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Developmental biology-inspired tissue engineering by combining organoids and 3D bioprinting

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Very few tissue-engineered constructs could achieve the desired results in human clinical trials. The main reason is their inability to recapitulate the cellular conformation, biological, and mechanical functions of the native tissue. Here, we highlight the future avenues of tissue regeneration combining developmental biology, organoids, and 3D bioprinting. A deep mechanistic insight into the embryonic level and recapitulating them would be the most promising strategy in next-generation tissue engineering. Rather than focusing on the adult tissue features, the latest developmental re-engineering strategies replicate the developmental phases of tissue development. Integrating developmental re-engineering with 3D bioprinting can regulate several signaling pathways. This would further help to fabricate mini-organ constructs for transplantation or *in vitro* screening of drugs using an organ-on-a-chip platform.

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Introduction

Organogenesis, one of the vital phases during embryo development, is dominated by interactions between cell-matrix proteins, cell migration, dynamics of transcription factors, and signaling pathways [1,2]. During embryonic development, cellular assembly occurs interactively with the surrounding matrix, forming the 'organ germ' [3]. To achieve such functionality, tissue engineers have strived to recapitulate intricate developmental biology signaling pathways and morphogenesis over the last few decades. But only a handful of tissue-engineered constructs could meet moderate accomplishment in human clinical trials. Currently, tissue engineering approaches are on the verge of experiencing a paradigm shift to recapitulate the initial stages of *in vivo* organogenesis instead of targeting the development of an engineered adult tissue/organ [4]. This morphogenesis is instrumented by a synchronized transmission of genetic machinery involving chemical, electrical, and mechanical stimuli [5]. These can affect cell proliferation, migration, survival, and differentiation [6,7,8]. Adopting 3-dimensional (3D) bioprinting strategies using organoids may open new possibilities to achieve patient-specific, defect-site-specific constructs. Moreover, 3D bioprinting provides spatiotemporal authority concerning the positioning of specific protein components and the orientation of the cells in the construct. During embryogenesis, 'organ germs' morphogenesis is governed through neighboring tissue interactions that provide definite microenvironments [9]. The process by which cells self-assemble during specific differentiation in the context of signals obtained from cell–cell adhesion, cellular morphology, the gradient of O_2–CO_2, and the surrounding microenvironment can be replicated by forming organoids following the developmental biology route. A crucial stage in this technology is the generation of self-organizing multicellular modules, however, it is challenging to carefully combine smaller modules into bigger tissue units [10]. Therefore, the functionalization of organ tissues can be greatly aided by the cooperative development of bioprinting and cellular self-organization technologies. For pluripotent stem cells (PSCs) or adipose-derived stem cells, bioprinting can pertain to a distinct spatial architecture design, such as the architecture of the native organs, so that the specific structure of organoids can be produced rapidly, highly precisely, and with high throughput. As a result, they can generate organoids faster and with better self-organization than single cells. Additionally, stem cell suspensions can self-organize into structures of millimeter scale with relatively moderate complexity. These structures can then be printed into more complex tissues and organs using the resulting geometry to guide organoid creation [2]. Hence, there is an essential requirement for combinatorial bioengineering approaches that define the break between organoid-based tissue culture models developed *in vitro* and *in vivo* morphogenesis. Such avenues could probably lead to a complex mechanistic understanding of developmental organogenesis, thereby widening modern avenues such as drug discovery and drug testing.

Toward this end, we discuss futuristic tissue-regeneration strategies by amalgamating the ideas of developmental biology, organoid formation, 3D bioprinting, and tailor-made microenvironments offered by specific biomaterials. Essentially, these perceptions would improve tissue engineering approaches only when we apprehend how these perspectives can regulate the involvement of a wide range of transcription factors and signaling pathways. This would ultimately help to develop an anatomically appropriate *in vitro* tissue model, thus bridging the gap between the cellular and organ levels.

Organ-regeneration strategies

Scaffold and bioreactor-based tissue engineering

The scaffold-cell-bioreactor system aims to recapitulate the *in vivo* microenvironment. Scaffolds act as a structural template for cell attachment, supplying chemical, biophysical, and architectural cues to the cells, thus modulating the gene expression and extracellular matrix (ECM) deposition. Their bioactivity can be augmented by making several amendments, such as adhering to cell-adhesion ligands either covalently or noncovalently to improve cell-matrix interactions and the inclusion of ancillary structural motifs to intensify degradation and tissue modeling [11].

Bioreactors can be combined with scaffolds to regulate culture conditions. For example, Hirt et al. [12•] noticed that perfused 3D culture obtained from human tumor cell lines gave rise to the fast generation of a tissue-like structure designated by a uniform cellular organization and enhanced cellular yields compared with the static 3D culture system. Interestingly, proliferation rates, apoptosis, and reaction to 5-fluorouracil in perfused 3D culture replicated those found in xenografts of similar cell types in immunodeficient mice [•12]. This commends the significance of perfusion flow while culturing tumor cells in 3D to effectively replicate the functional characteristics *in vivo* and ultimately assess the anticancer compounds. Despite these fascinating results, these strategies cannot replicate the complex anatomical features of adult tissues or many crucial aspects of organogenesis.

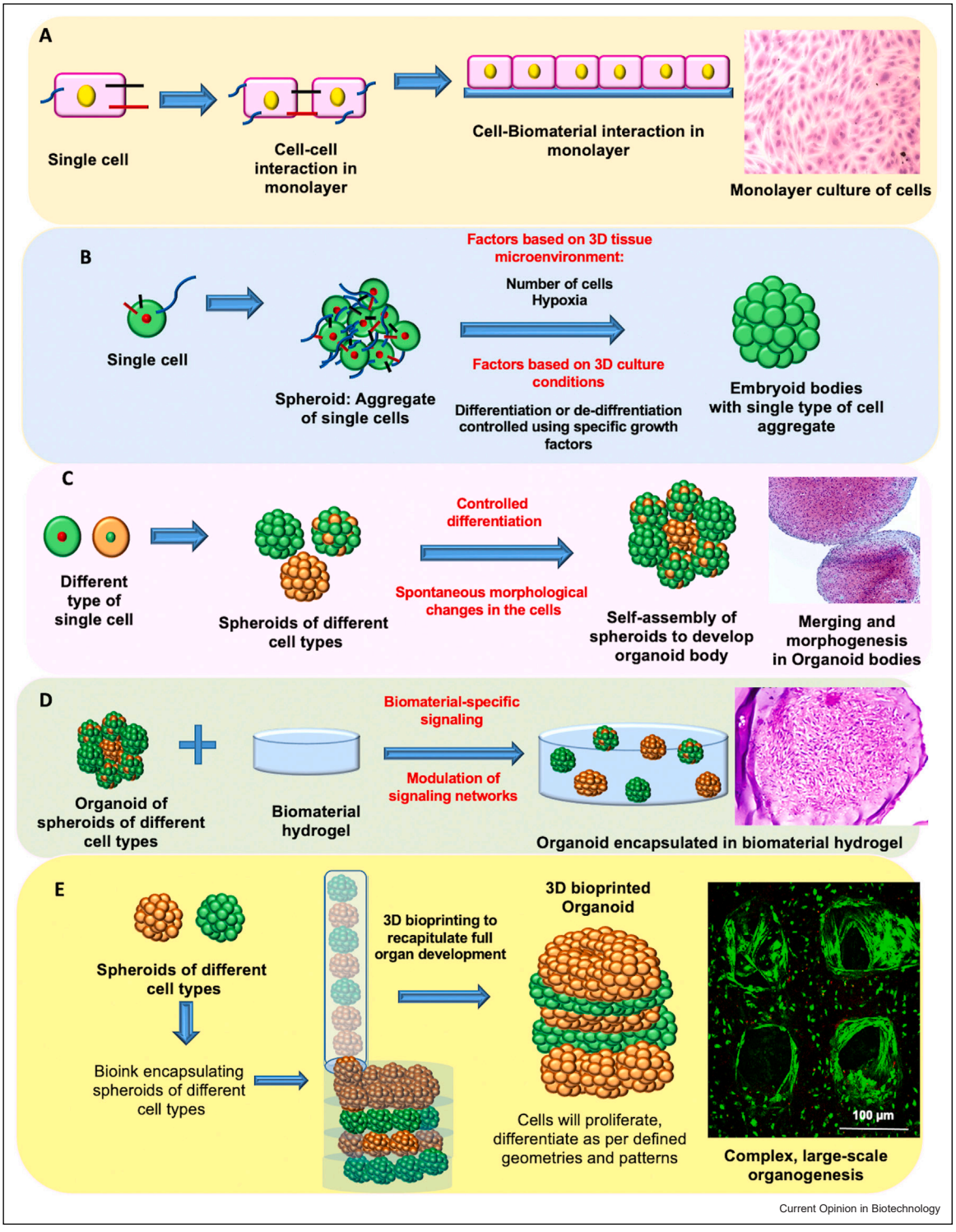
Strategies for the organoid formation

Organoids are aggregates of differentiating progenitor cells undergoing self-organization, replicating intrinsic cellular organization in a target tissue, cell–cell, and cell–ECM interactions. Previously, significant attempts have been made to induce differentiation of human embryonic stem cells or induced pluripotent stem cells (iPSCs) cultured as embryoid bodies [13]. For example, the defined number of stem cells (> 1000–1 million cells per well) were aggregated by various spheroid-formation techniques, such as microwells and hanging drops, and induced to differentiate into distinct cell types. Differentiated cells had varied morphologies with increased culture time, resulting in disorganized cell mass formation [14]. In culture conditions involving 3D organoids and growth factors, tissue-specific morphogens and stem cells (e.g. iPSCs) are embedded under certain conditions and experience differentiation and morphogenesis, giving rise to an organ-specific tissue. These organoids have tissue morphology and functionality similar to their *in vivo* counterparts [15].

Organoids can develop from the reaggregation of embryoid bodies, accompanied by cellular self-organization within the aggregate, without extrinsic scaffolding and mechanics [16]. An intriguing question is how to place organoids, where the agglomerated progenitor cells metamorphosize and self-assemble into a 3D anatomy representative of early organogenesis [17]. Several factors are crucial in driving effective organoid formation, such as cell–cell solid interactions brought about by E-cadherin, a cell-adhesion molecule, actin polymerization, and other proteins that aid cell linking [18]. Furthermore, during normal development, the morphogen signaling gradient is critical for placing and patterning complete embryos and tissues, resulting in the compartmentalization of the full-grown organ (Figure 1). Thus, the pursuit of modalities encompassing an amalgam of cytokines intended to produce such embryo development based on spatiotemporal morphogen signaling is the demand for present-day developmental biology-inspired tissue engineering.

Researchers are currently exploring the efficacy of Matrigel hydrogels, in which stem cells are encapsulated to form organoids spontaneously. However, Matrigel is a heterogeneous mixture of solubilized proteins of the basement membrane derived from mouse sarcomas, which carries the risk of pathogen and immunogen transfer, and suffers from batch-to-batch variation [19,20]. Attempts are being made to explore alternative biomaterials, for example, Lindborg et al. reported a hyaluronan–chitosan hydrogel blend supporting the formation of cerebral organoids exhibiting the generation of a neural rosette and a neural tube-like structure, which illustrated early carcinogenesis [21]. Meinhardt et al. used a pure polyethylene glycol (PEG) hydrogel containing single-mouse ESCs that compelled neural tube organoids to morphogenize rapidly, albeit at a notably lower rate than Matrigel hydrogels [22]. Gjorevski *et al.* reported that culturing intestinal stem cells in Matrigel developed lumen-containing colonies, but culturing cells in PEG gel failed [23]. However, chemically modifying the PEG gel improved cellular proliferation, indicating that manipulating chemical composition and matrix compliance plays a crucial role in tissue-specific directing morphogenesis. Hence, it would be interesting to investigate combining strategies involving organoid formation and response to biomaterials.

Figure 1



(a) Instead of a disordered cellular organization on the 2D/scaffold, the designed biomaterial can impart biochemical and mechanical feedback to individual cells to achieve apical–basal polarity and homotypic cell adhesion. **(b)** Uniform cellular spheroids can generate a better understanding of how precise cell numbers and 3D microenvironment can alter cell differentiation, expression of specific proteins**. (c)** Self-assembly of organoids and controlled differentiation can trigger cascades of endogenous cellular patterning during developmental biology. **(d)** Material-induced cues to provide chemical cues, mechanical cues to induce specific cellular transduction, activate/silencing-specific signaling pathways, and controlled hypoxia, enabling matrix remodeling either locally or globally. **(e)** Finally, developmental biology-motivated schematic with 3D bioprinting with organoids of different cell types would lead to 3D bioprinted construct. Live–dead assay depicted substantial migration of the encapsulated cells, leading to complete large-scale organogenesis.

Our group developed a human hair follicular organoid model for the first time [24]. Here, we developed a cellular spheroid comprising human dermal papilla cells, followed by coating the spheroid with silk fibroin–gelatin (SF–G) hydrogel, which in turn was embedded with two other cells (human hair follicle stem cells and keratinocytes). The SF–G hydrogel plays a pivotal role in cell proliferation by providing the required microenvironmental features. Second, it repressed bone morphogenetic protein (BMP) signaling and displayed a constitutively upregulated expression of β-catenin, these features have been inclusively noticed *in vivo* and are related to enhanced hair follicular regeneration.

In the last few years, decellularized ECM hydrogels have been exploited for organoid formation that can offer a setting that can control how cells grow. These gels bear the biochemical signature of tissue-specific ECM [25]. This study made organoids from human biopsies instead of using Matrigel. Human cholangiocyte organoids replaced highly variable tumor-derived basement membrane ECM [26]. Kim et al. [27] presented a defined ECM hydrogel produced from the small intestine that is supplemented with three ECM proteins necessary for intestinal development. Interestingly, in terms of organoid- formation efficiency, size, and gene-expression patterns of intestine-specific markers, intestinal organoids grew in these well-established ECM hydrogels.

Various blueprints for organoid formation have been employed to grow mini-organs *via* directed self-assembly using specifically designed organogenic cues. For instance, Eiraku et al. developed a double-layered optic cup-like organoid using self-organized 3D aggregates of mouse PSCs [28]. Similarly, Nelson et al. fabricated a 3D *in vitro* model of mouse mammary epithelial tubules using micropatterning technology to guide the 3D structure of the organoids [29]. Interestingly, multicellular tubular geometry and the available spatially regulated degree of autocrine-inhibitory morphogen concentration could control branch location throughout the morphogenesis of mammary epithelial cells and primary organoids.

Bioprinting

3D bioprinting is emerging as a new approach at the intersection of bioengineering and translational regenerative medicine. It has the potential to fabricate patient-specific and tissue-/organ-specific tissue-engineered constructs [30]. A pertinent question is how to secure optimum rheological features and printability index when the cells are mixed in bioink [31]. The promising prospects of this domain can be outlined from the point that, even in 2D culture techniques, the micropattern-based cellular arrangement could recapitulate gene- expression patterns akin to the early stages of embryonic organization and development [32••]. For example, Morgani et al. cultured mouse PSCs on micropatterned surfaces under specific morphogens such as fibroblast growth factor, ACTIVIN (NODAL), BMP, and Wnt, which could replicate the spatial pattern of embryo-like *in vivo*-regionalized patterning of the embryo [33]. Similarly, Rossi et al. demonstrated that it is possible to induce mouse embryonic stem cells (mESCs) to stably proceed through the essential stages of early heart organogenesis with an *in vivo*-like spatiotemporal fidelity [34•]. These axially structured embryonic organoids facilitate the development of first and second heart field compartments and cardiovascular progenitors. It would be highly intriguing if such perspectives could be combined with 3D bioprinting to replicate the formation of the primary pattern during developmental stages. Although bioprinting provides unprecedented control over cell placement in 3D, the success rate of the existing methods has been insubstantial. They become a constraint in replicating the sophisticated microarchitecture, cell-type diversity, and the functionality of the native tissues developed by cellular self-organization. To evade this problem, Brassard et al. developed a 3D bioprinting approach that employs stem cells, giving rise to organoids as a constituent that may be put directly into ECMs that promote spontaneous self-organization [16]. They fabricated centimeter-scale tissues by regulating the geometry and cellular density. These tissues had self-organizing attributes, for instance, lumens, bifurcated vasculature, and tubular intestinal epithelia. Discrete epithelial cells were printed consecutively to recapitulate the organ boundaries of the gastrointestinal tract, and supporting cells were laid down to regulate the spatiotemporal morphogenesis. This study thus illustrated the role of biofabrication combined with organoid technology to regulate the tissue self-organization to centimeter scale from millimeter, opening new possibilities in medication development, diagnostics, and regenerative medicine. Although spheroid cultures are highly promising because of their organotypic cellular densities. But it can be challenging to introduce heterogeneity and the function of multiple tissues by controlling their patterning over longer length scales. Toward this end, Daly et al. [35] formulated a bioprinting technique for the translation of spheroids into self-healing hydrogels permitting their patterning and combining into high cell-density microtissues with a predetermined spatial organization. They used spatially regulated cardiomyocyte and fibroblast cell ratios acquired from iPSCs in bioprinted cardiac microtissue models to mimic the structural and functional characteristics of scarred cardiac tissue that develops after myocardial infarction, such as reduced contractility and erratic electrical activity. This method relayed several

promising applications such as creating precise disease models and drug screening, especially when high cell densities and heterogeneity are crucial. Hence, we believe that combinatorial perspective integrating the concepts of developmental biology along with 3D bioprinting of organoids would be able to generate enormous potential to furnish the required instructive cues to trigger organogenesis pathways in a patient-specific fashion.

Among the critical signaling episodes during embryogenesis and adult tissue homeostasis, spatiotemporal levels of Wnt signaling hold great promise. Approximately 19 Wnt family genes are responsible for modulating cell proliferation, differentiation, migration, gene expression, apoptosis, preservation of cell polarity [36], and so on. Significance should be given to perceive how the bioink can regulate cellular signaling pathways. When integrated with 3D bioprinting, such insights can be expanded and exploited further to recapitulate cellular signaling events at the developmental level to produce 3D bioprinted tissue equivalents related physiologically and anatomically related. Toward this end, Chawla et al. [37•] successfully developed cartilage tissue equivalents by employing the mesenchymal stem cell MSC-encapsulated (SF–G) bioink, where a close resemblance in the signaling pathway was observed and hypertrophy was minimized in the 3D bioprinted construct concerning the signaling cascades encountered during embryonic cartilage development. In addition, it would be interesting to investigate the *in vivo* signaling crosstalk between Wnt and transforming growth factor β signaling, BMP signaling, and Hedgehog and Notch signaling, which occur in an *in vitro* network [38]. Furthermore, to precisely replicate the embryonic developmental program, the regulation of cell polarization, patterning, modulation of matrix arrangement, chemistry, and mechanical signals should be considered, promoting the structural reliability of 3D bioprinted constructs.

Nonetheless, numerous inexplicable challenges remain unresolved. For instance, the fabrication of fully vascularized 3D bioprinted structures with thickness and rigidity akin to native tissue/organs has yet to be realized, ensuring hassle-free integration with native tissue after implantation. None of the studies have replicated the complex anatomical architecture of the kidney, liver, and cardiac tissues. In such a scenario, an amalgamation of organoid- fabrication strategies, 3D bioprinting, and developmental biology-inspired *in vitro* strategies could be a probable solution to formulate 'organ germs' for organ replacement.

Developmental engineering

The traditional tissue engineering aspects have recently undergone a paradigm shift in the direction of 'developmental engineering' [39]. Current tissue engineering strategies are orchestrated to understand how these new blueprints can precisely imitate the *in vivo* developmental process *in vitro* [40]. The interaction between biochemical signaling and tissue mechanics choreographs tissue morphogenesis and patterning during development. Imitating these morphogenetic activities constitutes the core of the developmental tissue engineering paradigm [41]. Scotti et al. exploited the ability of human MSCs to develop bone tissue equivalents mimicking the *in vivo* endochondral program to generate a model to investigate the mechanisms of bone generation using transwell culture [42••]. Their strategy was to recapitulate the primary event of embryonic bone formation. Interestingly, when implanted subcutaneously into nude mice during different stages of chondrogenic differentiation, human MSCs constituted bone trabeculae, specifically during the development of hypertrophic tissue structure. It must be noted that the morphogenetic process encountered has a structural and molecular resemblance to temporal and spatial progression during embryonic bone development. The Indian hedgehog (IHH) signaling pathway is activated at earlier stages, essential for hypertrophic cartilage formation *in vitro*. To date, strategies employed for bone tissue engineering generally utilize direct differentiation protocols. Such bone models developed *via* a 'developmental engineering' prototype [42••] can produce improved grafts for bone renewal, ensuing woven or cancellous bone architecture, only when combined with bioprinting strategies. Notwithstanding, this research was based on approaches involving spheroids and transwells, as a result, it failed to simulate the anatomical architecture and the thickness of native bone tissue. Hence, to further widen this apprehension, in our laboratory, we employed a scheme in which the related endochondral ossification-inspired procedure was exploited and further merged with 3D bioprinting to fabricate bone tissue equivalents [43]. Imitating the course of the developmental biology-inspired endochondral ossification within the SF–G 3D bioprinted constructs loaded with MSC-activated Wnt/β-catenin, IHH, and parathyroid hormone signaling during osteogenic differentiation *in vitro*, resulted in enhanced potential for osteogenic differentiation of MSCs and amplified mineral deposition.

Although bioprinting of organoids is still far-fetched, research has been carried out to print PSCs using two strategies. The first involves bioprinting-undifferentiated PSCs, and the second requires bioprinting organoids or differentiated stem cells [44]. Nonmodular hydrogel inks, such as silk fibroin, PEG, and collagen/gelatin, have been explored for the bioprinting of organoids following the developmental pathway. For example, Gu et al. bioprinted iPSCs encapsulated in a bioink composed of alginate, carboxymethyl–chitosan, and agarose. The iPSCs showed *in situ* differentiation into self-assembling 3D embryoid bodies, demonstrating

the expression of three germ layers, indicating pluripotency after printing [45••]. Tissue engineers have attempted to extract cues from this approach and integrate those attributes with 3D bioprinting, resulting in the advent of the new discipline of organ printing, which holds the potential to sketch and develop engineered tissue/mini-organs for the restoration, repair, and replacement of impaired or damaged organs.

Taken together, the goal is to combine developmental biology and organoid-based bioprinting to develop anatomically clinically relevant 3D tissues using biomaterial-based cues or time-dependent release of agonists and/or antagonists from biomaterials. A fascinating application of this approach could be the generation of organ-on-chip platforms to test the efficacy of new drugs and toxicity testing.

Future perspectives

Integrating developmental biology and 3D bioprinting should be the ultimate direction to fulfil tissue engineers' demands to procreate the critical structure–-function interrelation of native adult tissues and organs. Nevertheless, how distant tissue engineers should recapitulate embryonic morphogenesis and organogenesis remains controversial. Logistics and monetary components give rise to a rational belief that printing organs will probably prohibit recapitulating all the features of functional living human organs. However, if successful, the generation of functional tissue-engineered mini-organs that are sufficiently vascularized and skilful to restore the essential functions of impaired or lost organs would accurately portray the technological aim and be an embodiment of prominent achievement.

The combinatorial perspectives of developmental engineering, organoids, and 3D bioprinting are promising. It would be immensely wrong if we did not state that numerous technological and elemental challenges still confine such a promising strategy. To mention some, organoid-formation mechanics based on high-throughput spheroids by microfluidic devices, procuring 3D bioprinters equivalent to those at the industry level, and optimization of 3D bioprinting for wide-ranging living tissues and mini-organs, and vascularization of the formulated thick anatomically pertinent tissue/mini-organs, innate and adaptive immune response to bioprinted constructs [46]. Nonetheless, many researchers are trying to center on such combinatorial policies over traditional scaffold-based counterparts and put forward confidence for additional research, reconnecting this new discipline in tissue engineering. 3D bioprinted counterparts should ideally be defined by considering the patient's genetic or epigenetic makeup and age-dependent immunological condition. These current developments in the regenerative field, involving the determination of novel regenerative factors accompanied by tissue engineering technology, may foster human organ and structural regeneration.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data were used for the research described in the article.

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