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REGENERATIVE BIOLOGY AND MEDICINE IN OSTEOPOROSIS (S BRYANT AND M KREBS, SECTION EDITORS)

Impact of Induced Pluripotent Stem Cells in Bone Repair and Regeneration

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Abstract

Purpose of Review The main objective of this article is to investigate the current trends in the use of induced pluripotent stem cells (iPSCs) for bone tissue repair and regeneration.

Recent Findings Pluripotent stem cell–based tissue engineering has extended innovative therapeutic approaches for regenerative medicine. iPSCs have shown osteogenic differentiation capabilities and would be an innovative resource of stem cells for bone tissue regenerative applications.

Summary This review recapitulates the current knowledge and recent progress regarding utilization of iPSCs for bone therapy. A review of current findings suggests that a combination of a three-dimensional scaffolding system with iPSC technology to mimic the physiological complexity of the native stem cell niche is highly favorable for bone tissue repair and regeneration.

Keywords Induced pluripotent stem cells (iPSCs) · Biomaterials · Osteogenic differentiation · Bone tissue engineering · Bone regeneration

Introduction

Limitations with Current Therapies

Bone defects are a common problem in orthopedics, which are often caused by trauma, tumor, and infection. Unfortunately, the treatment options for critical-size bone defects are very challenging. Bone is a dynamic and highly specialized form of connective tissue [1, 2] due to its unique capability of regeneration throughout one's life by a process known as bone remodeling [3]. For example, if the defect is minor, bone can heal within a few weeks and surgical interventions are not necessary. However, if the bone defect is of critical size, then a bone grafting procedure is required [4]. The critical-size bone defects are defined as those being more than 1.5 times greater than the diameter of the bone, and they do not heal by itself if left untreated [5]. Each year, millions of people suffer from various kinds of bone defects [6] and there is an urgent need for efficient bone graft transplantation.

There are several methods currently available for the treatment of critical-sized bone defects. One of the most commonly used methods is autologous bone graft transplantation, which is often considered as a gold standard in orthopedics, but the supply of autologous bone graft is limited [5, 7]. On the contrary, allogenic and xenogenic bone grafts are less preferred because of their clinical complications associated with the chances of immunological reactions and the transfer of pathogens. Thus, synthetic bone grafts have been introduced and are being used in clinical practices with promising results [8]. Although there has been some good progress in bone

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grafting using these synthetic materials, their long-term performance is generally not satisfactory [8, 9]. Therefore, there is an urgent need to develop alternative methods which could alleviate the problems above and accelerate the bone healing process, especially for large bone defects [10].

Potential Solutions and the Need for iPSCs

In the last few decades, tissue-engineered constructs have shown great possibilities for advancing orthopedic regenerative medicine. Being an interdisciplinary field, tissue engineering involves the combined use of stem/progenitor cells and biomaterials to aid in the regeneration of tissues and organs which lack self-regeneration potential [11–14]. Alternatively, cell-based therapies have also proved to deliver encouraging results in bone defect treatment [15]. For example, transplantation of mature cells (for example, osteoblasts) has often been used in bone defect treatment. However, transplantable autologous osteoblasts are not readily available by current experimental techniques and they may have donor site morbidity, and low proliferation capabilities [16]. Therefore, it becomes necessary to identify alternative cell sources for bone-related applications. The recent advancement of stem cell research has offered new hopes for bone regeneration, which gives us confidence that protective and regenerative therapies are possible [14, 17]. Among them, bone marrow–derived mesenchymal stem cells (BMSCs) have been considered as a promising source of stem/progenitor cells for clinical applications. Even though BMSCs are clinically being used for treating bone defects, they have their constraints, for example, limited availability and donor age-dependent proliferation potential. This lacunae in the field may be filled by identifying the right cell sources with abundant availability and high cell proliferative potentials [16, 17].

The use of induced pluripotent stem cells (iPSCs) in regenerative medicine for replacing defective cells and tissues has been researched over the past decade [5]. Nevertheless, some studies were published on the use of iPSCs for bone regeneration. For instance, Tang et al. have reported that human-iPSC-derived mesenchymal stem cells could be used in combination with calcium phosphate–based scaffolds for bone regeneration [18]. However, a recent report by Diederichs et al. suggests that a consistent quality of stem cell populations with high SOX9 protein induction is an essential indicator towards obtaining healthy cartilage tissues from iPSCs [19]. Therefore, iPSCs could be considered as a potent cell source for engineering bone tissues, certainly worthy of investigation and discussion.

The Scope of This Article

Considering the impact of iPSCs in regenerative medicine, we focus our attention here on iPSCs as the cell source for bone tissue engineering and analyze their bone regenerative capacity both in vitro and in vivo. For the benefit of the readers, the iPSC basic biology and the concept of bone tissue engineering are also discussed. However, the authors do not imply that iPSCs are the only choice of stem cells available for bone tissue engineering but the fundamental intention is to stimulate research on iPSCs in the context of tissue engineering and to gain insight into the state of the art of iPSCs for bone tissue engineering.

Induced Pluripotent Stem Cells (iPSCs): the Essentials and Overview of iPSC-Based Tissue Engineering

The principal objective of regenerative medicine is to replace non-functional/or damaged tissue. In order to achieve this goal, it is paramount to delineate the entire process of regeneration, including differentiated cells that can be converted into progenitor cells (which may have potential to replace the damages/non-functional cells); this process is termed as dedifferentiation of cells. On the other hand, the conversion of cells of one particular lineage into another cell type of a different lineage is widely known as transdifferentiation of cells. But in experimental conditions, where somatic cells can be induced to reprogram themselves by overexpression of four key transcription factors (OSKM, known as Yamanaka factors) to become pluripotent cells, this method is called cellular reprogramming. Studying the regenerative processes in both non-mammal (zebrafish, *Drosophila* fly models) and mammal models (rat, mice), natural or artificial processes, could emphasize the molecular and cellular signaling mechanisms behind cellular reprogramming, and then may be used to generate future regenerative therapies. The iPSCs are a sub-class of adult stem cells, which has emerged as a potent clinical alternative for embryonic stem cells (ESCs) by overcoming the ethical shortcomings of ESCs [20, 21]. The iPSCs have been shown to have similar characteristics and differentiation potential to ESCs that could be achieved by genetic reprogramming of adult somatic cells [22, 23]. For example, Yamanaka et al. have reported the reprogramming capability of murine fibroblasts into mouse embryonic fibroblasts or tail-tip fibroblasts by employing retroviral transduction of pluripotent-specific transcription factors, i.e., OCT4, SOX2, KLF4, and c-Myc. This study demonstrated the enhanced proliferative capacity and pluripotent behavior of reprogrammed cellular colonies [24]. Subsequently, a similar reprogramming strategy was tested with the lentiviral expression system to deliver four transcription factors namely OCT4, SOX2, NANOG, and LIN28 to fetal MRC5 lung fibroblast cells and new BJ-1 foreskin fibroblast cells which resulted in high reprogramming efficiency for fetal fibroblasts but only 0.01% for newborn fibroblasts [25]. Subsequently, the methodology was focussed towards increasing the reprogramming efficiency and reducing the number of

integrated vector sequences from the reprogrammed iPSCs. Since then, various methods have been used for the generation of iPSCs by reprogramming somatic cells, where the commonly used viral vectors for transcription factor delivery are retroviruses and lentivirus. Among them, lentivirus systems are best suited as delivery vehicles due to their ability to infect non-dividing and proliferating cells [26]. Furthermore, to prepare patient-specific human iPSCs (hiPSCs), cells were directly derived from the patients and reprogrammed. The differentiation of hiPSCs into tissue-specific cells is beneficial for cell-based therapies and patient-specific disease models for drug discovery and development [4].

Even though third generation lentiviral systems of somatic cells reprogramming are quite safe for pre-clinical studies, in recent years, there have been advancements in iPSC technology which could be attributed to a variety of reasons, for example, the advent of integration-free reprogramming approaches, disease modeling, and starting of preclinical trials [27]. Although iPSCs have not been considered as a model stem cell source due to their limitations such as poor reprogramming efficiency and tumorigenic potential, considerable research is going forward in this direction. As an alternative approach, various other methods have been explored for iPSC generation or to establish protocols for direct somatic cell application in the clinics. For instance, Jung et al. explored chemical biology for enhancing the reprogramming efficiency of adult stem cells to form iPSCs and increase their quality [4]. This study also investigated the cell phenotypes that govern the biological mechanisms related to iPSC generation. The study emphasizes small-molecule modulators that are capable of direct reprogramming adult cell types into clinically relevant cell types such as glial cells, neurons, and cardiomyocytes for translational research. However, Baek et al. reported that the extremely low-frequency electromagnetic field (EL-EMF) exposure could induce dynamic epigenetic changes which support effective somatic cell reprogramming. These dynamic epigenetic changes resulted from EL-EMF-induced activation of the histone lysine methyltransferase Mll2. Additionally, Kang et al. reported a direct conversion method for hiPSCs into osteoblasts by using adenosine molecules [28]. These alternative approaches may prevent the need for conventional vectors based on cell reprogramming and provide efficient methods for inducing epigenetic reprogramming.

Tissue engineering, in particular, iPSC-based tissue engineering, involves reprogramming of a patient's or donor's cells into iPSCs and then re-directing them to differentiate into the tissue-specific lineage, followed by culturing them onto a scaffolding system that provides structural and functional support to iPSC-derived tissues/organs, which could be then transplanted to the defective site (Fig. 1) [29]. iPSC-derived lineage-specific cells, appropriate scaffolds, and selected bioactive molecules are considered as vital components for the success of iPSC-based tissue engineering, as they can have a considerable impact on the cell-material interactions that guide tissue regeneration. Scaffolds provide structural support and a 3D microenvironment to the cells for cell attachment and subsequent tissue development. Besides scaffolds, cell sources are also one of the limiting factors for the success of tissue-engineered products. For the development of a viable tissue construct, specific cell types that show non-immunogenic behavior, high proliferative capacity, easy handling, and ability to differentiate into a variety of cell types with specialized functions are required. Cell sources utilized for tissue engineering include mature (non-stem) cells, adult stem cells, ESCs, and iPSCs, which can be autologous, allogeneic, or xenogeneic. Cell sources are available in a wide variety but are also found to be associated with their respective limitations. For instance, mature cells have low proliferation and differentiation capacity, whereas ESCs have high self-renewal and multilineage differentiation potential, but have limited access due to ethical and legislative issues [30]. To overcome these restrictions, iPSCs have been developed with ESC-like properties but surpassing the ethical issues. Besides the suitable cell type/source selection, scaffold design and bioactive molecule's mobilization that can facilitate cell-biomaterial interactions are also equally important factors. It has been proven that the addition of growth factors, bioactive molecules, or nanomaterials as bioactive signals can facilitate stem cell differentiation into the desired lineage. The interaction of cells with these engineered matrices is also a critical factor that governs the successful translation of functional tissue-engineered products.

State of the Art of iPSC-Based Bone Tissue Engineering and Regeneration

The ultimate goal of tissue engineering and regenerative medicine is to repair and regenerate damaged tissues or organs. Although MSCs have been used in a significant number of published studies for bone tissue engineering, ESCs and iPSCs remain as potential stem cell sources for researchers due to their pluripotent nature [23, 31] However, in vivo transplantation of ESCs and iPSCs raises several safety concerns including the risk of tumorigenesis. Although the unique combination of stem cell biology and biomaterials has enhanced the application of iPSCs in tissue engineering [32, 33], the process of designing and testing innovative biomaterials still limits the use of iPSCs in tissue engineering and clinical therapies.

Biomaterial–stem cell interaction can modulate the stem cell's fate. Recently, rapid prototyping techniques have been employed for designing customized 3D porous scaffolds suitable for bone defect regeneration. However, these porous scaffolds have been reported to be mechanically weak, which limits their use as bone grafts [34, 35]. Therefore, many research groups are focussing towards developing better synthetic scaffolds, which could best mimic natural bone composition and architecture for bone

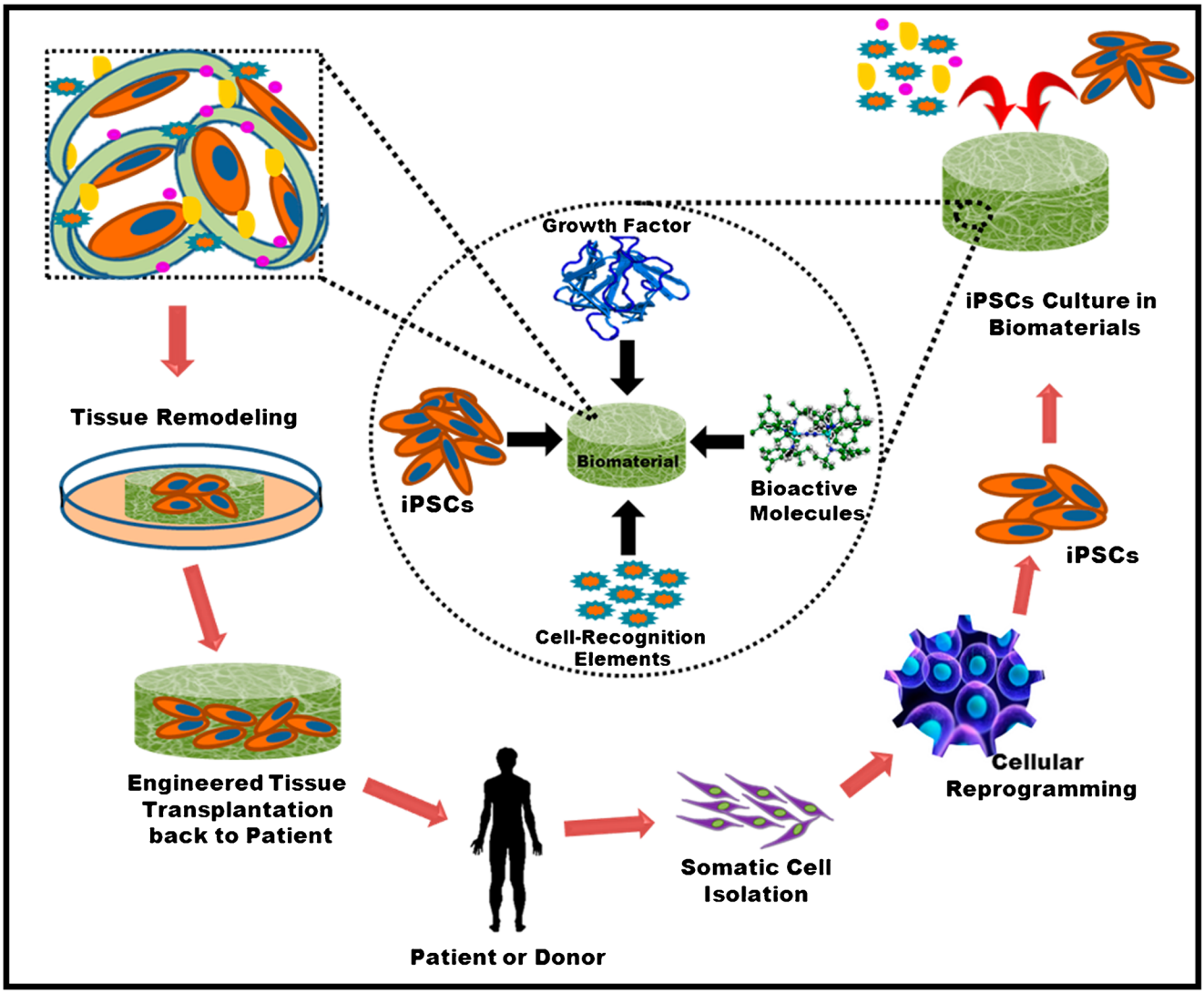

Fig. 1 Schematic representation of the use of iPSCs, as a cell source, for iPSC-based tissue engineering

defect regeneration. This could be achieved by processing innovative bioactive biomaterials or nanomaterials for bone tissue engineering in combination with biological molecules that can guide stem cells (or iPSCs) towards osteogenic differentiation [36•]. To this end, recent reports have demonstrated the ability of iPSCs to differentiate into osteoblasts or osteoclasts, implying that iPSCs could enhance the full aspect of bone remodeling and regeneration (Fig. 2).

In Vitro Perspective

Biomaterials such as calcium phosphate cement (CPC), which are similar to natural bone mineral, have been reported to treat complex-shaped bone defects with high osteoconductivity. For instance, Tang et al. reported that hiPSC-derived MSCs seeded onto CPC scaffolds could be used for bone regeneration in craniofacial, dental, and orthopedic repair applications [18]. The study employed iPSCs derived from reprogrammed adult marrow CD34+ cells by using a single episomal vector pEB-C5 which were cultured in vitro to form embryoid bodies (EBs), and MSCs were collected from the culture dish as cells migrated out of EBs during in vitro expansion. The iPSC-derived MSCs expressed the typical cell surface antigen profile of MSCs as confirmed by flow cytometry analysis and showed a mesenchymal differentiation ability by differentiating into chondrocytes, osteoblasts, and adipocytes. The iPSC-derived MSCs showed high cellular viability and osteogenic differentiation when seeded onto CPC scaffolds. The iPSC-derived MSCs cultured onto CPC scaffolds and supplemented with osteogenic medium revealed higher gene expression of osteogenic markers such as osteocalcin, collagen type I, alkaline phosphatase (ALP), Runx2 and other osteoblast-associated transcription factors than those samples cultured in control medium ($p < 0.05$).

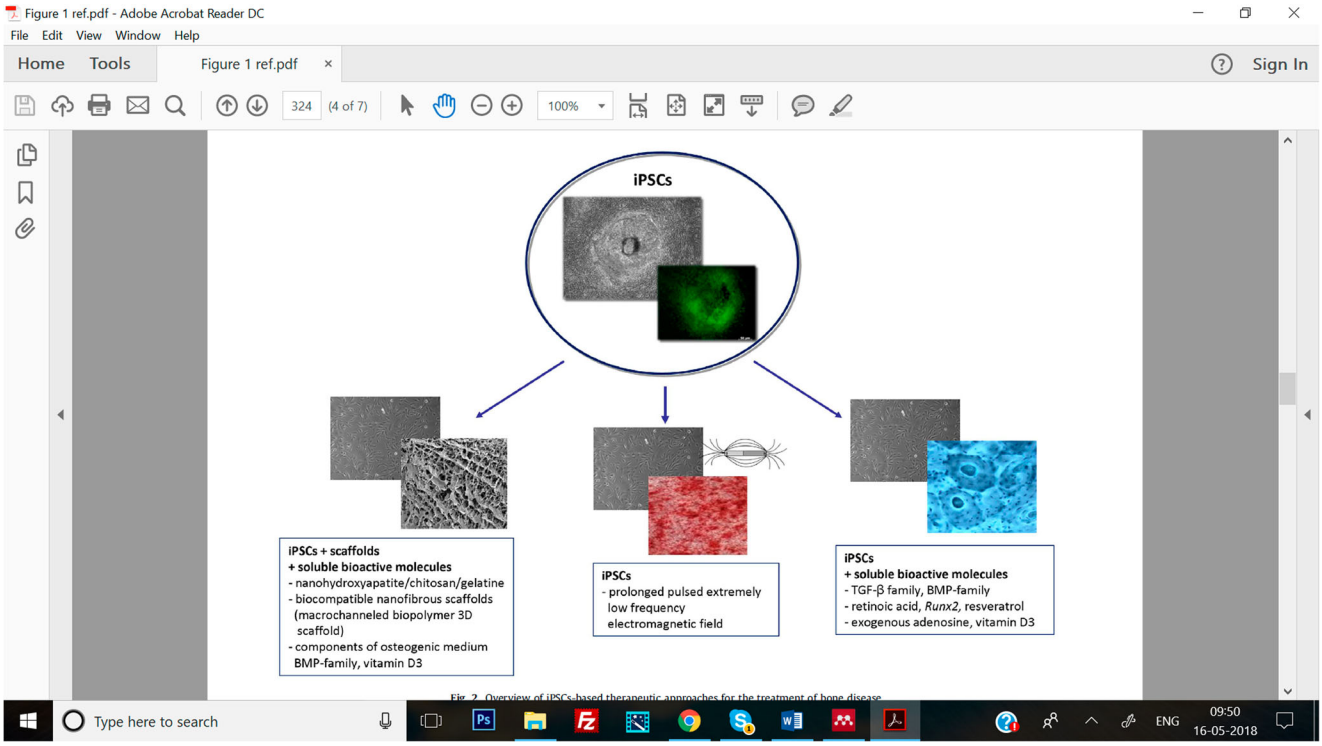

Fig. 2 Overview of iPSC-based therapeutic approaches for bone tissue engineering. Reprinted with permission from [37]. Used with permission from Elsevier

Additionally, in the same system, a ten times increase in ALP protein concentration was observed over that of the control group ($p < 0.05$). The study also stated that the bone mineral synthesized by iPSC-derived MSCs adherent to CPC scaffolds increased over time, and mineralization in the system supplemented with the osteogenic medium was 3–4-fold higher than the control group [18]. Similar to calcium phosphates, other essential trace bone mineral elements such as boron have also been evaluated for their effect on bone formation, growth, and health. In a report by Mondal et al., the polycaprolactone and borophosphosilicate glass (PCL/BPSG)–based hybrid biomaterial showed enhanced bone formation when cultured with iPSCs [38]. Cellular infiltration within the interior of the scaffold was observed. However, the osteogenic differentiation efficiency of the cultured iPSCs was dependent on the boron concentration in the hybrid scaffolds. Having said that, the scaffolds containing 2 mol% boron had a positive effect on osteogenic lineage expression for osteopontin (OPN), osteocalcin (OCN) and ALP [38].

Additionally, iPSCs have also been explored for chondrogenic differentiation. For instance, Mahboudi et al. studied the effect of nanofiber-based polyethersulfone (PES) scaffold on the iPSC chondrogenesis potential [39]. The study compared these scaffolds with the scaffold-free approach in vitro. However, after 21 days of culture, the results showed significant expression of collagen type II, collagen type X, and aggrecan, which are chondrogenic genes in PES scaffold–seeded hiPSC group in comparison with the expression levels in the scaffold-free group. In both groups, the expression of collagen I was observed to be downregulated, whereas SOX9 expression was upregulated, demonstrating the great efficiency of iPSCs and PES scaffolds for cartilage regeneration [39]. Various other types of scaffolds have also been tested for iPSC-based chondrogenesis. For example, electrospun 3D nanofibrous scaffolds composed of PCL/gelatin nanofibers cultured with iPSCs showed significant expression levels of chondrogenic genes than the control group [40•]. Being a potent cell source for osteo- or chondrogenesis, studies have also focussed on developing optimized protocols and address the shortcomings of iPSC chondrogenesis. It has been already reported that consistent quality of intermediate cell populations with high SOX9 protein induction is an essential indicator for achieving healthy cartilage differentiation from iPSCs [19]. To further validate this observation and compare results with MSC-driven chondrogenesis, Diederichs et al. investigated the SOX9 protein regulation during multiphase chondrogenic differentiation of two hiPSC cell lines which are comparable with MSC chondrogenesis. With intermediate mesenchymal progenitor cell (iMPC) generation, SOX9 protein was induced and reached variable levels in comparison with MSCs. It was found that the process of iMPC chondrogenesis was less efficient in comparison with MSCs chondrogenesis, whereas it was better in an iMPC cell line with higher SOX9 protein levels. Despite efficient Smad-2/3 phosphorylation, TGF-β-driven chondrogenic stimulation in iMPCs showed SOX9 protein

downregulation in contrast to high protein levels in MSCs. Unlike MSCs, in iMPCs, high levels of the SOX9 antagonize hsa-miR-145 protein levels which could be due to low SOX9 protein levels. These observations suggest altogether that considerable iMPC heterogeneity with variable SOX9 protein expression levels, altered condensation pattern, and low early SOX9 induction are the limiting factors for iPSC chondrogenesis [19].

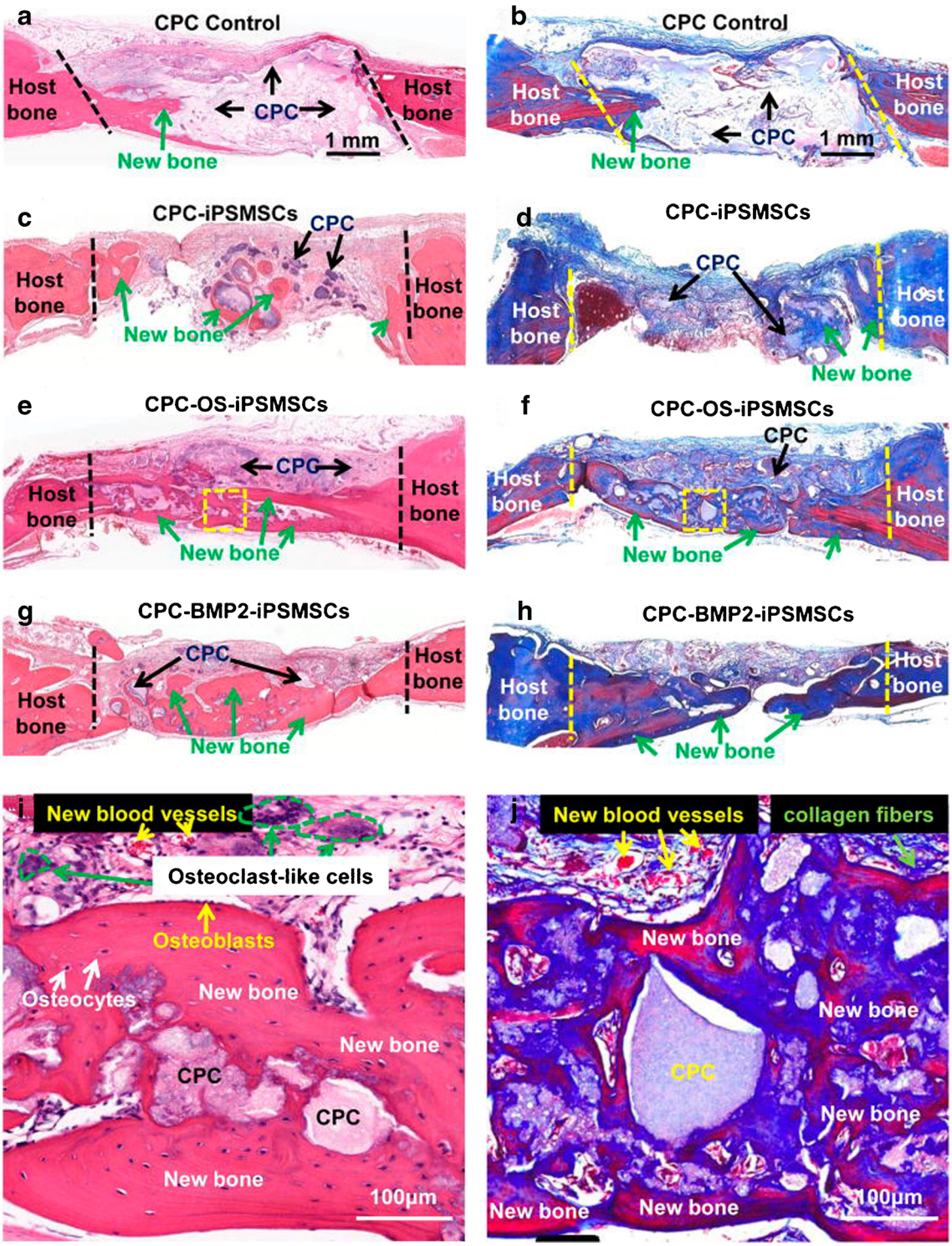

Fig. 3 Histological observations of bone regeneration in cranial bone defects in rats. CPC denotes calcium phosphate cement; OS-iPSCMSCs denotes osteogenic-mediated induced pluripotent stem cell-derived mesenchymal stem cells; BMP2 denotes bone morphogenetic protein 2. Reprinted with permission from [43]. Used with permission from Elsevier

In Vivo Perspective

Among others, one of the major challenges of iPSC applications in bone regeneration is the lack of safe and efficient methods or techniques for facilitating iPSC differentiation into osteogenic/chondrogenic lineages in vivo. Therefore, various research groups have tried to validate the applicability of iPSCs for osteogenic regeneration in animal models. For example, in a recent report by Wu et al., the first evidence of antibody-mediated differentiation of iPSC-derived mesenchymal stromal cells (iPSCMSCs), and osseous regeneration in vivo, was shown [41]. The subcutaneous implantation of iPSCMSCs and an anti-BMP2 antibody (3G7) showed significant vascularization and bone formation in vivo, whereas the sites with exogenous BMP2 revealed relatively lower vascularization and dystrophic calcification [41]. However, many researchers have investigated the effect of iPSCs on more conventional scaffolds for bone regeneration in animal models. Conventional scaffolds like CPC, which have already proved their potential with MSCs in animal models, were investigated with iPSCs. In a recent report by Kang et al., the reinforced composite scaffolds made of chitosan, whisker, and CPC when seeded with fifth-generation iPSC-derived MSCs proved better than the pure CPC scaffolds in vivo [42]. The composite scaffolds were implanted into Sprague Dawley rat models to establish the 8-mm-long skull bone defects and evaluated after 8 weeks. The percentage of new bone volume and the density of neovascularization in the composite scaffolds was observed to be significantly higher than those in the control group. The in vivo repair experiments showed that the new bone was mainly filled with the space of the scaffold material. Osteoblasts and neovascularization were surrounded by new bone tissue in the matrix, and osteoblasts were observed to be arranged on the new bone boundary [42]. To further modulate the physical behavior of hydrogels, a self-setting iPSC-derived MSCs and alginate/CPC composite–based injectable paste was reported for cranial defects [43]. The iPSC-derived MSCs (iPSCMSCs) were pre-osteoinduced (OS-iPSCMSCs) for 2 weeks or BMP2 growth factor transduced (BMP2-iPSCMSCs), followed by their encapsulation in fast-degradable alginate microbeads. The microbeads mixed with the CPC paste were used for filling cranial defects in nude rats. After 12 weeks, new bone area fraction (mean ± SD; $n = 5$) for the CPC-iPSCMSC group was (22.5 ± 7.6)% in comparison with the CPC-OS-iPSCMSCs (38.9 ± 18.4%) and CPC-BMP2-iPSCMSCs (44.7 ± 22.8%) (Fig. 3). The new bone fraction area for the control CPC group was 15.6 ± 11.2% which was much lower than other groups. These results clearly showed that the iPSCMSC-based CPC scaffolds could promote bone regeneration in vivo with accelerated scaffold resorption properties [43].

These scaffolds showed a significantly increased new bone formation in vivo, but could not provide adequate vascularization. Vascularization for a newly formed bone tissue construct is an important factor for maintaining cellular viability and functionality in vivo. Therefore, novel tri-culture CPC scaffolds were developed to elicit pre-vascularization [44]. Firstly, the human-derived iPSCMSCs seeded onto the CPC scaffold were osteoinduced, followed by the incorporation of HUVECs and pericytes to form a functional and stable

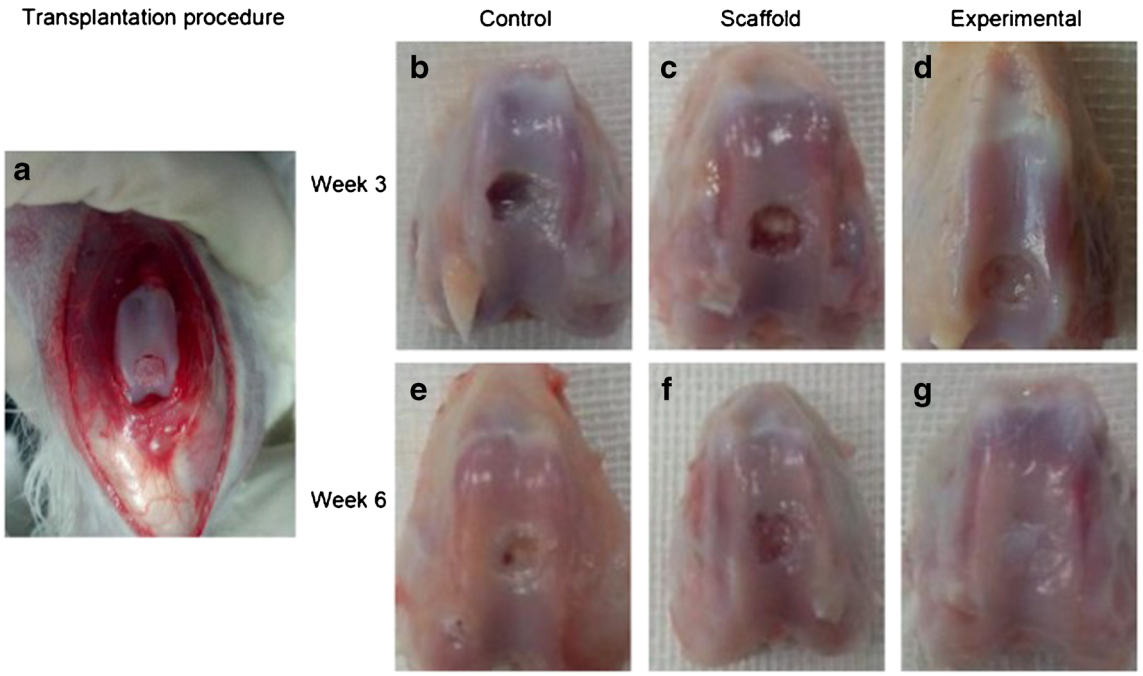

Fig. 4 Transplantation procedure for rabbit cartilage defects in control (B, E), scaffold only (C, F) and scaffold/hiPSC-MSC group (D, G) after 3 and 6 weeks. Reprinted with permission from [45•]. Figure used with permission from author (Open Access article)

vascular network that could mimic the natural vascular structures. The tri-culture construct was implanted in a nude rat model with the cranial bone defect for 12 weeks, and the results showed maximum new bone area fraction (45.3 ± 2.7%) and vessel formation (new blood vessel density) (50.7 ± 3.8 vessels/mm^2 in the tri-culture system) [44].

Besides bone regeneration, iPSCs have also been explored for cartilage and osteochondral regeneration in vivo. In a recent study, the MSCs derived from hiPSCs were seeded onto poly(lactic-co-glycolide) scaffolds followed by implantation into the cartilage defects of New Zealand white rabbits [45•]. After 3 and 6 weeks post-implantation, the experimental group exhibited high cartilage defect regeneration in comparison with the control groups. Post 6 weeks of implantation in the experimental group, cartilage-like tissue was observed along with no teratoma formation (Fig. 4) [45•]. These studies, along with others, prove the promising potential of iPSCs in bone repair and regeneration.

Concluding Remarks

The safety and efficacy of generated iPSCs need to be established beyond reasonable doubts to succeed in the field of translational regenerative medicine. While iPSC-derived stem cell resource is emerging as a substitutional cell supply, their cell intrinsic attributes of self-renewal and pluripotency after in vivo transplantation in small animal models often lead to tumorigenicity and genomic instability, which may result in low clinical utility. Rigorous studies in the iPSC field are being carried out to explore different technologies to generate integration-free iPSCs, rigorously characterize these cells, and widen their application for engineering physiologically relevant tissues and organs. New approaches towards generating iPSCs via episomal Sendai viruses, reprogramming mRNA, and other fine molecular tools for cellular reprogramming have also been experimentally tested. iPSC expansion on a bioreactor or 3D matrix that supports their self-renewal and pluripotency nature will be the next level of challenge in the field. Lastly, one needs to get well-differentiated cells from these iPSCs with minimal or acceptable contamination of pluripotent cells. Experimental designs are being developed towards the expansion of inter-converting procedures that can give rise to mature cell types, for example, skin, cardiac, cartilage, bone, and neural cell types. However, cell-matrix interactions are some of the defining factors for engineering physiologically functional tissues and/or organs; thus, the scientific focus is also required for the selection of a gradient scaffold support system for tissues where two different tissues need to be seamlessly organized. Although technological advancements have been made in cellular reprogramming of hiPSCs and have highlighted their therapeutic potential, relatively little is known about iPSC interaction with biomaterials during the in vitro lineage-specific differentiation process. Nevertheless, iPSCs and their derivatives have proven to be a potent renewable cell source for bone tissue engineering applications. Keeping the above points in mind, future research may focus on combining scaffold or biomaterials engineering with stem cell or iPSC technology to bio-mimic the physiological complexity of the stem cell niche in native tissues.

Compliance with Ethical Standards

Conflict of Interest Deepti Rana, Sanjay Kumar, Thomas J. Webster, and Murugan Ramalingam declare no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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