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EXPERT OPINION

Make no bones about it: cells could soon be reprogrammed to grow replacement bones?

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Recent developments in nuclear reprogramming allow the generation of patient-matched stem cells with broad potential for applications in cell therapies, disease modeling and drug discovery. An increasing body of work is reporting the derivation of lineage-specific progenitors from human-induced pluripotent stem cells (hiPSCs), which could in the near future be used to engineer personalized tissue substitutes, including those for reconstructive therapies of bone. Although the potential clinical impact of such technology is not arguable, significant challenges remain to be addressed before hiPSC-derived progenitors can be employed to engineer bone substitutes of clinical relevance. The most important challenge is indeed the construction of personalized multicellular bone substitutes for the treatment of complex skeletal defects that integrate fast, are immune tolerated and display biofunctionality and long-term safety. As recent studies suggest, the merging of iPSC technology with advanced biomaterials and bioreactor technologies offers a way to generate bone substitutes in a controllable, automated manner with potential to meet the needs for scale-up and requirements for translation into clinical practice. It is only via the use of state-of-the-art cell culture technologies, process automation under GMP-compliant conditions, application of appropriate engineering strategies and compliance with regulatory policies that personalized lab-made bone grafts can start being used to treat human patients.

Keywords: automation, bone engineering, clinical translation, embryonic stem cell, induced pluripotent stem cell, regulatory policies, skeletal reconstruction, stem cell therapy, tissue substitute

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Injured, malfunctioning and deteriorating tissues and organs represent a major medical problem with profound effects on the health status and quality of life of numerous patients. Current approaches to restore normal function of affected tissues and organs rely on pharmacotherapy, transplantation and implantation of medical devices, which have limited applicability, fail to provide optimal clinical solutions and can result in life-threatening outcomes [1]. The burden of tissue and organ deficiencies therefore poses a critical need for the development of new therapeutic solutions that can effectively and safely restore normal tissue structure and function.

Advances in stem cell isolation, characterization and biology during the past decades have fueled a revolution in tissue replacement therapies by providing the possibility to generate relevant cell types for a multitude of clinical applications. Beyond the use of somatic stem cells, which have been tested in the clinic and proven to have potential for regenerative medicine, great excitement is currently surrounding the field of somatic cell reprogramming to pluripotency, which allows

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the generation of hiPSCs with unlimited proliferation potential and ability to differentiate toward any specialized cells constituting the human body. In addition, Tachibama *et al*. recently reported the derivation of human embryonic stem cells (hESCs) [2] via transfer of adult fibroblast nuclei into enucleated oocytes, opening another possibility to derive patient-matched therapeutic cells [3]. Nevertheless, reprogramming of somatic cells to pluripotency by forced expression of specific transcription factors [4-6] has gained lots of attention, as it circumvents the ethical concerns associated with pluripotent stem cell derivation from human blastocysts, and provides an efficient way to generate patient-specific cells for applications in regenerative medicine. Although not identical, hiPSCs and hESCs have been demonstrated to be functionally equivalent [7] and display similar potential for advanced cell-based therapies. Future developments may even enable the generation of therapeutic cells via transdifferentiation of somatic cells toward desired phenotypes, bypassing iPSC formation.

Bone deficiencies associated with congenital and traumatic defects, or resulting from degenerative disorders and infectious diseases affect an increasing number of patients worldwide, with a combined annual US market for bone repair and regeneration therapies projected to reach 3.5 billion by 2017 [8]. Besides the implantation of alloplastic materials and transplantation of autogenous and allogeneic bone grafts, attempts to enhance repair of skeletal defects using stem/progenitor cells isolated from adult tissues alone or combined with different scaffolding materials and bone-inducing factors have been reported, and have demonstrated feasibility of a bone tissue engineering approach in specific clinical settings [9-14]. Nevertheless, no cases of large skeletal reconstructions in humans using engineered bone tissues matured *ex vivo* exist hitherto, but promising results are rapidly emerging from experimental and preclinical studies.

Engineering bone substitutes using pluripotent stem cells represents an appealing therapeutic strategy for structural and functional reconstructions of complex skeletal defects. In particular, the ability to derive autologous osteogenic, vascular and other cell lineages constituting healthy bone from hiPSCs for any patient in virtually unlimited numbers represents an unprecedented therapeutic resource [15-17]. Large numbers (100 millions to billions) of therapeutic cells are needed to reconstruct bone defects, and these might not be available from adult tissues for all the patients as recently reviewed [18]. The exact properties of progenitor cells derived from hiPSCs and hESCs in relation to adult skeletal progenitors/stem cells are currently under intense investigation. Encouragingly, some studies suggest that pluripotent stem cell-derived progenitors phenotypically resemble adult mesenchymal stem/stromal populations isolated from the bone marrow, which are commonly used for clinical tissue engineering of bone substitutes. In addition, these progenitors exhibit enhanced proliferation and functional potential compared to bone marrow cells [17,19,20], therefore representing a promising cell source for future clinical translation.

Attempts to grow several centimeter large bone substitutes *ex vivo* from adult stem cells have recently been successful by designing custom-shape scaffolds and matching anatomical perfusion bioreactors to support three-dimensional tissue formation [21]. We recently demonstrated that functional bone substitutes could similarly be engineered from pluripotent stem cell-derived progenitors by applying a scaffold – perfusion bioreactor culture model of bone development [22,23]. Pluripotency presents a challenge for directing specific lineages with high efficiency, and achieving pure, well-defined populations of therapeutic cells/tissues, but also offers the unprecedented possibility to recapitulate *ex vivo* the early phases of bone development. Our approach was to use a stepwise differentiation protocol, in which pluripotent stem cells were first induced toward the mesenchymal lineage and then coaxed to become osteogenic cells and form bone-like tissue. We induced several hiPSC lines with different genetic backgrounds, including those that did not harbor any exogenous genetic material. Osteogenic cells derived from hESC and hiPSC were then interfaced with mechanically compliant biomaterials – decellularized bovine trabecular bone scaffolds, and cultured under specific regime in perfusion bioreactors, previously optimized with adult stem cells [24]. Perfusion bioreactor culture nurtures the development of bone tissue by providing an appropriate physiological environment with stimulatory biophysical and biochemical signals. Cultivation of hESC/hiPSC-osteogenic progenitors in such conditions supported the formation of ~ 0.5 cm large, homogenous bone tissue, representing the first step toward the construction of clinical-size bone substitutes from hiPSCs [23].

As discussed, maturation of pluripotent cells into bone-forming cells and the depletion of undifferentiated cells with potential to form teratomas are fundamental to ensure the safety of cultured bone substitutes. We explored the molecular changes during the *ex vivo* formation of bone tissue from different hiPSC-mesenchymal progenitor lines, and found common alterations in the expression of several important genes involved in molecular pathways controlling cell proliferation and tissue maturation. A strong repression in the expression of CDC, POL, GINS, MCM, and CENP genes and associated factors, recognized to play a role in proliferation and oncogenesis, was concomitant with the upregulation of several genes involved in osteogenic differentiation and bone formation, including the MMP2, STAT3 and TGFB3 pathways. Common gene responses between the studied cell lines suggest that the selected culture conditions could be applied generally for engineering stable, mature bone grafts from hiPSCs of different patients. In fact, the *in vivo* implantation of lab-engineered bone substitutes up to 12 weeks did neither result in the formation of areas of uncontrolled proliferation nor differentiation toward other lineages. In the future, long-term preclinical studies are required to assess the phenotypic

stability of the engineered substitutes and the fate of transplanted cells, before they can be used to treat human patients. Emerging strategies for the identification and selective removal of residual undifferentiated pluripotent cells are also expected to contribute to the development of bone grafts for safe therapeutic use [25,26].

Another important aspect of the potential clinical use of lab-engineered bone substitutes from pluripotent stem cells deals with their abilities to integrate with the host tissue after transplantation and to support bone formation during defect regeneration. In essence, the engineered bone substitutes must integrate with the host tissue, be remodeled and orchestrate healthy and functional regeneration of the damaged skeletal region. Strategies for vascularization of engineered bone substitutes need to be developed to ensure graft survival and proper healing during therapeutic reconstruction. Previous works have demonstrated the fundamental role of the vascular compartment in promoting graft integration and supporting functional tissue regeneration [27]. The high regeneration potential of hiPSC opens the possibility to generate unlimited amount of vascular progenitors that could be used to engineer vascularized patient-matched bone substitutes to further enhance the healing of skeletal defects. In addition, development of new strategies to engineer biocompatible biomaterial scaffolds of any shape and size, and of appropriate chemical, architectural and mechanical properties to support functional regeneration, in a reproducible way, is crucial to meet the needs of specific clinical cases. Importantly, optimal biomaterial scaffolds should exhibit a resorption rate matching the rate of new bone formation, thus not compromising the mechanical properties of the transplanted tissue substitute during the healing period. Not least, engineering large and geometrically complex viable bone substitutes also requires designing customized bioreactors with anatomically shaped chambers, to accommodate the cell/scaffold constructs in a press-fit fashion [21]. Alternatively, large quantities of minute cell/scaffold constructs could be cultured in packed bed/column bioreactors, and then assembled for clinical reconstruction of larger skeletal defects [20]. However, the use of bioreactors for clinical applications largely depends on whether their beneficial effects on *in vitro* bone development and therapeutic efficacy of engineered bone will justify the cost. A detailed cost-benefit analysis of bone tissue engineering bioreactors was recently reported by Salter *et al*. [28].

Equally important, the immune properties of engineered bone substitutes must be studied to ensure immune tolerability for clinical applications. After transplantation, the responses of inflammatory and immune cells could compromise the survival and healing properties of the transplanted bone substitutes. Recent studies in mice showed that tissue-specific cells derived from pluripotent stem cells were not immunogenic in syngeneic conditions [29], opening the possibility that pluripotent stem cell-based products could be employed to treat skeletal defects in a personalized fashion. However, proper *ex vivo* models of immune response must be developed to investigate the immune properties of bone substitutes engineered from human cells, in order to accurately predict the clinical outcomes for safe therapeutic procedures.

Thus far, the studies suggest large variation in the efficiencies to form specific cell lineages from pluripotent stem cell lines of different individuals [30,31]. In hiPSCs, the problem of variability resulting from different genetic backgrounds and culture conditions is compounded by the differences in nuclear reprogramming protocols. It is therefore paramount to develop standardized procedures for the derivation, culture and differentiation of hiPSCs, and to assess the regenerative potential of different cellular subpopulations via their isolation/selection at different stages of culture, to most effectively engineer functional tissues for replacement therapies.

Additional barriers toward the clinical application of hiPSC-engineered bone (as well as other tissues) pertain to the development of adequate manufacturing and clinical procedures that meet international regulatory requirements and allow the generation of safe and effective tissue-engineered products for use in humans. The ability to reprogram cells using nonintegrating vectors, development of xeno-free culture conditions, storage of cells/engineered tissues, assessment of karyotype stability and cell phenotype after protracted cell expansion, seeding on biomaterials and culture in bioreactors, prevention of microbial contamination using environmentally controlled areas (clean rooms), process standardization and validation and quality control testing are among some of the most important challenges that must be addressed before personalized lab-made bone substitutes can be used to treat human patients [32]. Similarly important is the choice of valid animal models to conduct preclinical studies as a guide to efficacy and safety before tissue-engineered products are used to treat humans. Not least, compliance with regulatory policies and harmonization in the interpretation and application of technical guidelines and requirements for tissue-engineered products would enable companies to develop adequate marketing strategies, and therefore facilitate access and clinical translation of engineered bone for replacement therapies [33].

Production time and cost of customized bone substitutes will also play a role in enabling the large-scale production of replacement tissue for personalized clinical applications. It is evident that with the advances in bioprinting technologies, the potential to combine the bioprinting process with other biofabrication and rapid prototyping methods and the ability to automate each manufacturing step using closed-system bioreactors for real-time monitoring and controlling of tissue maturation would increase product consistency, and facilitate the transition from a research scale to a clinically applicable mass production of therapeutically safe and effective replacement tissues, in a reproducible, GMP-compliant and economically affordable fashion [34-36].

In conclusion, application of GMP-compliant state-of-the art/automated cell culture techniques in combination with advanced tissue engineering strategies in compliance with regulatory policies will probably enable the use of bone-engineered products for personal skeletal reconstruction in the future. Make no bones about it! Cells could indeed be reprogrammed to grow replacement bones.

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Declaration of interest

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