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Concise Review: Induced Pluripotent Stem Cells and Lineage Reprogramming: Prospects for Bone Regeneration

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ABSTRACT

Bone tissue for transplantation therapies is in high demand in clinics. Osteodegenerative diseases, in particular, osteoporosis and osteoarthritis, represent serious public health issues affecting a respectable proportion of the elderly population. Furthermore, congenital indispositions from the spectrum of craniofacial malformations such as cleft palates and systemic disorders including osteogenesis imperfecta are further increasing the need for bone tissue. Additionally, the reconstruction of fractured bone elements after accidents and the consumption of bone parts during surgical tumor excisions represent frequent clinical situations with deficient availability of healthy bone tissue for therapeutic transplantations. Epigenetic reprogramming represents a powerful technology for the generation of healthy patient-specific cells to replace or repair diseased or damaged tissue. The recent generation of induced pluripotent stem cells (iPSCs) is probably the most promising among these approaches dominating the literature of current stem cell research. It allows the generation of pluripotent stem cells from adult human skin cells from which potentially all cell types of the human body could be obtained. Another technique to produce clinically interesting cell types is direct lineage reprogramming (LR) with the additional advantage that it can be applied directly in vivo to reconstitute a damaged organ. Here, we want to present the two technologies of iPSCs and LR, to outline the current states of research, and to discuss possible strategies for their implementation in bone regeneration. STEM CELLS *2011;29:555–563*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The promise of regenerative medicine is to produce healthy tissues to replace diseased or damaged ones. Thus, it potentially opens new gateways for the treatment of degenerative diseases like Parkinson's, Alzheimer's, or Diabetes which remain a challenge for current medical practices. Among those, osteodegenerative diseases are rapidly gaining in importance. In particular, osteoporosis and osteoarthritis are major public health issues affecting a huge part of the elderly population. Besides these age-related bone diseases systemic disorders such as osteogenesis imperfecta or fibrous dysplasia manifest themselves in weak and fragile bones due to a defective bone matrix. Other cases require the replacement of large bone fragments. For example, maxillofacial surgeries of cleft palates and the loss of bone after surgical tumor excisions represent a high demand for clinically applicable bone tissue [1, 2].

To meet these issues, autologous bone grafting, that is, the replacement of damaged or missing bone tissue with the patient's own bone parts from healthy nonessential bones, which is widely regarded as the "golden standard" for alternative therapeutic approaches, has been practiced for decades. However, the limited availability of the patient's own bone tissue and the often accompanied donor site morbidity are inevitable drawbacks of the technique. Additionally, the transplantation of bone parts from allogeneic or xenogeneic origins bears the risks of immunological rejection and transmission of diseases.

Cell replacement therapies represent an alternative solution. The use of mature cells such as osteoblasts is associated with several disadvantages such as the limited availability, donor site morbidity, dedifferentiation, and restricted proliferation potential. Bone marrow-derived mesenchymal stem cells (MSCs), which are the developmental precursors of bone cells (apart from osteoclasts), have been discovered early for bone regeneration purposes and their therapeutic applicability has

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been assessed in case studies [3–5]. However, surgical bone marrow aspirations yield very limited numbers of MSCs. Moreover, the low proliferation rate of adult MSCs limits their expandability under culture conditions and their capacity to differentiate into new osteoblasts decreases with aging [6].

The derivation of pluripotent ESCs [7, 8] from the inner cell mass of preimplantation blastocysts has caused an unprecedented excitement among the scientific community. These cells have the unique character to self-renew indefinitely while maintaining the ability to give rise to all cells of the human body, called pluripotency. Thus, they potentially offer the possibility to produce clinically interesting cell types for cell replacement therapies. However, the broad excitement was at the same time followed by a basic ethical controversy regarding the fact that human embryos have to be destroyed during the isolation of ESCs.

Therefore, extensive studies over decades on a biological phenomenon called nuclear reprogramming in which one somatic cell type is converted into a different unrelated one through a switch of the gene expression pattern [9] resulted recently in the generation of ESC-like pluripotent cells by ectopic overexpression of only four genes in human fibroblasts, called induced pluripotent stem cells (iPSCs, pluripotent reprogramming) [10–12]. During the following months, an unparalleled wave of scientific contributions reported on the versatile advantages of iPSCs not only for regenerative medicine but also for drug discovery, toxicity testing, and academic research purposes.

At the same time another example of nuclear reprogramming has increasingly been investigated with therapeutic intentions. Easily accessible somatic cells such as fibroblasts or adipocytes could be directly converted to clinically relevant cell types on ectopic delivery of certain factors which are crucially involved in the embryonic development of the targeted cell type lineage reprogramming (LR). Thus, it potentially offers the generation of the addressed cell type directly in vivo as the absence of a pluripotent transition state excludes the risk of teratoma formation, the latter in contrast being an elementary characteristic of pluripotent stem cells [7, 8].

A schematic illustration of different therapeutic strategies for bone regeneration is shown in Supporting Information Figure S1.

Induced Pluripotent Stem Cells

Proceeding on the assumption that factors, responsible for the maintenance of the pluripotent state in ESCs, could induce pluripotency also in somatic cells upon ectopic overexpression, Kazutoshi Takahashi and Shinya Yamanaka identified only four of these factors, namely *Oct4*, *Sox2*, *Klf4*, and *c-Myc*, as being sufficient to reprogram mouse embryonic fibroblasts (MEFs) into a morphology highly comparable with ESCs, which they designated iPSCs [11]. The sensation was complete when the same group around Yamanaka reported the generation of human induced pluripotent stem cells (hiPSCs) with the same four factors [10]. Nearly simultaneously, James Thomson and coworkers equally reported the generation of hiPSCs from neonatal foreskin fibroblasts using another combination of reprogramming factors, by applying *Nanog* and *Lin28*, instead of *Klf4* and *c-Myc* [12]. Global gene expression profiles and DNA methylation patterns of iPSCs proved to be nearly indistinguishable from those of ESCs. They could maintain their self-renewal when cultured under ESC conditions and could be differentiated to cells of all three germ layers [13–15]. Moreover, they developed teratomas when injected into immunodeficient mice, and they could contribute to the development of chimeric mice, a major criterion for pluripotent stem cells.

After earlier attempts to derive autologous pluripotent cells by somatic cell nuclear transfer had failed [16], the discovery of iPSCs opened up new avenues to generate patient-specific pluripotent stem cells, and, in addition, resolved ethical concerns related to the destruction of human embryos. iPSCs could possibly be used to study embryonic development, to generate models of monogenetic diseases for research purposes, to design disease-specific cell-based assays for drug screening and toxicity tests, and not least to derive autologous tissue for cell replacement therapies.

However, prior to their potential applicability for clinical therapies, two inevitable impediments had to be surmounted. iPSCs appeared to evolve from the infected cells of origin very slowly (initially more than 3 weeks after infection) and resulted in a very low yield [10], which still could not be fully understood. Recently, an explanation was proposed by the hypothesis that iPSC reprogramming is a continuous stochastic process [17, 18]. On the other side, if iPSCs should be used for therapeutic purposes the cells must be free of genomic insertions of transgene sequences, which could cause serious genetic alterations if transplanted into patients.

Small molecule compounds were proposed as a possible solution to resolve both problems. Thus, a variety of so-called epigenetic modifiers was applied to support the loosening of the DNA state in differentiated cells [19, 20], so that silenced genes, like the pluripotency factors, could be reactivated faster. It was also found that, for example, inhibitors of the mitogen-activated protein kinase [21, 22], the glycogen synthase kinase-3β [23, 24], or the type 1 transforming growth factor (TGFβ) receptor [25], could replace some factors or increase the reprogramming kinetic and efficiency.

Further attempts to generate safer iPSCs by other means than retro- or lentiviral infections include the transient delivery of the reprogramming factors by adenoviruses [26], bacterial plasmids [27], piggyBac transposons [28, 29], and episomal vectors [30], with the last attempt resulting in the generation of hiPSCs free of any detectable genomic integration. Finally, it was shown that iPSCs could be generated if the reprogramming factors were delivered as recombinant proteins over a defined period of time, which probably represents the safest method to date [31, 32].

iPSCs and Drug Discovery

Very early, the broad versatility of iPSCs was discovered for pharmaceutical drug development and toxicity screening [33, 34].

Two main problems are associated with the conventional cell-based assays used in pharmaceutical research and development, that is, primary human cells carrying the disease of interest are often difficult or impossible to isolate, and they mostly do not proliferate sufficiently to obtain the required amount of cells for high-throughput screening approaches. Thus, they have to be cultured in mitogen-rich media, which force the cell to dedifferentiate to go back into the cell cycle. Hence, genetically immortalized cells, animal cell models, or tumor cells with high proliferation rates are applied frequently, which, however, do not imitate the human conditions optimally.

In contrast to that, iPSCs can be generated from a patient's skin sample, carrying a defined genetic mutation. Subsequently, the so-called disease-specific iPSCs can be expanded and differentiated to the cells of interest, which will display the pathological characteristics of the disease. Thus, numerous disease-specific iPSCs have been generated for

various clinically highly challenging diseases including Parkinson's disease, amyotrophic lateral sclerosis, and type 1 diabetes [35–37].

However, to our knowledge, no iPSC model of a monogenetic bone disease has been derived so far. Prominent systemic bone disorders include for example McCune-Albright syndrome, with a mutation in the *GNAS* gene, encoding the guanine nucleotide-binding protein, multiple hereditary exostoses, with mutations in the *EXT-1* and *EXT-2* genes, osteogenesis imperfecta, with mutations in the collagen type 1 encoding genes *COL1A* and *COL1B* in over 90% of the cases, or hypophosphatasia, with mutations in the alkaline phosphatase encoding gene *ALPL*. A comprehensive and reliable cataloging of genetic bone diseases can be found at the NIH's "Genetics Home Reference" (http://ghr.nlm.nih.gov/conditionCategory/bones-muscles-and-connective-tissues). These bone disease-specific iPSCs could not only be used for pharmaceutical high-throughput screening assays and osteotoxicity tests but would also offer a unique opportunity to study the pathological mechanisms of the diseases.

iPSCs and Cell Replacement Therapy

In the meantime, seminal proof-of-principle studies demonstrated how iPSC-based therapies could be performed in the future [38–41]. In these studies, two different strategies were proposed, one applies healthy iPSC-derived cells, and the other uses autologous patient-specific cells after the mutation was repaired by gene technological methods.

Hanna et al. used iPSCs to treat a humanized mouse model of sickle cell anemia [38]. They reprogrammed fibroblasts of the diseased mouse to iPSCs, corrected the mutant gene by homologous recombination, differentiated the pluripotent cells to hematopoietic progenitors, and transplanted them back into the mouse. The therapy resulted in a substantial improvement of the symptoms. A similar attempt was reported for the treatment of Fanconi anemia [39]. In this case, skin fibroblasts were isolated from a human patient suffering from the disease. The mutant gene was replaced, and subsequently the skin cells were reprogrammed to iPSCs. The "cured" iPSCs could be differentiated to hematopoietic progenitor cells, which could be cultured in vitro, stably maintaining the disease-free state.

In two other studies, healthy iPSC-derived dopaminergic neurons [40] and endothelial cells [41] were used to treat rat and mouse disease models of Parkinson's disease and hemophilia A. In both cases, the transplanted cells functionally integrated into the host organisms resulting in clearly improved disease patterns.

A conceptual model for the use of iPSCs for research purposes and cell replacement therapies is given in Figure 1.

Lineage Reprogramming

In principle, the strategy of pluripotent reprogramming is based on the creation of one "super-potent" cell from which all somatic cells could be obtained. In contrary to that, LR differs in its approach basically in two concepts, that is, it does not require a total epigenetic reset to the pluripotent state, and it is potentially suited for in vivo cell conversions (Supporting Information Figure S2).

LR In Vitro

The proof-of-concept that adult cells could be directly converted to other somatic cell types was given when Davis et al. [42] discovered that *MyoD*, a key regulatory transcription factor during muscle development, could convert MEFs to myoblasts upon ectopic overexpression.

Many years after the initial reports on *MyoD* [43, 44], scientists refocused their attention on LR in extensive studies on the interconversion capabilities of cells within the hematopoietic lineage [45, 46], probably due to the advanced knowledge about hematopoietic development, the broad diversity of cells within a common lineage, and the easy accessibility of blood cells. One outstanding finding in hematopoietic LR was definitely the generation of macrophage-like cells by transdifferentiation from skin fibroblasts with only two factors [47]. These initial results prompted soon analog attempts to obtain cell types of acute clinical relevance.

Aiming at the derivation of functional neurons, Kondo et al. were able to convert oligodendrocyte progenitor cells to a precursor state, similar to that of multipotent central nervous system stem cells, without genetic manipulations [48]. The converted cells could self-renew and give rise to neurons, type 1 astrocytes, and oligodendrocytes. An impressive result was published very recently, when Vierbuchen et al. reported the generation of functional neurons from fibroblasts by ectopic expression of the three transcription factors *Ascl1*, *Brn2*, and *Myt1l* [49], which they termed induced neuronal cells. The group was able to convert embryonic as well as postnatal fibroblasts.

On the other side, Takeuchi et al. identified the cardiac transcription factors *Gata4, Tbx5*, and a cardiac-specific subunit of the Brg/Brahma-associated factors chromatin remodeling complexes, *Baf60c*, as being capable of inducing transdifferentiation of mouse mesoderm, including the noncardiogenic posterior mesoderm and the extraembryonic mesoderm of the amnion, to beating cardiomyocytes [50]. And only very recently it was reported that functional cardiomyocytes could even be obtained from dermal fibroblasts by LR with a similar combination of only three factors: *Gata4*, *Mef2c*, and *Tbx5* [51].

LR In Vivo

Two outstanding studies on in vivo LR outline the principles of the approach. In one attempt, the authors reported the restoration of hearing in deaf guinea pigs [52]. A main reason for the loss of auditory function is the permanent degeneration of cochlear sensory (hair) cells. Hence, de novo generation of sensory cochlear cells by forced conversion from nonsensory epithelial cells of the cochlea by ectopic expression of *Atoh1* had been reported in vitro and in vivo [53, 54]. On the basis of these initial results, Izumikawa et al. demonstrated an eventual therapeutic potential by showing that hearing could be improved substantially in mature deaf inner ears of mammals on direct in vivo reprogramming of nonsensory cells by adenoviral overexpression of *Atoh1* [52].

In another approach, the group around Douglas Melton converted pancreatic exocrine cells to insulin-producing β-cells in adult mice by injection of an adenoviral pool containing the pancreatic factors *Ngn3*, *Pdx1*, and *Mafa*, demonstrating the principles of a potential therapy for the rehabilitation of insulin production in type 1 Diabetes [55].

iPSCs and Bone Regeneration

Clinical demand of healthy bone tissue for transplantation therapies and reconstructive surgery becomes manifest basically in two distinct scenarios. First, for the densification and strengthening of porous, weak, and fragile bone matrix in systemic bone disorders, affecting the entire skeleton. In this case, the bone-forming cells have to be transplanted systematically to reach all bone parts, for example, by intravenous

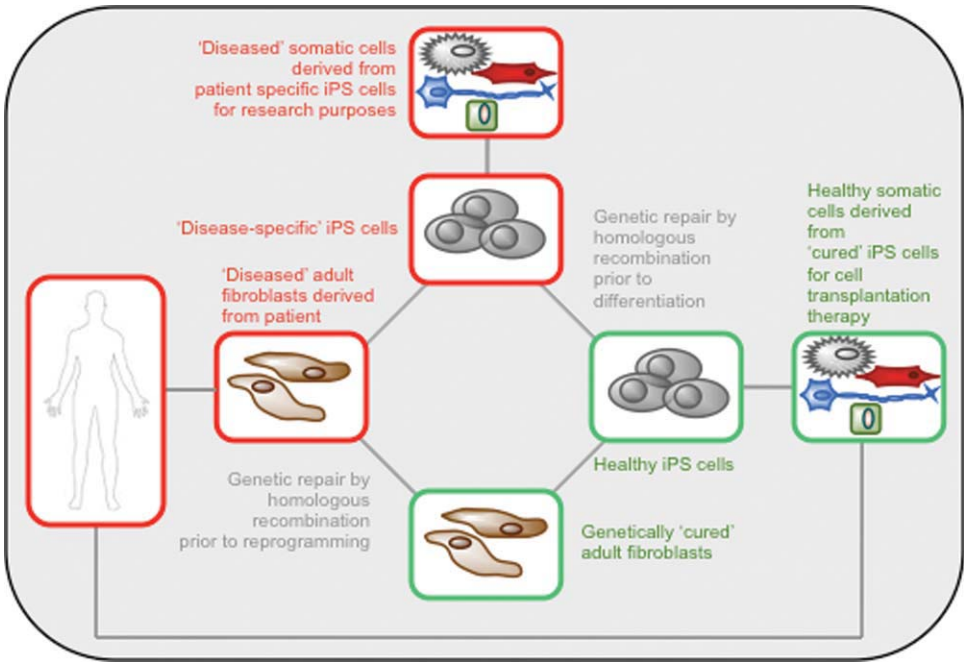

Figure 1. iPSCs for research purposes and cell replacement therapies. Disease-specific iPSCs, obtained from patients with defined genomic mutations, offer in vitro research models of pathologic tissue formation. Genetic repair of the mutations by homologous recombination before differentiation enables the generation of healthy patient-specific cells for transplantation therapies. Abbreviation: iPSC, induced pluripotent stem cell.

injection [56, 57]. In the second case, an acutely affected, locally defined bone part has to be remodeled completely because of damage or loss, caused by accidents or surgical tumor excisions [2, 3, 58, 59]. Because of the missing carrier matrix the mechanical stability and structural constitution have to be ensured by artificial, biocompatible cell delivery scaffolds.

Osteogenic Potential of iPSCs In Vitro

The differentiation of ESCs to bone cells has been adapted from the protocols for the osteogenic differentiation of MSCs. The basic components of the commonly used osteogenic medium are fetal bovine serum, ascorbic acid, β-glycerophosphate, and dexamethasone (Dex) [60]. Additional enhancing supplements include certain bone morphogenetic proteins (BMP) [61, 62], or the calcium-regulating hormone vitamin D3 (VitD3) [63].

Importantly, the timing of osteogenic supplementation was shown to be relevant. Dex, for example, was found to have an increasing osteogenic effect, when added at later stages of differentiation [64]. If VitD3 was applied instead of Dex it had to be used after the embryoid body (EB) formation step [63]. When BMP2 was added during earlier stages of differentiation it even decreased osteogenesis [65]. In contrast, BMP4 was found to be required continuously [61].

Another strategy, probably based on the cell-cell interactions and secretion of supporting factors, is the differentiation in coculture with primary bone cells [66] or the use of cell extracts from osteogenic cells [67].

Apart from that, the EB formation step has been a disputed element of many differentiation protocols. Although it could be shown that the omission of the EB formation resulted in an accelerated osteogenic differentiation of mouse ESCs based on marker expression and calcification [68, 69], it remains unclear whether the EB formation step leads to a more efficient osteogenic differentiation of human ESCs (hESCs). In one study, the absence of EB formation resulted in a slightly delayed calcium deposition [60], whereas Karp et al. reported an earlier and more efficient osteogenesis without EBs [70]. It is certain, though, that the EB formation step is not necessarily required during osteogenic differentiation.

On the basis of differentiation protocols for ESCs, recently the generation of bone matrix-forming osteoblasts has been reported from mouse and human iPSCs (Fig. 2). In one approach, exogenous overexpression of the key osteogenic transcription factor *Runx2* by adenoviral transduction enhanced the osteogenic differentiation of mouse iPSCs [71]. *Runx2*-transduced iPSCs exhibited after differentiation more than 50% higher alkaline phosphatase activity than nontransduced cells, and the level of calcium was about eightfold higher. The authors suggest adeno vector-mediated transient delivery of *Runx2* as a tool for application to safer regenerative medicine using iPSCs. However, they do not show any evidence that the transgenic sequence did not permanently integrate into the host genome, like polymerase chain reaction analysis with genomic DNA, or Southern blot analysis. In another attempt, resveratrol, a natural polyphenol antioxidant found largely in skins of red grapes, nuts, and red wine, was found to have a promoting effect on osteogenic differentiation of mouse iPSCs, based on mineralization, and osteogenic marker expression [72]. The group also attempted to evaluate the in vivo bone formation by iPSCs, which were cultured in osteogenic medium for 7 days prior to transplantation into nude mice, when the mice were fed with resveratrol. Six weeks after transplantation they observed a higher expression of osteopontin in the transplants isolated from the mice, which were fed with resveratrol, compared with the control mice, and conclude that resveratrol effectively promotes osteogenic differentiation in vivo. However, the article does not contain any evidence of mineralization within the transplant, and from the provided histologic analyses, neither typical bone histology nor osteoblasts and osteocytes can be recognized.

Furthermore, the derivation of MSC-like cells from mouse iPSCs was equally reported [73]. In one study, the authors emphasized the promoting effect of TGF-β1 and retinoic acid

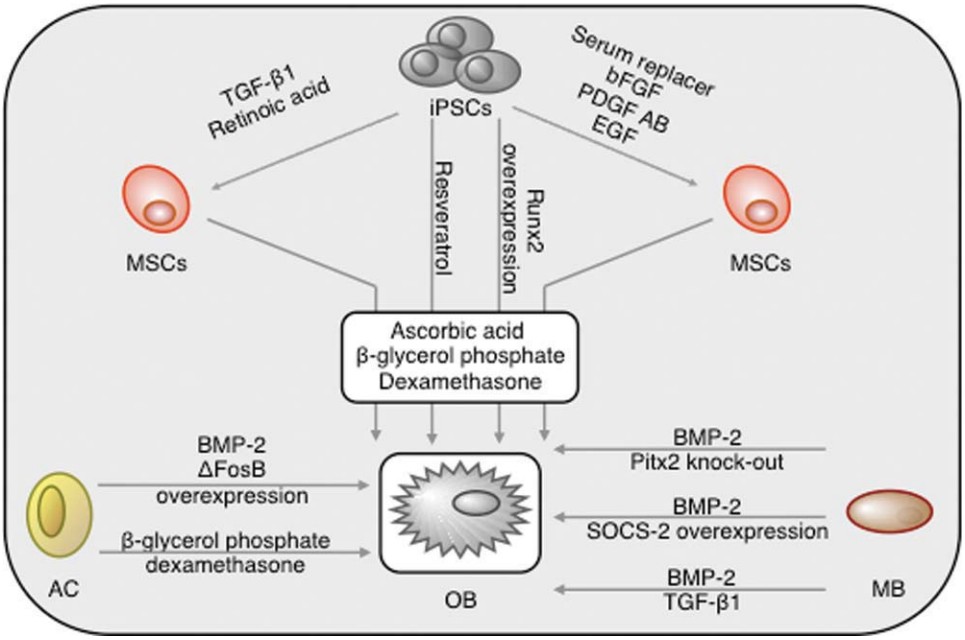

Figure 2. Derivation of osteoblasts by iPSC-based and lineage reprogramming. Osteoblasts and MSCs have been differentiated from mouse and human iPSCs. Additionally, osteoblasts could be obtained by direct lineage reprogramming from myoblasts and adipocytes through specific reprogramming factors. Abbreviations: AC, adipocytes; bFGF, basic fibroblast growth factor; BMP2, bone morphogenetic protein 2; EGF, epidermal growth factor; iPSCs, induced pluripotent stem cells; MB, myoblasts; MSCs, mesenchymal stem cells; OB, osteoblasts; PDGF AB, platelet-derived growth factor AB; SOCS-2, suppressor of cytokine signaling 2; TGF-β1, transforming growth factor beta 1.

on MSC differentiation. The derived cells were characterized by the expression of MSC-specific surface markers and the ability to further differentiate into adipocytes and osteoblasts upon exposure to the respective culture conditions. Osteogenic differentiation was assessed by positive von Kossa staining, alizarin red staining, and the expression of osteogenic markers.

The potential therapeutic relevance of a strategy aiming at the derivation of MSCs from iPSCs, was demonstrated by Lian et al. [74], who derived functional MSCs from human iPSCs, which they could isolate, purify, and maintain under common MSC culture conditions. A subsequent transplantation into a mouse model of limb ischemia resulted in a substantial attenuation of the symptoms by promotion of vascular and muscle regeneration. Interestingly, they reported that iPSC-derived MSCs showed better results than adult bone marrow MSCs, due to their superior survival and engraftment after transplantation, and a higher capability to differentiate into the appropriate tissue. Unfortunately, the authors do not compare the iPSC-derived MSCs to bone marrow-derived MSCs regarding their potential to form new bone in vivo upon transplantation.

Toward this end, Bilousova et al. showed only very recently that mouse iPSC-derived mesenchymal tissue could differentiate into functional osteoblasts in vitro, which when cultured on a gelatin scaffold could give rise to mineralized bone tissue with vascular supply in vivo on transplantation in syngenic mice [75]. However, the iPSC-derived MSCs were not purified and maintained separately to be compared with bone marrow-derived MSCs regarding their osteogenic potential in vivo and analog results still need to be obtained using human iPSCs.

Osteogenic Potential of iPSCs In Vivo

Until recently, the published data offered only some indications of bone-like and mineralized tissue formation by hESCs in vivo [64, 76, 77]. Small, mineralized, von Kossa-positive patches were observed, where also osteocalcin was expressed. However, bone histology was missing [77], or very vague [64, 76], with no evidence of osteocytes and osteoblasts, and insufficient proof of tissue origin.

More recent investigations, however, support convincingly the osteogenic potential of hESCs and iPSCs in vivo [78, 79]. Arpornmaeklong and coworkers first derived MSCs from the hESC line BG01, characterized by the expression of MSC-specific surface antigens, and the ability to further differentiate into adipogenic, chondrogenic, and osteogenic tissue [78]. Subsequently, they induced osteogenic differentiation of the hESC-derived MSCs upon culture in osteogenic medium for 28 days. Before transplantation into calvarial defects of nude mice, osteoprogenitors were enriched by fluorescence-activated cell sorting based on the expression of alkaline phosphatase and a previously transfected green fluorescent protein (GFP)-construct for cell tracking. Histologic analysis convincingly demonstrated new bone formation within the cranial defect, and staining of human-specific nuclear antigen and GFP revealed that the newly formed bone originated from the transplanted cells.

The by far most extensive in vivo bone formation by hESC-derived cells was described very recently by Robey and coworkers [79], who elaborately tested different differentiation conditions in vitro for prolonged periods of time ranging from 7 to 14 weeks prior to transplantation into immunocompromised mice. In vivo development of the transplants was followed up to 20 weeks after transplantation. Extensive new bone formation was demonstrated by histologic analyses, where osteoblasts and osteocytes could be recognized, and intense green fluorescence of highly mineralized bone matrix. The human origin of the cells forming new bone was determined by in situ hybridization for human-specific *alu* repetitive DNA sequences. Importantly, the group identified a correlation between certain culture conditions and an accumulated teratoma formation in 12- to 20-week-old transplants, and despite multiple conditions tested, the group has not achieved consistent bone formation by hESC progeny.

The in vivo bone formation by iPSCs was equally demonstrated [80]. The authors investigated the capabilities of human iPSCs for periodontal tissue regeneration in nude mice. They reported that EMD, a gel containing an enamel matrix-derived protein complex from the amelogenin family, substantially promoted new in vivo alveolar bone and cementum formation with regenerated periodontal ligament between them, after they transplanted iPSCs on silk scaffolds into a mouse periodontal fenestration defect model.

LR and Bone Regeneration

To propose a general strategy for the derivation of bone cells by LR, a few key issues have to be addressed.

Cells to Be Derived

Two different methods were proposed, that is, either the generation of the core cell type, which would directly reconstitute the function of the organ [49], or the derivation of its progenitor cell [48].

Osteoblasts are progenies of MSCs and constitute the rudimentary bone matrix-secreting cell type, which progressively transform into osteocytes [81]. MSCs, together with hematopoietic stem cells, are derived from the mesoderm, and constitute the two main postnatal stem cell populations residing in the bone marrow [82–84]. MSCs are also the progenitors of myoblasts, chondrocytes, adipocytes, and fibroblasts [85, 86]. Hence, osteoblasts and MSCs are the core cell types in osteogenesis, and thus represent the key cell types to focus on for bone regeneration purposes [87].

Cells to Be Converted

One might assume that developmentally adjacent cell types are more amenable to reprogramming toward each other because a shared developmental history would result in a similar epigenetic state. Thus, it has been shown that mature cells are more difficult to reprogram to pluripotency than immature cells [88, 89], and initial studies on LR were successfully performed within the same lineage. Hence, the derivation of osteoblasts is probably most promising from cells of the MSC lineage, such as chondrocytes, myoblasts, adipocytes, or fibroblasts [86].

Accordingly, it has been found that myoblasts could be forced to convert into osteoblasts instead into myocytes, after the mechanism was elucidated that leads to a split up in differentiation after an initial common development from MSCs [90–93]. Furthermore, it was reported that human adipocytes could be directly reprogrammed into osteoblasts [94–96] (Fig. 2).

Reprogramming Factors

To identify key developmental regulators of osteogenesis as potential reprogramming factors considerable understanding of the alterations in gene expression during osteogenic commitment could be won from gene expression analyses during ESC differentiation to osteoblasts, particularly because stages earlier than the MSC phase could be analyzed [87, 97]. hESC differentiation to osteoblasts has been studied extensively [98], and crucial transcription factors regulating osteoblast differentiation have been identified together with their specific function [99–103] such as *Runx2*, *Osterix*, *β-catenin*, *Foxc1*, *Msx1*, *Msx2*, *Dlx5*, *Dlx6*, *Twist*, *AP1(Fos/Jun)*, *Knox-20*, *Sp3*, *Atf4, Alx4*, etc. Among those supremacy has been awarded to the early osteogenic marker *Runx2* and the late osteogenic regulator *Osterix* [104–107], with particularly *Runx2* playing a master regulatory role and being indispensable for osteoblast differentiation [107]. Additionally, extracellular factors, which often play important roles in development, and were identified to promote osteogenic differentiation in vitro, such as *TGFβ*, *BMP*, *IGF*, etc., could potentially be tested as genetically noninvasive reprogramming factors [108].

Based on these findings, recently, it has been demonstrated that human adipocytes could successfully be converted to osteoblasts on lentiviral overexpression of *BMP-2* and delta FBJ murine osteosarcoma viral oncogene homolog B (Δ*FosB*) [94]. The choice of factors was justified by the ability of *BMP-2* to induce *Runx2* expression during in vitro differentiation, and the fact that Δ*FosB* supported osteoblast maturation and inhibits adipogenesis [94]. Additionally, a transdifferentiation of human adipocytes to osteoblasts solely through a defined culture medium was reported, dispensing genetically invasive methods entirely [95, 96].

It has been known for a while that BMP could also convert the differentiation program of myoblasts to an osteogenic commitment [90]. Later it was reported that the transdifferentiation from myoblasts to osteoblasts is based on *TGFβ1*- and *BMP-2*-induced expression of the key osteogenic transcription factor *Runx2* [92]. Further mechanistic studies revealed that suppressor of cytokine signaling *SOCS-2* potentiates [93], and that paired-like homeodomain transcription factor *Pitx2* inhibits *BMP*-induced transdifferentiation of myoblasts to osteoblasts [91]. Thus, an additional alternative to ectopic overexpression of reprogramming factors is the knockout of crucial factors that stabilize the state of the initial cell type or the implementation of both techniques simultaneously.

Bone Regeneration by LR In Vivo

For in vivo LR, on the one side, other cells have to be available in the same organ that could be reprogrammed to the cell type of interest, and on the other side, an appropriate "niche" is required for the newly generated cells, particularly, for progenitor cells [52, 55]. There are several cell types within the compact bone organ, emerging from the mesodermal lineage, which could possibly be reprogrammed into osteoblasts, particularly, fibroblasts from the outer fibrous layer, and adipocytes from the yellow marrow.

As shown above, several studies already reported the successful conversion of adipocytes to osteoblasts in vitro [94, 95, 109]. However, also mature osteoblasts could undergo adipocyte transdifferentiation under appropriate culture conditions [110]. Notably, an increased fat tissue amount and decreased bone tissue volume has been found to correlate with progressed age [111, 112]. To confront these age-related loss of bone mass, both, new derivation of osteoblasts from progenitor cells, and the active functionality of already existing osteoblasts, are key requirements [95]. Thus, for example, the age-related limitations of MSC to generate new osteoblasts [113], could be improved by artificial "rejuvenation" upon overexpression of human telomerase reverse transcriptase in human MSCs [114, 115].

An alternative approach to cure age-related loss of proper functioning osteoblasts could be direct reprogramming of the increased amount of fat tissue to osteoblasts in vivo. For example, a senescence-accelerated mouse model could be used for an in vivo screening assay to identify potential osteogenic reprogramming factors capable of improving the osteoblast/adipocyte-ratio within bones [116, 117].

Remaining Challenges

IPSCs and LR put themselves forward as promising technologies for the regeneration of bone tissue. The derivation of

MSCs and osteoblasts was reported using both methods, and their capabilities to reconstitute bone tissue in vivo were equally demonstrated. However, a few remaining challenges have to be resolved prior to their potential implementation in clinical therapies.

Cell reprogramming protocols have to be optimized toward the highest possible kinetic and conversion efficiency. Toward this end, different methods were applied [118]. Still the conversion of fibroblasts to iPSCs takes considerably longer than LR of fibroblasts to cardiomyocytes or neural cells, which appeared only 3 days after viral induction [49, 51].

Equally, the differentiation protocols of iPSCs to MSCs and osteoblasts have to fulfill clinical safety standards, which require high-quality homogenous cell populations without the risk of remaining teratogenic cells. Therefore, apart from the expansion of undifferentiated iPSCs in stirred suspension bioreactors, additional focus will have to be directed at the large-scale differentiation, enrichment, and selection protocols for the generation of homogenous osteoblast populations [119].

Apart from that, viral overexpression of the reprogramming factors has to be replaced by safer, genetically noninvasive means, such as proteins, growth factors, cytokines, or small molecules, as already shown [31, 32, 48, 96]. For example, reversine, a small molecule compound, was found to be able to reprogram myoblasts into a MSC-like state, which could be subsequently differentiated into osteoblasts [120].

Another major impediment of stem cell therapy is the development and optimization of cell delivery scaffolds and the adjustment of the protocols from tissue culture plastic dishes to 3D scaffolds. There are several requirements that crucially determine the efficiency of bone formation, that is, the size and distribution of pores, the surface appearance, and the mechanical properties of the material. Currently proposed scaffolds include those made of inorganic materials, organic or synthetic polymers, or of mixed materials (composite scaffolds). Additional issues, such as vascularization, anatomical shapes, and the delivery of growth factors, will remain challenges for bone tissue engineering in the future.

Finally, two other questions of immense importance, particularly for in vivo LR remain open, that is, how should the reprogramming factors be delivered to the organ of interest, and how to address certain cells specifically, without harming other tissues? Local microinjections of either gene expression vectors or virus suspensions are two proposed techniques [52, 55], which could possibly be optimized to safer solutions.

Conclusion

In summary, iPSCs and LR represent two promising technologies in regenerative medicine, and investigative efforts are intensively addressing the remaining issues prior to future therapeutic applications. We believe that here presented studies are important contributions to the derivation of healthy bone tissue for therapies in osteoregenerative medicine, and we are optimistic that they will soon encourage further investigative efforts in this field.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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