-platelet at day 21 was significantly higher than other CPC groups
Liu et al. ³⁶	In vitro	Human iPSC BC1 line (derived from bone marrow CD34+ cells)	—	EB formation: DMEM knock out serum replacement, NAAs, L-glutamine, β -Mercaptoethanol (10 days) MSC generation: DMEM-LG, 10% FBS, L-glutamine Osteogenic induction: BMP2 lentiviral	RGD-chitosan-CPC	—	-BMP2-transduced iPSCs on RGD-CPC -osteo-induced iPSCs on RGD-CPC	BMP2-transduced iPSCs expressed significantly higher levels of BMP2, Col1A1 and OCN ALP activity of BMP2-transduced iPSCs was significantly higher Alizarin Red staining showed significantly more mineralization in BMP2-transduced iPSCs

(Continous)

TABLE 1 (Continued)

Authors	Type of study	Cell Source for iPSCs	Transduction	Treatment	Scaffolds	Animal Model	Test Groups	Results
Diederichs et al. ⁵⁵	In vitro	BMSCs	Lentiviral vector Oct3/4, Klf4 and Sox2, c-Myc	EB formation: DMEM knockout serum replacement, NAAS, L-glutamine, β -mercaptoethanol (3 days) MSC generation: DMEM, 10% FBS, L-glutamine, RA(8 days) Osteogenic induction: OM (7 days)	—	—	-Osteo-induced iPSCs -Osteo-induced BMSCs	Alizarin Red staining verified matrix mineralization in osteo-induced iPSCs Osteo-induced iPSCs Col1a1 at similar levels as BMSCs, but OCN and BSP remained lower
Wang et al. ⁵⁸	In vitro	Human iPSC BC1 line		EB formation: ultralow attachment cell culture flask (10 days) MSC generation: DMEM-LG, 10% FBS Osteogenic induction: -OM -BMP2 transduction (lentivirus human BMP2)	Fast-degradable alginate microbeads+CPC paste	—	-iPSC-MSCs -Osteo-induced iPSCs -BMP2-transduced iPSCs	iPSC-MSCs encapsulated in CPC-microbeads maintained good viability. Osteo-induced and BMP2-transduced iPSCs demonstrated earlier upregulation of ALP, Runx2, Col1a1 and OCN Alizarin Red staining showed higher degree of mineralization in osteo-induced and BMP2-transduced iPSCs
	In vivo					5 mm calvarial defect athymic nude rats	-iPSCs on CPC -Osteo-induced-iPSCs on CPC -BMP2-transduced iPSCs on CPC -CPC	New bone area fraction at 12 weeks was higher in osteo-induced-iPSCs and BMP2-transduced iPSCs
Xie et al. ⁶¹	In vitro	Prepared Mouse iPSC	—	EB formation: DMEM, 10% FBS, 10^{-7} M RA (4 days) MSC generation: DMEM/F12, 10% FBS (7 days) Osteogenic induction: OM (2 weeks)	HA/Collagen/Chitosan nanofibers	—	-Osteo-induced iPSCs on HA/Collagen/Chitosan -Collagen/Chitosan -Osteo-induced iPSCs on HA/Chitosan -Osteo-induced iPSCs on Chitosan -Osteo-induced iPSCs on TCP	Osteo-induced iPSCs on HA/Collagen/Chitosan showed the highest cell viability and attachment Runx2, OCN, ALP and Col were significantly expressed in osteo-induced iPSCs cultured on HA/Collagen/Chitosan ALP activity was significantly elevated in osteo-induced iPSCs on HA/Collagen/Chitosan CT represented that defects fully repaired in osteo-induced iPSCs on HA/Collagen/Chitosan and bone mineral density was elevated significantly in this group Histological examination revealed robust, thick and large new bones in osteo-induced iPSCs on HA/Collagen/Chitosan
	In vivo					3 mm calvarial defect in ICR mice	-Chitosan -HA/Chitosan -HA -/Collagen/Chitosan -Osteo-induced iPSCs on HA/Collagen/Chitosan -Empty defect	

(Continous)

TABLE 1 (Continued)

Authors	Type of study	Cell Source for iPSCs	Transduction	Treatment	Scaffolds	Animal Model	Test Groups	Results
Liu et al. 70	In vitro	Human iPSC BC1 line		EB formation: DMEM/F12, 20% knockout serum replacement, minimum essential medium NEEA, L-glutamine, β -mercaptoethanol (10 days) MSC generation: - Culture of EBs on 0.1% gelatin-coated plates -Mechanical separation of MSCs which migrated out of EBs -lentiviral transduction of iPSC-MSCs with NELL1 and RFP -Osteogenic induction: OM (21 days)	RGD grafted CPC	—	RFP-iPSC-MSCs seeded on RGD-CPC scaffold cultured in OM NELL1-iPSC-MSCs seeded on RGD-CPC scaffold cultured in OM	Expression of Runx2, OCN at 14 days and Col1a1 at 7 days was higher in NELL1-iPSC-MSCs Mineral synthesis was increased in NELL1-iPSC-MSCs at 21 days
Sheyn et al. 47	In vitro In vivo	Human dermal fibroblasts	Episomal plasmid vector OCT4, SOX2, KLF4, L-MYC, LIN28, and p53 shRNA	EB formation: DMEM/F12, 17% knockout serum replacement, minimum essential medium NEEA, β -mercaptoethanol (8 days) MSC generation: DMEM, 10% FBS, L-glutamine+10 ng/ml TGF- β 1(2 days) Osteogenic induction: -OM (7 or 14 days) (In vitro) -BMP6 nucleofection (In vivo)	- Collagen type I biodegradable scaffolds (radial defect model)	- Ectopic model in thigh muscle in NOD/SCID mice 1.5 mm radial defect in NOD/SCID mice	-BMSCs -early iPSC-MSCs (aiMSC) -late iPSC-MSCs (tiMSC) -BMP6-overexpressing BMSCs -BMP6-overexpressing aiMSC -BMP6-overexpressing tiMSC -BMP6-overexpressing BMSCs -BMP6-overexpressing aiMSC -BMP6-overexpressing tiMSC -acellular collagen sponge (control mice)	ALP activity was significantly higher in aiMSC on days 7 and 14 BMSCs and tiMSCs expressed significantly higher levels of ALP than aiMSC on day 7 aiMSC and tiMSC expressed significantly higher levels of Col1a1 than BMSCs on day 14 Bone volume of the ectopic bone induced by BMSCs and aiMSC was significantly higher than tiMSC after 4 weeks tiMSC-treated group had significantly higher bone volume than BMSCs and aiMSC

(Continues)

TABLE 1 (Continued)

Authors	Type of study	Cell Source for iPSCs	Transduction	Treatment	Scaffolds	Animal Model	Test Groups	Results
Jeon et al. ⁶⁹	In vitro	Prepared human iPSCs line	—	EB formation: ultralow attachment cell culture flask MSC generation: DMEM, 10% FBS, 1% Glutamax, FGF- β (10 days) Osteogenic induction: -OM (3 weeks) -Osteogenic supplements: dexamethasone, β -glycerolphosphate, 1 α ,25-dihydroxy vitaminD3	PLGA/PLLA scaffold with 0%, 1% and 5% w/v HA (HA-0), (HA-1) and (HA-5)	-	- Mono-cultured hiP-SC-MSC with osteogenic supplements - Mono-cultured hiP-SC-MSC without osteogenic supplements - Co-cultured hiPSC-MSCs and hiPSC-macrophages with bone supplements - Co-cultured hiPSC-MSCs and hiPSC-macrophages without bone supplements	- Expression of OCN was higher for both hiPSC-derived cultures under the osteogenic supplements - Co-culture without osteogenic supplements exhibited large multinucleated osteoclasts and significant increases in the expression of OC-related genes
In vivo								
Subcutaneous in athymic nude mice								
Egusa et al. ⁵⁰	In vitro	Mouse gingival fibroblast from adult C57BL/6J mice	Retroviral vector Oct3/4, Klf4 and Sox2	EB formation: DMEM, 15% FBS, L-glutamine, NEAA, β -mercaptoethanol, (2 days) Osteogenic induction: OM (30 days)	—	—	- Osteo-induced hiP-SC-MSCs on scaffold - Co-cultured hiPSC-MSCs and hiPSC-macrophages without bone supplements on scaffold	- Co-cultured cells significantly produced more maturation of bone-specific matrix than mono-cultured cells in the same dose of HA - Co-cultured cells on HA-5 composite scaffolds produced more maturation of bone-specific matrix and mineralization than HA-1 and HA-0 scaffolds significantly
Nasu et al. ⁴⁶	In vitro	BMSCs (BM-dermal fibroblast (DF-iPSCs))	pMXs retroviral vector Oct3/4, Klf4 and Sox2, c-Myc	EB formation: DMEM, 10% knockout serum replacement, 10% FBS, activin-A (5 days) Osteogenic induction: OM, 100 ng/ml Activin-A	—	—	- Osteo-induced BM-iPSCs - Osteo-induced DF-iPSCs	More calcium deposition was observed in osteo-induced iPSCs Expression of Runx2, DLX5 and OCN was significantly higher in osteo-induced iPSCs iPSC colony forming was fewer in BM-iPSCs There were no significant differences in osteogenic potential between two groups

(Continous)

TABLE 1 (Continued)

Authors	Type of study	Cell Source for iPSCs	Transduction	Treatment	Scaffolds	Animal Model	Test Groups	Results
Kato et al. ³²	In vitro	Human iPSCs (line 201B7)	—	EB formation: DMEM/F-12, 20% knockout serum replacement, NEAA, L-glutamine, 2-Mercaptoethanol (6 days), followed by 2 μ M thiazovivin (1 hour) without FGF- β Osteogenic induction: OM, followed by FGF- β , IGF-1 and TGF- β (2 weeks), followed by 1,25(OH) $_2$ vitamin D3 (2 weeks)	—	—	- Osteo-induced iPSCs - Osteo-induced BMSCs	Osteo-induced iPSCs treatment with 1,25(OH) $_2$ vitamin D $_3$ showed increased in expression of Col1a1 and OCN and decreased in expression of TNAP and Runx2 iPSCs were more reactive to 1,25(OH) $_2$ vitamin D $_3$ than MSCs
Ochiai-Shino et al. ³³	In vitro	Human iPSCs (line 201B7)	—	EB formation: DMEM/F-12, 20% knockout serum replacement, NEAA, L-glutamine, β -mercaptoethanol (6 days), followed by 2 μ M thiazovivin (1 hour) Osteogenic induction: -OM plus FGF- β , TGF- β $_1$, IGF-1 and BMP-2/7(2 weeks)	—	—	—	Number of TNAP $^+$ cell by adding β -FGF- β , TGF- β , IGF-1 were increased TNAP $^+$ cell showed high level of TNAP, OSX, BSP, OCN, Runx2 and Col1a1
Ardehshir-ylajimi et al. ⁶²	In vitro	Prepared human iPSCs line	—	EB formation: DMEM/F12, 15% knockout serum replacement, NEAA, L-glutamine, β -Mercaptoethanol (4 days) Osteogenic induction: OM (21 days)	PES Tissue culture Polystyrene (TCPS)	—	Osteo-induced iPSCs on scaffolds	Higher ALP activity and higher expression of Runx2, Col1, OCN and ON were observed in osteo-induced iPSCs on PES
Duan et al. ⁴⁹	In vitro	Human iPSC-cell line (derived from foreskin fibroblasts) Mouse iPSC-cell line	—	EB formation: -mTeSR1 for hiPSCs -DMEM with FBS, L-glutamine, NEAA, β -Mercaptoethanol for mouse iPSCs Osteogenic induction: -OM -EMD-stimulated media (DMEM/F12 with 20% FBS, 30 μ g/ml EMD)	Apatite-coated silk fibroin	—	- iPSCs induced in OM - iPSCs induced in EMD-stimulated media - iPSCs in basic media (DMEM/F12 with 20% FBS)	Expression of Runx2 greatly increased in iPSCs induced in EMD-stimulated medium and OM. Expression of OC decreased in iPSCs in EMD-stimulated medium Col1 expressed significantly in iPSCs treated with OM Alizarin Red staining showed almost no calcium nodules in EMD-stimulated group
	In vivo				2 $\times$ 1.5 mm 2 periodontal fenestration defect in nude mice		- Silk scaffold - Silk scaffold+EMD - Osteo-induced iPSCs on silk scaffold+EMD	Histological and radiographical analysis showed higher new bone formation area in iPSCs cultured on silk scaffold +EMD Higher expression levels of OCN, OSX and Runx2 were observed in iPSCs cultured on silk scaffold +EMD

(Continuous)

TABLE 1 (Continued)

Authors	Type of study	Cell Source for iPSCs	Transduction	Treatment	Scaffolds	Animal Model	Test Groups	Results
Kao et al. ⁶⁵	In vitro	Prepared Murine germ line-competent iPSCs	—	EB formation: DMEM, 15% FBS, 1% NEAA, β -mercaptoethanol Osteogenic induction: -OM (2 weeks) -OM (2 weeks) followed by 20 μ mol/L resveratrol	—	—	- iPSCs induced in resveratrol treated OM - iPSCs induced in OM	Alizarin Red staining showed that the addition of resveratrol to the osteogenic medium significantly increased calcium accumulation in iPSCs Expression of Runx2, OCN and IBSP was significantly higher in resveratrol-treated group Higher expression of OPN in grafts from mouse fed with resveratrol than mouse fed with control vehicle Expression of Oct-4, Nanog, Klf-4 and c-Myc was significantly suppressed in grafts from mouse fed with resveratrol
In vivo						Subcutaneous implantation in nude mice (BALB/c strain)	- Mouse fed with resveratrol (per os at 30 μ g/g animal/day) - Mouse fed with control vehicle	
Ko et al. ³⁰	In vitro	Human iPSCs line (SC80 2A -1)		EB formation: DMEM/F12, 20% (v/v) knockout serum Replacements, NEAA, β -Mercaptoethanol, FGF- β +10 ⁻⁷ M ATRA (10 days) Osteogenic induction: OM (2 weeks)	—	—	- Osteo-induced iPSCs- Osteo-induced BMSCs	Expression of Col1a1 and BSP in osteo-induced iPSCs was lower at day 7 In day 14 higher expression of Runx2 was observed in osteo-induced iPSCs ALP staining and Alizarin Red staining showed that osteoblastic differentiation and mineral deposition were significantly weaker in osteo-induced iPSCs No significant difference between the defects treated with osteo-induced iPSCs and those treated with osteo-induced BMSCs Better bone regeneration was observed on HA/ β -TCP side than fibrin glue side
In vivo					HA/ β TCP Fibrin glue	4 mm calvarial defects in Sprague-Dawley rats (fibrin glue on left defects and HA/ β -TCP on right side)	- Osteo-induced iPSCs on scaffolds - Osteo-induced BMSCs on scaffolds - Empty	
					4 mm long defect on radius in Sprague Dawley rats (with fibrin glue)	- Osteo-induced iPSCs on fibrin glue - Osteo-induced BMSCs on fibrin glue - Fibrin glue		No significant difference between the defects treated with osteo-induced iPSCs and those treated with osteo-induced BMSCs

(Continous)

TABLE 1 (Continued)

Authors	Type of study	Cell Source for iPSCs	Transduction	Treatment	Scaffolds	Animal Model	Test Groups	Results
Hayashi et al. ⁶⁴	In vitro	Prepared Mouse iPSCs (iPS-MEF-Ng-20D-17)	—	EB formation: suspension culture (5 days) Osteogenic induction: OM (3 weeks) X-ray irradiation: 2 Gray	—	—	-Irradiated osteo-induced iPSCs -Non-irradiated osteo-induced iPSCs	No difference was observed in the expression of OCN and Runx2 between two groups
	In vivo					3 mm calvarial defects in SCID mice 5 mm calvarial defects in Wister rats	-iPSCs -Osteo-induced iPSCs -Irradiated osteo-induced iPSCs	Histological and radiographical evaluation showed higher bone regeneration in Osteo-induced and irradiated osteo-induced iPSCs No tritoma formation was reported in irradiated osteo-induced iPSCs
Tashiro et al. ⁶⁷	In vitro	Prepared mouse iPSCs lines (20D17, 38C2, and stm99-1)	—	EB formation: DMEM, 15% FCS, 1% NEAA, β -Mercaptoethanol, 2 mM L-glutamine, 100 nM RA Osteogenic induction: -OM, ATRA (3 days) followed by transduced with adenovectors (Ad-CA-LacZ or Ad-CA-Runx2)	—	—	-Osteo-induced iPSCs -Ad-CA-LacZ transduced osteo-induced iPSCs -Ad-CA-Runx2 osteo-induced iPSCs	Ad-CA-Runx2 osteo-induced iPSCs exhibited higher ALP activity and higher level of calcium deposition than non-transduced or Ad-CA-LacZ transduced osteo-induced iPSCs Expression of Runx2, OSX, BSP, OCN and Col1 was higher in Ad-CA-Runx2 osteo-induced iPSCs
Ye et al. ⁶⁸	In vitro	Prepared mouse iPSCs (clone ST-8)	—	EB formation: DMEM, 15% ESC qualified FBS, β -mercaptoethanol + NEAA, L-glutamine (5 days) Transduction: pBABE-hygro-SATB2 or empty Vector (pBABE-hygro) Osteogenic induction: OM (3 weeks)	Silk	—	-pBABE-Hygro-SATB2 transduced osteo-induced iPSCs -pBABE-Hygro Vector-transduced osteo-induced iPSCs -Osteo-induced iPSCs	pBABE-Hygro-SATB2 transduced osteo-induced iPSCs showed higher calcium deposition and ALP activity Expressions of OSX, Runx2, BSP and OCN were higher in pBABE-Hygro-SATB2 transduced osteo-induced iPSCs
	In vivo					4 mm calvarial defect in nu/nu mice	-Silk Scaffold -iPSCs on silk -Osteo-induced iPSCs on silk -pBABE-Hygro Vector-transduced osteo-induced iPSCs on silk -pBABE-Hygro-SATB2 transduced osteo-induced iPSCs on silk	Micro-CT image showed significant higher mineral density in pBABE-Hygro-SATB2 transduced osteo-induced iPSCs on silk Higher expression of Runx2, OCN and BSP was observed in pBABE-Hygro-SATB2 transduced osteo-induced iPSCs on silk

(Continous)

TABLE 1 (Continued)

Authors	Type of study	Cell Source for iPSCs	Transduction	Treatment	Scaffolds	Animal Model	Test Groups	Results
Ji et al. ⁴²	In vitro	Human gingival fibroblasts	Lentiviral vector Oct3/4, Klf4 and Sox2, c-Myc	EB formation: ES medium Osteogenic induction: OM	nanoha/ chitosan/ gelatin	—	- Osteo-induced iPSCs on sphere nanoHA/ chitosan/gelatin - Osteo-induced iPSCs on rod nanoHA/ chitosan/gelatin	Cell proliferation rate was higher on sphere nanoHA/ chitosan/gelatin Higher expression of ALP, Col1, Runx2, OCN and OPN was observed in Osteo-induced iPSCs on sphere nanoHA/ chitosan/gelatin
Ji et al. ⁴³	In vitro	Human gingival fibroblasts	Lentiviral vector Oct4, Klf4 and Sox2, c-Myc	EB formation: ES medium Osteogenic induction: OM (4 weeks)	nanoha/ chitosan/ gelatin with different ratio of HA: HCG-111:1 wt/vol%	—	- Osteo-induced on HCG311 - Osteo-induced on HCG111	Expression of OCN and OPN was significantly higher in osteo-induced iPSC cultured on HCG311 Expression of ALP and Col1 was significantly lower in osteo-induced iPSC cultured on HCG311 No difference in expression of Runx-2 between two groups
	In vivo				nHA, HCG-311: 3 wt/vol% nHA	Subcutaneous implantation in specific-pathogen-free BALB/c nude mice	- Osteo-induced on HCG311 - Osteo-induced on HCG111 - HCG311 - HCG111	Osteo-induced on HCG311 generated larger new bone Runx2 and OCN were expressed higher in osteo-induced on HCG311
Ma, M.S. et al. ⁶⁶	In vitro	Mouse iPSC line		EB formation: Knockout DMEM, 15% FCS, NEEA, L-glutamine, β -mercaptoethanol, LIF (4 days), 10^{-7} M ATRA (4 days) Osteogenic induction: OM and rhBMP4 (4 weeks)	—	—	- Osteo-induced mESCs - Osteo-induced miPSCs	Both cells showed high level of osteogenesis shown by Alizarin Red staining and ALP Expression of Runx2, BSP and Col1a1 was higher in osteo-induced iPSCs
Ardeshtari/Ajimi et al. ⁶³	In vitro	Prepared human iPSCs lines	—	EB formation: DMEM/F12, 15% knockout serum replacement, NEEA, L-glutamine+ β -Mercaptoethanol without human FGF- β (5 days) Pulsed extremely low frequency electromagnetic fields (ELF-EMF) placed in a CO2 incubator (5 days) Osteogenic induction: OM (2 weeks)	—	—	- Osteo-induced iPSCs - Osteo-induced iPSCs subjected to ELF-EMF - iPSCs subjected to ELF-EMF - iPSCs	Higher ALP activity and Alizarin Red staining were observed in osteo-induced iPSCs subjected to ELF-EMF Expression of Col1, OCN, ALP, MMP1, ON and OSP was highest in Osteo-induced iPSCs subjected to ELF-EMF

(Continous)

TABLE 1 (Continued)

Authors	Type of study	Cell Source for iPSCs	Transduction	Treatment	Scaffolds	Animal Model	Test Groups	Results
Ardeshty et al. ³⁹	In vitro	Prepared human iPSCs line	—	EB formation: DMEM/F12, 15% knockout serum replacement, NEEA, L-glutamine, β -mercaptoethanol Osteogenic induction: OM	-TCPS -Electrospun nanofibrous PES	—	-Osteo-induced iPSCs on nanofibrous PES scaffold -Osteo-induced iPSCs on TCPS -undifferentiated iPSCs (control)	higher ALP activity was observed in Osteo-induced iPSCs on nanofibrous PES scaffold Expression of Runx2, OCN, ON, Col1 was higher in osteo-induced iPSCs on nanofibrous PES scaffold
Wu et al. ⁵⁹	In vitro In vivo	Mouse tail tip fibroblasts from Alox5-knockout (Alox5-KO-iPSCs) and wild-type (Wild-iPSCs) mice	Leniviral vector Oct4, Klf4 and Sox2	EB formation: DMEM, 15% FBS, NEAA, L-glutamine, β -mercaptoethanol followed by treatment with RA (2 days) and without RA (2 days) Osteogenic induction: OM	HA/Collagen/Chitosan	— 5 mm calvarial defect in SD rats	-Alox5-KO-iPSCs -Wild iPSCs -Empty -HA/Collagen/Chitosan -Alox5-KO-iPSCs on HA/Collagen/Chitosan -Wild-iPSCs on HA/Collagen/Chitosan	Expression of ALP, OCN, OPN, Runx2 was decreased in Alox5-KO-iPSCs Micro-CT revealed decreased bone formation in Alox5-KO-iPSCs cultured on HA/Collagen/Chitosan compared with wild-iPSCs on HA/Collagen/Chitosan
Okawa et al. ⁵¹	In vitro	Mouse gingival fibroblasts (GF)	Retroviral transduction Oct3/4, Sox2, and Klf4	EB formation: -ES medium (2 days) -ES medium+ 10^{-6} M ATRA GF-iPSCs constructs: EBs seeded on pNIPAAm gel mold in ES medium+ 10^{-6} M ATRA (2 days) Osteogenic induction: OM (30 days)	—	—	—	Significant upregulation of RUNX2, OCN, Col1a1 and OX was observed in collected ball-like cell constructs Histologic analysis showed ball-like cell constructs consisted of an inner region of unstructured cell mass and an outer osseous tissue region that was surrounded by osteoblast progenitor-like cells
	In vivo					Subcutaneous in immuno-deficient mice		Extensive ectopic bone formation surrounded by teratoma tissue was observed in histologic examination

(Continues)

TABLE 1 (Continued)

Authors	Type of study	Cell Source for iPSCs	Transduction	Treatment	Scaffolds	Animal Model	Test Groups	Results
Yin et al. 48	In vitro	Human gingival fibroblasts	Episomal plasmid vector Oct4, Sox2, Klf4, L-myc, Lin28 and p53 shRNA	EB formation: DMEM/F12, 20% FBS, β -mercaptoethanol, L-glutamine, NEAA (5 days) Periodontal cell generation: -EMD medium (DMEM/F12, 20% FBS+30, 60 or 90 μ g/ml of Endogain) (14 days) -GDF-5 medium (DMEM/F12, 20% FBS+100, 200 or 400 ng/ml of recombinant human GDF-5) (14 days)	—	—	-iPSCs treated with EMD medium -iPSCs treated with GDF-5 medium -iPSCs treated with control media (DMEM/F12, 20% FBS)	Expression of OCN and BSP significantly enhanced after 14 days of treatment with EMD and GDF-5

Ad, adenovirus; ALP, alkaline phosphatase; α MEM, alpha minimum essential medium; ATRA, all-trans retinoic acid; FGF- β , basic fibroblast growth factor; BMP6, bone morphogenetic protein 6; BSP, bone sialoprotein; CA, cytomegalovirus enhancer/ β -actin promoters; Myc, myelocytomatosis oncogene; Col1a1, collagen type 1 pro alpha 1; CPC, calcium phosphate cements; DLX5, distal-less homeobox 5; DMEM, Dulbecco's modified Eagle medium; DMP1, dentin matrix protein 1; EB, embryo body; EMD, enamel matrix derivative; FBS, foetal bovine serum; FCS, foetal calf serum; FEPP, fibronectin-like engineered polymer protein; Fn, fibronectin; Flt-1, vascular endothelial growth factor receptor 1; GDF-5, growth/differentiation factor-5; GFP, green fluorescent protein; HA, hydroxyapatite; HA/TCP, hydroxyapatite/tricalcium phosphate; hBMSCs, human bone marrow stromal cells; hiPSCs, human-induced pluripotent stem cells; hMSCs, human mesenchymal stem cells; hUCMSCs, human umbilical cord mesenchymal stem cells; IBSP, integrin-binding sialoprotein; ICR, Institute of Cancer Research; iPSCs, induced pluripotent stem cells; Klf4, Kruppel-like factor 4; KO-DMEM, knockout Dulbecco's modified Eagle medium; LacZ, elongation factor-1a promoter-driven β -galactosidase; LIF, leukaemia inhibitory factor; MEF, murine embryonic fibroblast; mESCs, mouse embryonic stem cells; miPSCs, mouse-induced pluripotent stem cells; MMP1, matrix metalloproteinase 1; MSC, mesenchymal stem cell; MSCV, murine stem cell virus; NEAA, non-essential amino acids; NELL1, Nel-like protein 1; NOD mice, non-obese diabetic mice; OCN, osteocalcin; OCT3/4, octamer-binding transcription factor 3/4; OM, osteogenic medium; ON, osteonectin; OSP, osteoprotegerin; OSX, osterix; OPN, osteopontin; PES, polyethersulfone; PLGA, poly(lactic-co-glycolic acid); PLLA, poly(l-lactic acid); PMEDSAH, poly[2-(methacryloyloxy) ethyldimethyl-(3-sulfopropyl) ammonium hydroxide]; RA, retinoic acid; RGD, arginine-glycine-aspartate; rhBMP4, recombinant human bone morphogenic protein 4; RFP, red fluorescent protein; Runx2, Runt-related protein 2; SATB2, special AT-rich sequence-binding protein 2; SCID mice, severe combined immune deficient mice; Sox2, sex-determining region Y-box 2; Spp1, secreted phosphoprotein 1; TCPS, tissue culture polystyrene; TGF- β 1, transforming growth factor β 1; TNAP, tissue-non-specific alkaline phosphatase.

TABLE 2 iPSC-osteoprogenitors obtained without EB formation step

Authors	Type of study	Cell source for iPSCs	Transduction	Treatment	Scaffolds	Animal model	Test groups	Results
Dogaki et al. ²⁵	In vitro	Prepared mouse iPSCs line (iPS-MEF-Ng-178B-5)	—	MSC generation: serum-reduced MSC medium, FGF- β (direct plating method) Osteogenic induction: OM (2 weeks)	—	—	-Osteo-induced iPSCs -Osteo-induced mBMSCs -iPSCs	Osteo-induced iPSCs showed significantly higher ALP activity compared with iPSCs Expression of ALP, Runx2, OSX and OCN was significant in osteo-induced iPSCs
Zou et al. ²⁶	In vitro	Human primary fibroblasts (IMR90)	mRNA reprogramming Oct4, Sox2, and Klf4	MSC generation: DMEM-LG, L-glutamine, 10% FBS Osteogenic induction: OM	PCL PHT	—	-Osteo-induced iPSCs on PCL -Osteo-induced iPSCs on PHT	Significantly higher ALP activity and higher calcium contents were observed in osteo-induced iPSCs on PHT Higher expression ALP was observed in osteo-induced iPSCs on PHT
	In vivo					Subcutaneous implantation in NMRI-nude mice Subcutaneous	-Osteo-induced iPSCs on PCL -Osteo-induced iPSCs on PHT -PCL -PHT	Micro-CT showed that ectopic bone formation in osteo-induced iPSCs cultured on PHT was more uniform than other groups
de Poppo et al. ²⁴	In vitro	Prepared human iPSCs lines from dermal (11C, 1013A) and bone marrow (BC1)	—	MSC generation: KO-DMEM FBS, GlutaMAX, NEAA, β -mercaptoethanol, Osteogenic induction: OM	Decellularized bone cylinders	—	-Osteo-induced 11C iPSCs -Osteo-induced 1013A iPSCs -Osteo-induced BC1 iPSCs -Osteo-induced ESCs	Osteo-induced iPSCs derived from 11C displayed poor osteogenic differentiation in perfusion bioreactor, BC1-engineered bone constructs displayed regions with the highest cell density and new bone-like matrix, approaching the density of native tissue
	In vivo					Subcutaneous implantation in nude mice	-Osteo-induced 1013A iPSCs -Osteo-induced BC1 iPSCs -Osteo-induced ESCs	Bone constructs engineered using osteo-induced BC1 iPSCs and ESCs displayed higher mineral content
Ishiy et al. ⁵⁶	In vitro	SHED (iPSCs-SHED) Human dermal fibroblasts (iPSCs-FIB)	Retroviral vector Oct3/4, Klf4 and Sox2, c-Myc	MSC generation: DMEM, 10% FBS, NEAA, FGF- β Osteogenic induction: OM	—	—	-Osteo-induced iPSCs-SHED -Osteo-induced iPSCs-FIB -Osteo-induced SHED	ALP activity and matrix mineralization was significantly higher in osteo-induced iPSCs-SHED

(Continues)

TABLE 2 (Continued)

Authors	Type of study	Cell source for iPSCs	Transduction	Treatment	Scaffolds	Animal model	Test groups	Results
Kang et al. 45	In vitro	Human dermal fibroblasts	mRNA reprogramming (mRNA-iPSCs) or polycistronic lentiviral vector (Lenti-iPSCs) Oct3/4, Klf4 and Sox2, c-Myc	MSC: DMEM-LG+L-glutamine,+FBS (for Lenti-iPSCs) Osteogenic induction: OM and Vitamin D	—	—	-Osteo-induced mRNA-iPSCs -Osteo-induced Lenti-iPSCs -Osteo-induced BMSCs	More homogeneous and robust calcium staining were observed in Osteo-induced Lenti-iPSCs than Osteo-induced mRNA-iPSCs Robust expression of Runx2, ALP and OCN was observed in Osteo-induced BMSCs Strong expression of Col1a1 was observed in both osteo-induced iPSCs. Osteo-induced Lenti-iPSCs has an efficacy of osteogenesis similar to BM-MSCs
Kang et al. 44	In vitro	Human fibroblast (IMR90)	mRNA reprogramming	MSC generation: DMEM-LG,10 FBS, L-glutamine Osteogenic induction: OM and Vitamin D	PCL PCL-nHA	—	-Osteo-induced iPSCs on PCL -Osteo-induced iPSCs on PCL-nHA -iPSC-MSCs on PCL -iPSC-MSCs on PCL-nHA	Expression of OCN was higher in osteo-induced iPSCs cultured on PCL-nHA Substantial increase in ALP activity level and expression of Runx2, Col1a1 and OCN was observed in both scaffolds seeded by osteo-induced iPSCs
Hynes et al. 41	In vitro	Gingival fibroblasts (GF-iPSCs) PDL cells (PDL-iPSCs) Prepared human lung iPSC	Retroviral vector Oct3/4, Klf4 and Sox2, c-Myc	MSC generation: α -MEM, 10% FCS, L-ascorbate- β -phosphate+L-glutamine, NEAA, HEPES Osteogenic induction: OM	HA/TCP	—	-Osteo-induced PDL-iPSC -Osteo-induced GF-iPSCs -Osteo-induced lung iPSCs	Alizarin Red staining showed that osteo-induced PDL-iPSC generated the highest level of calcium deposition Significant increase in expression of BMP2 and Runx2 were observed in all Histological analysis demonstrated that all three Osteo-induced iPSCs had the capacity to form mineralized tissue in vivo Osteo-induced PDL-iPSC cultured on HA/TCP formed physiological mature bone structures as demonstrated by Anti-BrdU staining
	In vivo					Subcutaneous implantation in NOD/SCID mice	-Osteo-induced PDL-iPSC on HA/TCP -Osteo-induced GF-iPSCs on HA/TCP -Osteo-induced lung iPSCs on HA/TCP	

(Continues)

TABLE 2 (Continued)

Authors	Type of study	Cell source for iPSCs	Transduction	Treatment	Scaffolds	Animal model	Test groups	Results
Hynes et al. 71	In vitro	Prepared human foreskin-derived iPSCs	—	MSC generation: α -MEM, 10% FCS, L-ascorbate- β -phosphate, L-glutamine, NEAA, HEPES Osteogenic induction: OM	—	—	—	Osteo-induced iPSCs demonstrated high level of Alizarin Red staining Increased levels of BSP 2, OPN and Runx2 was observed in osteo-induced iPSCs Histomorphometric analysis showed that the percentage of new bone regenerated in Undifferentiated iPSCs clotted with fibrinogen and thrombin was the highest
Lepage et al. 53	In vitro	Equine fibroblasts (H3-B)	Piggy-bac transposon based vector Oct3/4, Klf4 and Sox2, c-Myc	MSC generation: DMEM-HG, 10% FBS, NEAA, ALK inhibitor, FGF- β Osteogenic induction: OM (10 days)	—	2 $\times$ 3 mm ² Periodontal fenestration defect in athymic nude rats (CBH-mu)	-Undifferentiated iPSCs clotted with fibrinogen and thrombin -Fibrinogen and thrombin -Empty	Von Kossa and Alizarin Red staining revealed the high osteogenic differentiation of equine iPSCs
Hong, S.G. et al. 54	In vitro In vivo	Rhesus macaque dermal fibroblasts, BMSCs and CD34 ⁺ cells	polycistronic lentiviral vector Oct3/4, Klf4 and Sox2, c-Myc	MSC generation: α -MEM, 20% non-heat-inactivated FBS, 10 ng/ml FGF- β , 10 ng/ml PDGF, and 10 ng/ml EGF Osteogenic induction (In vitro): StemMACS OsteoDiff Medium (14 days)	HA/TCP	Subcutaneous implantation in Rhesus macaques	-Rhesus BMSCs on HA/TCP -Rhesus iPSC-SCs on HA/TCP -HA/TCP	ALP staining showed differentiation towards the osteogenic lineage Histological analysis revealed robust bone formation at 8 weeks from rhesus iPSC-SCs on HA/TCP

ALK, anaplastic lymphoma kinase; ALP, alkaline phosphatase; α MED, alpha minimum essential medium; FGF- β , basic fibroblast growth factor; BMSCs, bone marrow stromal cells; BMP2, bone morphogenic protein 2; BSP2, bone sialoprotein 2; c-Myc, cellular myelocytomatosis oncogene; DMEM, Dulbecco's modified Eagle medium; DMEM-HG, Dulbecco's modified Eagle medium-high glucose; DMEM-LG, Dulbecco's modified Eagle medium-low glucose; EGF, epidermal growth factor; ESCs, embryonic stem cells; FBS, foetal bovine serum; FCS, foetal calf serum; HA/TCP, hydroxyapatite/tricalcium phosphate; HEPES, 4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid; hiPSCs, human-induced pluripotent stem cells; iPSCs, induced pluripotent stem cells; iPSC-SCs, iPSC-derived mesodermal stromal-like cells; Klf4, Kruppel-like factor 4; mBMSCs, mouse bone marrow stromal cells; MSC, mesenchymal stem cell; NEAA, non-essential amino acids; nHA, nano-hydroxyapatite; NMRI, Naval Medical Research Institute; OCN, osteocalcin; OCT4, octamer-binding transcription factor 4; OM, osteogenic medium; OSX, osterix; OPN, osteopontin; PCL, poly (caprolactone); PDL, periodontal ligament; PDGF, platelet-derived growth factor; PHT, PCL scaffold functionalized with natural polymer hyaluronan and ceramic TCP; Runx2, Runt-related protein 2; SCID, severe combined immunodeficient; SHED, stem cells from human exfoliated deciduous teeth; Sox2, sex-determining region Y-box 2.

TABLE 3 Direct use of iPSCs for bone regeneration

Authors	Type of study	Cell source for iPSCs	Transduction	Treatment	Scaffolds	Animal model	Test groups	Results
Phillips et al. ²⁹	In vitro	Human skin fibroblast (AG20443) and AG08395) hBMSCs	Retroviral vector Oct3/4, Klf4 and Sox2, c-Myc (for fibroblasts)	Osteogenic induction: OM	HA/TCP particles	—	-Osteo-induced iPSCs from skin -Osteo-induced BMSCs	Expression of Runx2 was high in both cell types. Expression of ALP was the highest in osteo-induced BMSCs Col 2 was higher in Osteo-induced iPSCs from skin
	In vivo		StemCCA lentiviral reprogram-ming kit (for BMSCs)			Subcutaneous implantation in nude mice (NIH-Ly st ^{tg} Foxn1 Btk ^{kid} or Nod. Cg-Prkdc ^{scid} L2RG ^{tm1wjl} SzJ)	- Osteo-induced iPSCs from fibroblast - Osteo-induced iPSCs from BMSCs - Osteo-induced BMSCs	No difference was observed in bone regeneration in osteo-induced iPSCs; however, amount of new bone regenerated was higher in osteo-induced BMSCs
Jin et al. ²⁷	In vitro	hiPSCs (SC101A)	—	Osteogenic induction: OM	PCL	—	—	Osteo-induced iPSCs showed high level of OSN expression
	In vivo					Subcutaneous implantation in athymic mice	-Osteo-induced iPSCs on PCL -PCL	Histological evaluation and OCN immunostaining revealed the bone regeneration in osteo-induced iPSCs cultured on PCL
Levi et al. ²⁸	In vitro	Human adipose-derived stromal cells	Lentiviral vector Oct3/4, Klf4 and Sox2, c-Myc	Osteogenic induction: OM with or without rhBMP-2	HA-PLGA	—	-Osteo-induced iPSCs in OM containing rhBMP2 -Osteo-induced iPSCs in OM containing rhBMP2	Alizarin Red staining showed significantly greater extracellular matrix mineralization in osteo-induced iPSCs in OM containing rhBMP2 Expression of OCN Runx2 was higher in osteo-induced iPSCs in OM containing rhBMP2
	In vivo				4-mm calvarial defect in nude mice		-HA-PLGA -HA-PLGA+BMP2 -HA-PLGA with h-iPSCs -HA-PLGA+BMP2 with h-iPSCs -HA-PLGA with h-ESCs -HA-PLGA+BMP2 with h-ESCs	Pluripotent cells placed onto a HA-PLGA+BMP-2 microniche within the larger context of a skeletal defect macroniche are capable of contributing to new bone formation with a greatly reduced propensity to form teratoma in vivo
						Subcutaneous implantation in nude mice	- Osteo-induced iPSCs on HA-PLGA+BMP2 - Osteo-induced ESCs on HA-PLGA+BMP2 - HA-PLGA	Histological analysis revealed de novo bone formation by both cell types

(Continous)

TABLE 3 (Continued)

Authors	Type of study	Cell source for iPSCs	Transduction	Treatment	Scaffolds	Animal model	Test groups	Results
Ardeshirylajimi et al. ⁴⁰	In vitro	Prepared human fibroblast iPSC line	—	Osteogenic induction: OM	—	—	<ul style="list-style-type: none"> - Osteo-induced iPSCs - osteo-induced human AdSCs 	<p>Significantly higher ALP activity was observed in osteo-induced iPSCs</p> <p>Alizarin Red staining showed higher calcium deposition in osteo-induced iPSCs</p> <p>Expression of Runx2 was higher in osteo-induced AdSCs</p> <p>Expression of OCN and ON was higher in osteo-induced iPSCs</p>
Ardeshirylajimi et al. ⁶²	In vivo	hiPSC line	—	EB formation: DMEM/F12+15% knockout serum replacement+NEEA+ L-glutamine+β-mercaptoethanol without human FGF-β Osteogenic induction: OM	PES Plasma treated (P-PES)	8 mm calvarial defect in Sprague Dawley rats	<ul style="list-style-type: none"> - Osteo-induced iPSCs on P-PES - P-PES - Empty 	<p>Complete regeneration was observed in osteo-induced iPSCs cultured on P-PES</p>
Kang et al. ⁷²	In vitro	hiPSC line (IMR90p18)	—	Growth medium: DMEM, with 10 v/v% FBS without osteogenic induction factors	GelMA-coA6ACA hydrogel for 2D culture GelMA-co-A6ACA-co-PEGDA macroporous hydrogels for 3D culture	—	<ul style="list-style-type: none"> - hiPSCs on mineralized 2D culture - hiPSCs on non-mineralized 2D culture - hiPSCs on mineralized 3D culture - hiPSCs on non-mineralized 3D culture - Cell culture grade glass coverslips coated with Gelatin (control) 	<p>Significant upregulation of OCN, Runx2 and SPP1 was observed in cells on mineralized matrices</p>

ADSCs, Adipose-derived stem cells; ALP, alkaline phosphatase; A6ACA, acryloyl 6-aminocaproic acid; c-Myc, cellular myelocytomatosis oncogene; ESCs, embryonic stem cells; GelMA, gelatin methacrylate; HA, hydroxy apatite; HA/TCP, hydroxy apatite/tricalcium phosphate; Klf4, Kruppel-like factor 4; OCN, osteocalcin; OCT3/4, octamer-binding transcription factor 3/4; OM, osteogenic medium; ON, Osteonectin; PEGDA, poly(Ethylene Glycol)-diAcrylate; PLGA, poly-L-lactic acid; rhBMP2, recombinant human bone morphogenetic protein 2; Runx2, Runt-related protein 2; Sox2, sex-determining region Y-box 2; Spp1, secreted phosphoprotein 1; 2D, two dimensional; 3D, three dimensional.

Integrating viral vectors (IVVs), including retroviruses^{21,29,41,46,50–52,56,57} and lentiviruses,^{28,42,43,54,59} were the vectors most commonly used for reprogramming cells into iPSCs in the included studies. IVVs deliver and maintain persistent expression of transduction factors. However, since multiple proviral copies integrate with the cell genome, they are accompanied by the possibility of genomic instability and chromosomal aberration.^{19,20,78} Polycistronic viral vectors were used in the study by Kang et al.⁴⁵ Although this vector may be associated with leaky expression, it may also overcome the issues associated with IVVs, because a single promoter regulates the transgenes.^{79–82} The study by de Peppo et al.²⁴ demonstrated that the Sendai virus is an appropriate carrier, since it retains high reprogramming efficiency without incorporating into the genome. Also, since it would gradually be depleted from cytoplasm after several passages, it would be proper for clinical applications.

Adenovirus vectors have not been utilized for iPSCs reprogramming in the included studies. However, in their study, Tashiro et al. used these vectors to transduce runt-related transcription factor 2 (Runx2 (gene, a key osteogenic transcription factor, in to mouse embryonic fibroblast-iPSCs from Nanog-reporter.⁶⁷ Their study demonstrated a reasonable transduction efficiency for Runx2. Previous studies showed that adeno-vectors can be used for cell reprogramming without genomic integration,^{83–85} However, reprogramming with these methods has been shown to have low efficiency.

Non-viral vectors such as plasmid or episomal vectors^{23,37,38,47,48,52,58} also used in seven studies, and they were demonstrated to be reasonable carriers because they do not integrate in to genome. However, their reprogramming efficiency is estimated to be low.^{86–90} The mRNA reprogramming method was used in three studies.^{26,44,45} This method can successfully generate transgene-free iPSCs; however, its labour-intensive approach may occur as a limitation.⁹¹ Ko et al. directly delivered reprogramming proteins fused to cell-penetrating peptide to induce pluripotency.³⁰ According to other studies, this method eliminates genome manipulation and DNA transfection,^{85,92} and it appears to be simple and efficient.⁹³ Lepag et al. successfully generated iPSCs from equine fibroblasts by the Piggy-Bac transposon-based approach.⁵³ This efficient method eliminates reprogramming factors and reduces genomic modifications.^{94–96} However, since chromosomal rearrangement may occur at the excision site due to transposition, clonal expansion of iPSC colonies may be required in order to purely isolate the transfected cells.

Four combinations of transduction factors were used in the included studies. The combination of Oct3/4, Sox2, Klf4 and c-Myc was most commonly utilized.^{21,22,28,29,41–43,45,46,52–55,57} Some studies removed c-Myc, an oncogene, from this combination^{26,50,51,59} so as to reduce tumorigenicity. However, by eliminating c-Myc, reprogramming efficiency was significantly reduced.⁷⁸ The combination of Oct3/4, Sox2, Klf4 and Lin28 was used exclusively with episomal vector pEB-C5.³⁷ It is suggested that this combination is not inherently mutagenic, and these methods successfully generated iPSCs from different cell sources.^{23,24,36–38} Furthermore, Duan et al. used the combination of Oct4, Sox2, Lin28 and Nanog to reprogramme human fibroblasts.⁴⁹ It was demonstrated in the literature that this combination is sufficient

for reprogramming human somatic cells.⁹⁷ Yin et al. used a combination of six factors: Oct4, Sox2, Klf4, Lin28, L-Myc and tumour protein P53 (TP53), which has been shown to generate a significantly greater colony of iPSCs.⁴⁸ Moreover, Nakagawa et al. showed that L-Myc can increase the number of iPSC clones without increasing tumorigenic potential.⁹⁸

Although different reprogramming methods and various combinations of transcription factors may have variable efficiency, it seems that all of them may provide an adequate amount of iPSCs for bone regenerative purposes.³⁵ However, the utilization of non-integration methods, specifically cell-penetrating peptides, may be more reasonable in future studies, since their approach is easy to use and shows a lack of permanent integration into the host genome. In particular, for clinical applications, genome integrating vectors will gradually be replaced by non-integration methods.⁹⁹ It has been demonstrated that different induction methods are capable of inducing pluripotency with various level of efficacy; however, it was not feasible to precisely compare their efficacy because of the lack of reported data regarding transfection efficiency.

4.3 | Treatment approaches used for osteo-induction of iPSCs

4.3.1 | iPSC-derived MSCs for bone regeneration

Researchers and clinicians have grown increasing interest in using MSCs in clinical stem cell treatment strategies during last decades. However, a critical problem with these strategies is the identification of both available and reliable sources of MSCs. Obtaining BMSCs from patients is overshadowed by its invasive, expensive and sensitive procedure. In addition, since BMSCs generally experience culture senescence after certain passage number,¹³ their capacity is limited to expansion in culture; therefore, they are not promising candidates for generating of large numbers of cells for clinical uses. Thus, the establishment of alternative stem cells sources is essential.

The discovery of iPSCs has led to a promising and attractive alternative for acquiring larger populations of adult stem cells to use in regenerative medicine and cell therapies. It has been demonstrated that the characteristics of human iPSCs-MSCs are similar to those of MSCs derived from adult tissues such as bone marrow, fat, umbilical cord, etc, both in vitro and in vivo.¹⁰⁰ Interestingly, iPSCs-MSCs have indicated not only an advance in growth^{74,101} but also higher telomerase activity and less senescence compared with adult MSCs.^{74,101} In addition, Lian et al. demonstrated that functional MSCs derived from human iPSCs can be isolated, purified and maintained in conventional MSC culture.⁷⁴ To generate iPSCs-MSCs, two main strategies are employed, one with and one without the EB formation step, through the dissociation of iPSC colonies into single cells. EBs are cell aggregates that spontaneously generate all three germ layers.¹⁰² EB formation is recognized as an intermediate step in the differentiation of ESCs and iPSCs into MSCs such as pre-osteoblasts.⁶⁰ EBs cultured on gelatin-coated flasks are enriched for MSC-like cells through various methods.^{57,103}

4.3.2 | iPSC-MSCs obtained with EB formation step

From all the studies included in this review, 29 articles had generated EBs from iPSCs prior to differentiation. The routine protocol for obtaining EBs from iPSCs is to culture the iPSCs in ESC medium from which FGF has been depleted. FGF should be removed from the medium since it enhances cell commitment towards the mesodermal lineage.¹⁰⁴

Several methods have been applied to enhance the osteogenic potential of EB-derived MSCs. Eight studies have applied modifications in either the MSC culture medium or OM.^{21,22,32,33,48,49,52,65,104} The study by Bilousova et al. suggested that treating EBs with retinoic acid (RA) increases cell commitment to the mesenchymal lineage and leads to in vitro and in vivo bone regeneration when cultured on a gelatin scaffold.²¹ Two studies by Li et al. also suggested that treating EBs by TGF- β and RA leads to greater MSC population as well as greater osteogenic potential.^{22,52} It has been demonstrated that MSCs secrete bone morphogenetic protein -4 (BMP4) and TGF- β 1 to their culture medium and treating EBs with hMSC-derived culture medium enhances the mesenchymal commitment and calcium deposition.¹⁰⁴ In the study by Ochiai-Shino et al., TGF- β , IGF-1, FGF- β (TIF) were added to the OM following the generation of EBs.³³ They demonstrated that TIF treatment leads to a greater number of tissue-non-specific alkaline phosphatase (TNAP)-positive cells and the higher expression of osterix (OSX), bone Sialoprotein (BSP), OCN, Runx2 and Col1a1 in TNAP⁺ cells. In the study by Kato et al., vitamin D3 was added to the OM. This study showed a significant increase in the expression of OCN and decreased expression of TNAP following the addition of vitamin D3, which is indicative of differentiating to terminal osteocytes.³² They also demonstrated that iPSCs are more responsive to vitamin D3 treatment than BMSCs, and this treatment resulted in the late phase osteoblast generation after 14 days with a dose-dependent pattern.³²

The study by Duan et al. suggested that treating EBs with basal medium supplemented with enamel matrix derived (EMD), containing protein complex from the amelogenin family, results in the significant expression of Runx2 compared with treatment with conventional osteogenic or basal medium. However, OCN expression was decreased after 20 and 30 days with no calcium nodule formation indicating that EMD may inhibit cell maturation despite the significant bone repair in a mouse periodontal fenestration defect model.⁴⁹ Similarly, Yin et al. demonstrated that treating iPSC-derived EBs with EMD and growth/differentiation factor-5 (GDF-5) could significantly increase expression of OCN and BSP bone markers.⁴⁸

Kao et al. investigated the effect of resveratrol (trans-3,5,4'-trihydroxystilbene) in osteogenic potential of iPSCs.⁶⁵ They demonstrated that iPSCs induced in OM plus resveratrol showed a higher level of mineralization. Also, the expressions of Runx2, OCN and IBSP were significant in treatment with resveratrol. A further in vivo study showed that the expression of OPN in grafts from mice fed with resveratrol was greater than that in mice fed with the control vehicle. The expression of Oct4, Nanog, Klf4 and c-Myc was significantly suppressed in grafts from mice fed with

resveratrol, indicating a possible effect of resveratrol in decreasing tumorigenicity.⁶⁵

Six studies showed the enhanced osteogenic differentiation of iPSCs by transduction with Runx2,⁶⁷ bone morphogenetic protein-2 (BMP2),^{23,36} BMP6,⁴⁷ NEL-like protein 1 (NELL1)⁷⁰ and special AT-rich sequence-binding protein 2 (SATB2).⁶⁸ In the study by Tashiro et al. osteo-induced iPSCs were transduced with Runx2; the results showed that not only the ALP activity of Runx2-transduced iPSCs was more than 50% higher but also the level of calcium deposition was 8-fold higher compared with non-transduced cells.⁶⁷ The study by Ye et al. demonstrated that osteo-induced iPSCs transduced with SATB2, a nuclear matrix protein regulating osteoblast differentiation, showed higher ALP activity and enhanced matrix mineralization in vitro.⁶⁸ Also, SATB2-transduced iPSCs showed up-regulation of SATB2, OSX, Runx2, BSP and OCN and down-regulation of HoxA2 in vitro. SATB2-transduced iPSCs seeded on a silk scaffold for bone regeneration in critical-sized calvarial defects in nude mice showed 59.58 $\pm$ 7.00% bone formation at 5 weeks post-operatively. Scaffolds seeded with non-transduced iPSCs cultured with and without osteogenic supplements revealed 26.94 $\pm$ 7.10% and 5.37 $\pm$ 1.01% bone formation, respectively. Significant expression of BSP and SATB2 was determined in new bone treated with SATB2-transduced iPSCs. Two studies used BMP2 lentivirus for osteogenic induction.^{23,36} The study by Liu et al. showed high expression levels of Col1 and OCN in BMP2-induced iPSC-MSCs cultured on RGD-chitosan-CPC.³⁶ Wang et al. compared the osteogenic capabilities of iPSCs induced either in OM or transduced with BMP2 both in vitro and in vivo.²³ A high degree of mineralization was observed in both osteo-induced and BMP2-transduced iPSCs, and no significant difference was reported.²³

Other protocols are utilized to enhance bone regeneration after obtaining pluripotent EBs. Hayashi et al. irradiated osteo-induced iPSCs. The results of their study showed that although bone formation potential is not influenced by irradiation, the non-irradiated groups formed teratomas even after osteogenic induction.⁶⁴ The study by Ardeshtyrlajimi et al. showed that treatment with OM in an extremely low-frequency electromagnetic field (ELF-EMF) leads to a greater proliferation rate, ALP activity and expression of Col1, OCN, MMP1, ON and OSP.⁶³ Due to scarce literature in this field, further studies are essential to reach a conclusion regarding the effect of irradiation and electromagnetic field on the osteogenic potential of iPSCs as well as their capability to form teratomas.

Six studies evaluated the effect of cell environment and various scaffolds on the osteogenic potential of iPSCs.^{30,38,39,42,43,61} The study by Ji et al. showed that seeding osteo-induced iPSCs on sphere-shaped nano-hydroxyapatite/chitosan/gelatin (nHA/CG) with more uniform pores and smoother surface results in greater expression of ALP, Col1, Runx2, OCN and OPN compared with rod-shaped nHA/CG.⁴² Also, higher mineralization was observed both in vitro and in vivo in osteo-induced iPSCs cultured on sphere-shaped nHA/CG.⁴² In a similar study by Ji et al., different concentrations of HA were incorporated in nHA/CG scaffolds.⁴³ Their results demonstrated greater bone volume and higher Runx2 and OCN expression when 3% HA was incorporated vs 1% HA although 1% HA scaffold had smoother

and significantly larger pore width.⁴³ It has been demonstrated that co-culture of iPSCs-derived osteoblasts and osteoclasts on composite scaffolds (PLGA-PLLA-HA) with higher HA content positively affects bone formation.⁶⁹ In two studies by Ardeshirylajimi et al., the osteogenic potential of osteo-induced iPSCs seeded on PES scaffolds was evaluated *in vitro*.^{39,62} Both studies showed a high level of ALP activity and expression of Runx2, OCN, ON and Col1 in osteo-induced iPSCs on PES scaffolds. The study by Xie et al. demonstrated that hydroxyapatite/collagen/chitosan (HA/Col/CTS) scaffolds are both osteoinductive and osteoconductive, and when they were loaded with osteo-induced iPSCs, the almost complete repair of calvarial defects was observed.⁶¹ Thein Han et al. compared the osteogenic capability of osteo-induced iPSCs on CPC containing five different bifunctional agents, RGD (Arg-Gly-Asp) peptides, fibronectin (Fn), fibronectin-like engineered polymer protein (FEPP), extracellular matrix Geltrex and platelet concentrate.³⁸ The modification of CPC with RGD, Fn and platelet concentrate has been shown to increase the expression of ALP, Runx2 and Col1. Also, greater mineralization was observed with modification with given bifunctional agents. In addition, in the study by Okawa et al. instead of using scaffolds, cells were cultured on thermoresponsive poly-N-isopropylacrylamide (pNIPAAm) gel mold cell chambers and 3-D ball-like cell structures were generated, and it was suggested that these 3-D scaffold free cell structures have osteoinductive properties *in vivo*.⁵¹

Taken together, administering various materials and modifications including using nanosize effect and osteoconductive and osteoinductive properties of HA and Col,⁶¹ increasing HA portion in HA/CG scaffold⁴³ manufacturing sphere-shaped nano-HCG rather than the rod-shaped ones⁴² and biofunctionalizing scaffolds with platelet derivative³⁸ has demonstrated to enhance osteogenic differentiation of iPSCs.

Considering the morbidities associated with obtaining BMSCs and the legal/ethical issues associated with applying ESCs for bone regeneration, several studies have assessed whether iPSCs have comparable osteogenic potential to that of MSCs and ESCs in order to utilize these cells as a promising alternative source. From the included studies, seven studies compared the osteogenic capability of iPSCs and other types of stem cells. Studies by Egusa et al.,⁵⁰ and Ko et al.,³⁰ Diederichs et al.⁵⁵ and Sheyn et al.⁴⁷ compared the osteogenic potential of iPSCs and BMSCs. The study by Egusa et al. demonstrated that the expression profiles of Runx2, osterix and BSP were similar between BMSCs and iPSCs. However, Distal-Less Homeobox 5 (DLX5) and OCN levels were higher in osteo-induced iPSCs. Furthermore, it was demonstrated that iPSCs generated HA crystals similar to BMSCs; however, the underlying mechanisms seemed to be different between the groups.⁵⁰ The study by Ko et al. showed that the expression of osteogenic markers including Col1 and BSP was lower and delayed in osteo-induced iPSCs. On the contrary, Runx2 level was significantly greater in iPSCs than in BMSCs.³⁰ However, no significant difference were observed in bone regeneration *in vivo*.³⁰ Sheyn et al. demonstrated that MSCs derived from EBs in later stages and transduced with BMP6 were more potent than BMSCs in healing of radius segmental defect and MSCs obtained from iPSCs had greater proliferation rate.⁴⁷ The differences

in the patterns of osteogenic marker expression in these two studies^{30,50} may be attributed to the difference between RA treatment and osteogenic treatment periods. In addition, the transduction genes and transfection methods were different between these studies. Egusa et al.⁵⁰ did not utilize C-Myc to induce pluripotency; they transduced the cells by retroviral induction. On the contrary, Ko et al.,³⁰ transduced fibroblasts by peptides. In addition, human gingival fibroblasts have been shown to have poor osteogenic potential compared with gingival-derived iPSCs.⁴³

Comparing the osteogenic potentials of iPSCs and ESCs revealed that levels of Runx2, OCN, OPN and Col1a1 were significantly up-regulated in iPSCs; however, the basic levels of osteogenic markers were greater in undifferentiated iPSCs compared with ESCs. This may be attributed to the epigenetic memory of iPSCs, since they were derived from fibroblasts with a mesenchymal lineage origin.⁶⁶ In another study, the osteogenic potential of iPSCs was compared with hBMSCs and human umbilical cord MSCs (hUCMSC). No significant difference in mineralization was detected among these cells, both *in vitro* and *in vivo*.²³ These studies suggest that osteogenic potential of iPSCs may be comparable or superior that of MSCs and ESCs.

4.3.3 | iPSC-MSCs obtained without EB formation step

An alternative strategy for obtaining iPSC-MSC is to dissociate iPSC colonies without the EB formation step. Nine studies have reported successful derivation of MSC-like cells from iPSCs with the omission of the EB formation step^{24-26,41,44,45,53,56,71}; however, in three studies, the MSC medium was supplemented with basic fibroblast growth factor (FGF- β).^{25,53,56} The assumption for adding FGF- β to the MSC medium is decreasing undifferentiated cells by increasing Erk and subsequently inhibiting pluripotency.¹⁰⁵

Several studies have also evaluated the effects of various scaffolds in the osteogenic potential of iPSCs.^{24,26,44} Kang et al. reported the interactions of human fibroblast-derived iPSCs with PCL and PCL-nHA scaffolds.⁴⁴ They showed that Runx2, ALP, Col1A1 and OC were highly expressed in both scaffolds. However, the expression of these genes was detected at different time points in two scaffolds. In addition, the increase in OC gene expression was greater in the PCL-nHA scaffold than in the PCL scaffold. Zou et al. compared the osteogenic potential of iPSCs on PCL and PHT scaffolds. Results of their study showed significantly higher ALP activity levels and higher calcium deposition in osteo-induced iPSCs on PHT.²⁶

Some studies have demonstrated the comparable osteogenic differentiation potential of osteo-induced iPSCs obtained without the EB formation step to that of MSCs from other tissue sources.^{24,45,56} In the study by Kang et al., human fibroblast-derived iPSCs demonstrated mesenchymal characteristics similar to adult BMSCs.⁴⁵ Kang et al. conducted reprogramming using either mRNA reprogramming (mRNA-iPSCs) or transduction with polycistronic lentiviral vector (Lenti-iPSCs). The results of their study revealed that more homogeneous and robust calcium staining was observed in osteo-induced Lenti-iPSCs than osteo-induced mRNA-iPSCs. In addition,

osteo-induced Lenti-iPSCs had an efficacy of osteogenesis similar to osteo-induced BMSCs.⁴⁵ Similarly, the study by de Poppo showed similar mineralization levels by osteo-induced iPSCs derived from BMSCs and osteo-induced ESCs.²⁴ In contrast, Ishiy et al. showed that the osteogenic capability of SHED-derived iPSCs was greater than that of osteo-induced SHED.⁵⁶

4.3.4 | Direct use of iPSCs for bone regeneration

Direct osteogenic differentiation of iPSCs⁶ was observed in six studies.^{27–29,40,62,72} Three main approaches have been used to enhance the osteogenic differentiation of iPSCs for bone regeneration purposes: (1) Induction in OM, including dexamethasone, ascorbic acid and β -glycerophosphate, which is a simple and conventional method; (2) application of growth factors and cytokines such as BMP2 added to OM; and (3) using calcium phosphate or mineralized-based scaffolds such as HA/TCP, which works as a micro-niche for mineralization induction.

Two studies used the OM approach for osteogenic induction.^{27,40} In the study by Ardashirylajimi et al., osteogenic induction of iPSCs derived from human fibroblast was compared to adipose-derived mesenchymal stem cells (AdSCs).⁴⁰ They demonstrated the superior osteogenic differentiation potential of iPSCs compared with AdSCs; they reported a significantly higher ALP activity at 7, 14 and 21 days of osteo-induced iPSCs. Gene expression analysis showed that Runx2 was significantly higher in AdSCs after 7 and 14 days; however, ON and OCN were expressed at a significantly higher rate in iPSCs.⁴⁰ Another *in vivo* study conducted by Jin et al. the OM approach used for osteogenic induction of iPSCs.²⁷ Histological evaluation and OCN immunostaining revealed that bone regeneration occurred in osteo-induced iPSCs cultured on PCL with no signs of teratoma formation within 4 weeks of subcutaneous transplantation into nude mice. In their study, iPSCs were maintained in OM for 2 weeks prior to implantation with neither gene transduction nor treatment with growth factors.²⁷

One study enhanced the osteogenic differentiation of iPSCs using recombinant human BMP2 (rhBMP-2).²⁸ Levi et al. studied the osteogenic capabilities of iPSCs by addition of rhBMP2 in OM *in vitro*; they later compared the bone regeneration potential of human iPSCs and human ESCs seeded onto HA-coated PLGA releasing rh-BMP2 scaffolds as a micro-niche into the context of a 4 mm critical-sized calvarial defect in mice as a macro-niche.²⁸ *In vitro*, they showed that when rh-BMP2 was added to the OM, not only was the expression of pluripotent genes Oct4, Nanog and Sox2 reduced after 7 days but also the osteogenic markers Runx2 and OCN were significantly up-regulated. Their *in vivo* study showed a greater amount of bone regeneration in hiPSCs or hESCs cultured HA-PLGA releasing BMP-2. Also, immunostaining revealed the higher expression of Runx2 and OCN in ESCs or iPSCs seeded on HA-PLGA+rhBMP-2 compared with the HA-PLGA scaffold without rhBMP2. No teratoma was observed when human iPSCs were seeded onto HA-PLGA or HA-PLGA+BMP-2 scaffolds among 27 animals, in contrast, two cases of teratomas were detected in 15 animals receiving human ESCs seeded on these scaffolds.²⁸

The study by Kang et al. demonstrated that seeding iPSCs on mineralized gelatin-methacrylate-co-acryloyl 6-aminocaproic acid-co-poly(ethylene glycol)-diacrylate macroporous hydrogel on a growth medium lacking any growth factors and osteogenic-inducing small molecules resulted in osteogenic differentiation.⁷² The 3D culture of mineralized matrices showed significant up-regulation of Runx2, OCN and secreted phosphoprotein 1 (SPP1) compared with non-mineralized matrices. It was confirmed by immunohistochemical staining for OCN which displayed progressive increases in OCN with further culturing; in fact, osteoblast differentiation was found only in iPSCs on mineralized matrices. Moreover, they showed that the expression of Nanog has down-regulated significantly in iPSCs on both mineralized and non-mineralized matrices and in 2D cultures; this indicates that the pluripotency of the cells was not maintained using the matrices.⁷²

4.4 | Limitations

Several limitations were observed in the included studies which resulted in heterogeneity in reported data and made a reasonable comparison between studies difficult: (1) Several studies did not report the duration of cell incubation in either EB medium, MSC medium or osteogenic medium; (2) most of the included studies did not report the transfection efficiency which made a comparison between transduction methods not feasible; (3) only three studies reported/evaluated teratoma formation which is an important criteria in evaluating the safety of iPSCs for future clinical applications; and (4) there is no study in comparing given three main approaches used for iPSC osteo-induction for bone regeneration.

5 | CONCLUSION

The current review is systematical review regarding the application of iPSCs in bone regeneration. Despite the limitations in the included studies which made the comparison difficult, some general conclusions can be drawn: (1) Studies included in this study have provided evidence that iPSCs can serve as a potent stem cell source for bone regeneration; (2) the majority of studies showed that osteo-induced iPSCs have an osteogenic capability equal or superior to other MSCs; (3) according to most of the studies, no significant difference was observed in the osteogenic potential of iPSCs derived from various sources; (4) addition of resveratrol to OM and irradiation after osteogenic induction has been shown to reduce teratoma formation in animal models; (4) transfection with Lentiviral BMP2 results in higher mineralization compared with osteo-induction in OM and (5) addition of TGF- β , IGF-1 and FGF- β to the OM following the generation of EBs showed an increased osteogenic capability of iPSCs.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

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