

Perspective on the Evolution of Cell-Based Bone Tissue Engineering Strategies

M. Jakob F. Saxer C. Scotti S. Schreiner P. Studer A. Scherberich
M. Heberer I. Martin

Departments of Surgery and Biomedicine, University Hospital Basel, Basel, Switzerland

Key Words

Bioreactor systems • Bone regeneration • Cell-based bone graft substitutes • Developmental engineering • Intraoperative graft engineering • Stromal vascular fraction • Tissue engineering

Abstract

Despite the compelling clinical needs in enhancing bone regeneration and the potential offered by the field of tissue engineering, the adoption of cell-based bone graft substitutes in clinical practice is limited to date. In fact, no study has yet convincingly demonstrated reproducible clinical performance of tissue-engineered implants and at least equivalent cost-effectiveness compared to the current treatment standards. Here, we propose and discuss how tissue engineering strategies could be evolved towards more efficient solutions, depicting three different experimental paradigms: (i) bioreactor-based production; (ii) intraoperative manufacturing, and (iii) developmental engineering. The described approaches reflect the need to streamline graft manufacturing processes while maintaining the potency of osteoprogenitors and recapitulating the sequence of biological steps occurring during bone development, including vascularization. The need to combine the assessment of efficacy of the different strategies with the understanding of their mechanisms of action in the target regenerative processes is highlighted. This will be crucial to identify the nec-

essary and sufficient set of signals that need to be delivered at the injury or defect site and should thus form the basis to define release criteria for reproducibly effective engineered bone graft substitutes.

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Introduction

Unlike other tissues, bones possess the capacity for regeneration, remodeling and repair in response to injury in adults and in children alike [1, 2]. The most common cause of bone regeneration is fracture healing, which occurs through intramembranous and/or endochondral ossification. The majority of bone injuries and fractures can heal without formation of scar tissue within a certain timeframe. The healing process restores the bone with its original physicochemical properties leading to newly formed tissue indistinguishable from the adjacent healthy bone. However, in some cases, bone regeneration is compromised. Generally, the risk is reported to be around 5–10%, reaching up to 40% in patients with typical risk factors like smoking or diabetes [3–5]. Several biomechanical and biological factors are associated with the development of a delayed union or non-union. This occurs when the required bone regeneration exceeds the natural potential for self-healing, as in large bone defects occurring after trauma, infection, tumor resection [6] or skel-

etal abnormalities, or when the regenerative process is compromised due to various reasons, e.g. avascular necrosis, osteoporosis and other local or systemic pathologies. In all these cases, the supply of additional bone grafts is required for repair.

The current clinical gold standard to treat bone defects, e.g. in orthopedic and trauma surgery, consists in the transplantation of autologous bone grafts used as osteogenic substitutes. Typical examples of autologous bone grafts are structural grafts like the corticocancellous iliac crest or vascularized fibular grafts, and non-structural cancellous bone grafts that can be harvested either locally (e.g. in spinal or hip replacement surgery) or from the iliac crest. Unfortunately, all these techniques are associated with the limited availability of autologous material [7, 8] and considerable morbidity at the donor site, such as additional blood loss, which might reach significance in patients with co-morbidities, chronic pain, iliac wing fractures or infections [9–13]. Allo- or xenografts are available, but despite the development of screening methods for contamination and a production procedure following good manufacturing practices, concerns about pathogen transmission and immunogenicity of such grafts still remain [14]. Synthetic and natural polymeric scaffolds can potentially avoid such risks but they lack most of the actual osteoinductive or osteogenic properties of autologous bone chips [15]. Another approach is the use of recombinant growth and differentiation factors, e.g. recombinant human bone morphogenetic protein-2 (BMP-2), which are able to induce osteogenesis by resident progenitor cells, but which have been associated with aberrant bone formation [16], neurotoxicity [17], cancer development [18], high costs and a short half-life, rendering fine-tuning of the local application difficult [19, 20].

Tissue engineering has emerged as an attractive alternative, possibly improving the results of current techniques. Engineering of bone graft substitutes is typically based on bone marrow-derived autologous osteoprogenitors (mesenchymal stromal cells, MSC) expanded in plastic dishes and combined with osteoconductive scaffolds in order to generate living grafts, possibly analogues of autologous bone. Despite validation of this approach in several small- and large-size animal models, as well as in a few clinical cases [21], its routine clinical implementation is challenging, and very few studies were able to demonstrate its efficacy in bone matrix production, either because it did not occur or because no biopsy to document it could be analyzed [22–25]. The lack of demonstrated reproducibility and cost-effectiveness is reflected in the

limited number of clinical cases of bone defects treated using cell-based grafts [26].

The next sections of this paper describe and discuss three alternative approaches currently pursued by the authors to evolve classical tissue engineering paradigms towards possibly more effective products: (i) bioreactor-based manufacture, aimed at the development of automated and controlled bone graft production; (ii) intraoperative graft manufacture, which does not require a graft production phase, since the graft is assembled during the surgical procedure, and (iii) developmental engineering, which relies on the biological rationale of recapitulating normal bone regeneration (fig. 1).

Bioreactor-Based Production

The issues of manufacturing, reproducibility, quality and costs have been addressed in other biotechnology sectors by the introduction of automated and controlled production environments, typically referred to as ‘bioreactors’. Bioreactors have been proposed as a possible tool to develop cell-based therapeutic approaches towards a broader clinical adoption [27]. The possibility of controlled culture conditions would improve the regulation of the bioprocess and minimize graft product variability. Monitoring systems would be instrumental to implement traceability and safety compliance, whereas process automation would target standardization, scaling-up and possibly cost-effectiveness. The latter represents the critical and ultimate target among the various criteria for reimbursement policies by healthcare systems.

The industrialization within bioreactors of a previously established and validated manufacturing process, however, is expected to require a revalidation of the resulting products, including new preclinical and clinical tests, and likely resulting in robotic systems which merely replicate the sometimes ineffective manual procedures. The challenge is thus to adopt effective automation within bioreactors early in the development of a clinical program. This strategy requires a higher investment upfront but also a streamlined pathway to the clinical use and possibly the introduction of more effective manufacturing processes [28].

As an example of the considerations above in the specific context of bone tissue engineering, we have demonstrated that the use of perfusion-based bioreactor systems for the seeding and culture of MSC within porous 3D scaffolds allows to entirely bypass the phase of cell expansion in monolayers, typically associated with a progres-

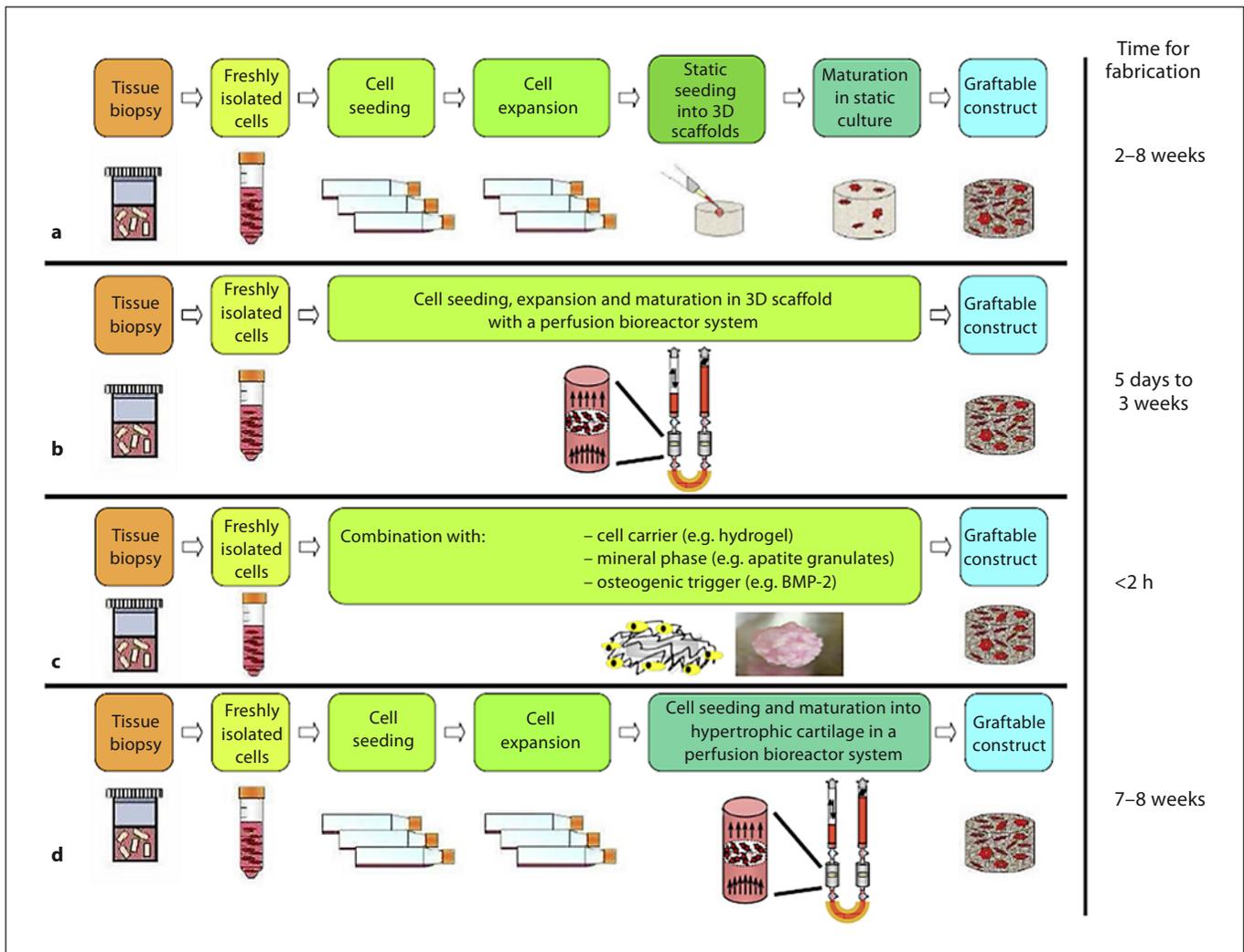


Fig. 1. Schematic drawing depicting the canonical tissue engineering approach (a); the streamlined bioreactor-based approach (b); the intraoperative approach (c) and the developmental engineering approach (d).

sive loss of osteogenic differentiation capacity [29]. The possibility to avoid MSC expansion in monolayers on stiff plastic not only streamlined the manufacturing process but also supported a superior maintenance of progenitor properties in MSC expanded under 3D perfusion culture [30]. In particular, probably due to the interaction with an environment better resembling the native stromal tissue, 3D-expanded MSC retained a higher clonogenicity (i.e. the capacity to form clonal strains under low-density plating conditions) compared to the state-of-the-art control established using cells from the same donors [30]. Moreover, grafts generated under 3D perfusion and implanted ectopically in nude mice generated higher and

more spatially uniform amounts of bone tissue, with superior reproducibility across different donors.

Cells from the stromal vascular fraction (SVF) of adipose tissue were also tested in the perfusion bioreactor system for the possibility of a streamlined manufacturing of osteogenic grafts. One advantage in using SVF as opposed to bone marrow cells lies in the superior amount of mesenchymal progenitors which can be available under minimally invasive conditions (1 ml of a lipoaspirate contains about 100-fold higher numbers of clonogenic mesenchymal progenitors than 1 ml of bone marrow). The large availability of cells allowed the generation of osteogenic grafts under perfusion culture in 5 days as opposed

to the typical 3 weeks necessary for bone marrow-derived MSC [31]. One additional important advantage of SVF cells consists in the presence (among the heterogeneous mix of phenotypes) of endothelial lineage cells. These cells, which are typically lost in monolayer cultures but are preserved during 3D perfusion cultivation, were shown to contribute to the formation of blood vessels rapidly anastomosing with those from the recipient site [31]. The resulting process of inosculation supported an enhanced engraftment of large grafts (1 cm in diameter/1-cm-thick cylinders) and a deeper formation of bone osicles compared to conditions excluding endothelial cells [32].

The results presented above urge for the development of bioreactor systems compatible with good manufacturing practices, possibly relying on the principle of 3D perfusion culture and allowing for clinical tests of the innovative strategies and streamlined manufacturing processes. The task is not trivial, as it requires the critical consideration of scientific, technical, regulatory and commercial challenges [28, 33]. Among these are (i) the identification and validation of online quality control tests which may be predictive of functional performance, (ii) the qualification of in-process monitoring and control systems, (iii) the compliance to yet ambiguous safety and regulatory guidelines, (iv) the development of friendly interfaces between the complex manufacturing system and the clinicians, and (v) the need of large upfront investments combined with the uncertainties of the markets, reimbursement policies and overall clinical adoption.

Intraoperative Manufacturing

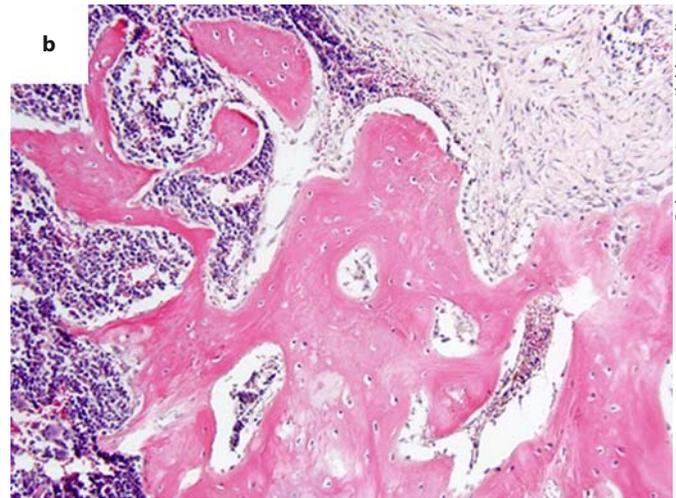
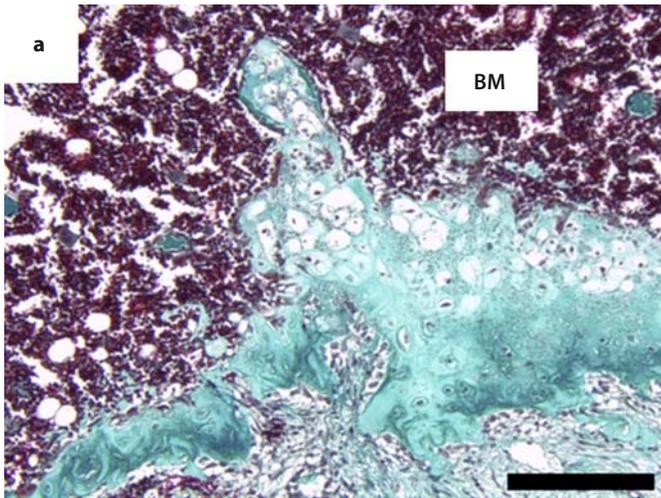
The intraoperative approach derives from the concept of cell and tissue transplantation. It exploits the fact that the engineering of an autologous bone graft can become more practical and less costly if the manufacturing process, including isolation of autologous cells, generation of the graft and its implantation in the bone defect, can be combined with the procedure intended to surgically treat the bone defect itself. It allows eliminating the resource- and time-consuming *in vitro* expansion phase; instead, the patient's body is used as an *in vivo* bioreactor system to support the expansion and the differentiation of cells freshly harvested and directly transplanted into the bone defect.

The critical point in this scenario is the challenging task of identifying a suitable autologous cell source. These cells have to possess an intrinsic osteogenic capacity in

vivo without prior culture or osteoinduction and need to be available in sufficient quantities directly upon isolation. The use of freshly isolated bone marrow-derived MSC, which were possibly harvested using a reamer-irrigator-aspirator [34, 35], concentrated by immunoselection [36] or modified genetically [24], was proposed in such bone repair approaches. Despite the data collected so far in animal models about bone formation by implanted cells, a major limitation remains: clonogenic MSCs (referred to as fibroblastic colony-forming units), which are considered the functional cells establishing a progeny and responsible for bone tissue formation *in vivo* [37], are found at a very low frequency among bone marrow cells (1 in 10,000 nucleated cells) [30]. Therefore, the reproducibility in the collection of sufficient numbers of MSC from various patients which are effective for direct grafting still requires confirmation.

In contrast, the freshly isolated SVF of human adipose tissue might represent a better cell source for a one-step surgical procedure thanks to an up to 100-fold higher number of fibroblastic colony-forming units per volume of tissue sample compared to human bone marrow [31, 38]. Moreover, adipose tissue is available in large quantities and its harvest is associated with low morbidity [39]. Two studies [40, 41] tested the impact of SVF cells implanted intraoperatively in a goat spinal fusion model and demonstrated a superior bone healing with SVF cells than in cell-free scaffolds. Overall, healing was comparable to that achieved with the gold standard of autologous bone grafting plus titanium cage. However, this orthotopic model could not investigate the direct osteogenic contribution of the cells implanted. To achieve that, our group tested human SVF cells seeded onto porous hydroxyapatite scaffolds using fibrin gel in an ectopic nude mouse model. The study that freshly isolated SVF cells can ectopically generate osteoid structures but not frank bone tissue [42]. More recent results indicate that low doses of BMP-2 are sufficient to instruct unmanipulated SVF to form bone tissue even at an ectopic site [unpubl. data], thus highlighting the importance of local growth factor concentrations at an orthotopic site.

Interestingly, SVF cells not only include progenitors capable of osteogenesis, but also constitute a relevant reservoir of vascular progenitors [43]. We showed that neither mesenchymal CD34+CD31- nor endothelial CD34+CD31+ cells from SVF were able to form vascular structures alone, but that their combination inside SVF resulted in a robust assembly of vascular structure *in vitro* [32]. A study from our group confirmed that the vasculogenic potential of SVF cells does not require any *in vitro* pre-



Color version available online

Fig. 2. **a** Masson's trichrome staining of hypertrophic cartilage tissue undergoing remodeling. BM = Bone marrow. Scale bar = 200 μ m. **b** H&E staining of bone tissue obtained through endochondral ossification with a developmental engineering approach.

conditioning of SVF cells, but reproducibly and robustly takes place upon implantation of SVF cells *in vivo* [42].

In conclusion, the intraoperative generation of osteogenic grafts with intrinsic vasculogenic properties using SVF cells has been proved to be feasible. A safe clinical translation of this paradigm now requires further validation in animal models (e.g. with orthotopic implantation and/or in immunocompetent animals) and the definition of quality control markers of the cells to predict their clinical performance prior to their implantation.

Developmental Engineering

A possible hint to identify an alternative improvement in current bone tissue engineering approaches has grown out of developmental biology studies. As a matter of fact, most tissue engineering strategies for bone regeneration resemble intramembranous ossification, i.e. the direct osteogenic differentiation of mesenchymal progenitors using a mineralized surface as 'priming' substrate [25], while most bones develop by endochondral ossification, which consists of remodeling a hypertrophic cartilaginous template into bone. Indeed, upon *in vivo* implantation of *in vitro* generated hypertrophic cartilage templates, it is now possible to recapitulate and finalize the endochondral route [44–46]. This very promising approach, initially demonstrated with embryonic stem cells [45], has recently been duplicated with the more clinically

compliant human MSC [44] (fig. 2). Most importantly, the underlying morphogenetic process was structurally and molecularly similar to the temporal and spatial progression of bone development in embryos. Overall, these studies paved the way for the design of clinically relevant strategies for bone regeneration and gave a proof of concept that it is possible to recapitulate embryonic processes with human MSC in order to engineer functional tissue. However, besides being more 'biomimetic', the endochondral route presents several practical advantages compared to the classic intramembranous ossification-like approaches, such as (i) higher vasculogenic potential thanks to the production of vascular endothelial growth factor by hypertrophic chondrocytes; (ii) osteoinductivity thanks to the production of BMPs, and (iii) higher chances of survival in the hypoxic environment after implantation, because cartilage cells are equipped with poor environmental conditions for survival [46].

The term 'developmental engineering' has recently been introduced into the scientific community by Lenas et al. [47, 48] for this innovative approach to tissue regeneration. The application of typical concepts of developmental biology represents a paradigm change from the original, simplistic tissue engineering strategy of 3D cell growth on a scaffold [47]. This new paradigm consists in engineering 'processes' recapitulating embryonic events rather than 'tissues'. Recapitulating *in vitro* the complexity of tissues and organ development may be a nearly impossible challenge. However, by activating the right de-

developmental mechanism, it is possible to trigger a self-maintained and self-regulated process thanks to the principle of 'path dependence', namely the dependency of each developmental stage from the previous one [47]. In a bone-developmental engineering perspective, this means that the engineered graft would be a 'committed cartilage template', duplicating the critical stage of bone development by inducing the processes of vasculogenesis, remodeling and ultimately bone formation (fig. 2).

Several issues remain to be addressed before a clinical exploitation of this approach can be planned. Especially the long in vitro culture makes a clinical translation difficult. However, this limitation can be overcome by the use of decellularized hypertrophic cartilage templates: these tissues may be engineered with an extracellular matrix rich in growth factors, which may be sufficient to trigger the remodeling of the tissue by host osteoclasts and formation of bone over the remodeled template by host osteoprogenitors. Experimental studies are currently exploring this strategy which can possibly lead to off-the-shelf bone substitutes inducing bone regeneration by priming endochondral ossification.

Conclusion

In this review, we presented recent advances in bone tissue engineering focusing on three different mainstays of bone-regenerative strategies: (i) bioreactor-based tissue manufacture; (ii) intraoperative strategies, and (iii)

developmental engineering (fig. 1). However, many other experimental strategies are currently explored in parallel and none of them seems at the moment superior to the others and suitable to regenerate bone in the different clinical scenarios. On all accounts, different clinical indications (defect size, patient type, weight-bearing vs. non weight-bearing bones, trabecular bone vs. cortical bone, or etiology of the bone defect) may require different types of grafts, with different modes of manufacturing and action. Therefore, all these approaches are promising in principle and will help in selecting the bone-regenerative strategies of the future.

Research will have to be based on and develop from a more fundamental understanding of the biological processes during tissue regeneration in order to push the transition of the field from tissue engineering (implantation of ex vivo manufactured tissues) to regenerative medicine (inducing self-regeneration processes). From a clinical standpoint, the possibility to effectively embed the master signals regulating tissue regeneration in a bioactive, off-the-shelf product seems to be the most attractive approach at present. For this reason, the definition of requirements to induce regeneration (e.g. the need of living cells; extracellular matrix components/specific peptides; cytokines, or gradients) will be of paramount importance to foster this transition. Then all this knowledge has to be applied in immunocompetent models which take inflammatory signals into account, which are now widely recognized to regulate and control bone regeneration processes.

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