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Towards Organs on Demand: Breakthroughs and Challenges in Models of Organogenesis

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Abstract

In recent years, functional three-dimensional (3D) tissue generation in vitro has been significantly advanced by tissue-engineering methods, achieving better reproduction of complex native organs compared to conventional culture systems. This review will discuss traditional 3D cell culture techniques as well as newly developed technology platforms. These recent techniques provide new possibilities in the creation of human body parts and provide more accurate predictions of tissue response to drug and chemical challenges. Given the rapid advancement in the human induced pluripotent stem cell (iPSC) field, these platforms also hold great promise in the development of patient-specific, transplantable tissues and organs on demand.

Keywords

spheroids; organoids; miniature organs; organs-on-chips; 3D bioprinting; blastocyst complementation

Introduction

Irreversible organ failure remains a worldwide concern as demand for transplantable organs far outpaces the available supply. To overcome this shortage, xenotransplantation, where tissues or organs from animals are transplanted into humans has been tested with limited success since the early 1900's. The most successful attempt, a renal transplant from a chimpanzee to a 23-year-old woman late in 1964, was reported to restore kidney function for at least 6 months (1). Factors affecting the outcome of transplantation across the xenogeneic barrier include both humoral (innate) (2) and cell-mediated (innate/adaptive) (3) immune responses. Despite the development of strategies to achieve immunological tolerance, including thymus transplantation (4) and induction of mixed chimerism through donor bone marrow transplantation (5), xenotransplantation is not yet a clinical reality (6). Indeed, while

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Conflict of Interest

Maria Giovanna Francipane and Eric Lagasse declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

organ rejection can be avoided, the risk of an infectious or zoonotic disease spreading from animals to humans raises further concerns about the clinical application of xenotransplantation (7).

Apart from the demand for replacement organs to use to treat irreversible organ failure in civilian, there is a need of tissues or organs for wounded soldiers returning from battle. Indeed, the increase in Improvised Explosive Device (IED) in current conflicts has often resulted in massive wounds for warriors involving neural, cardiopulmonary, musculoskeletal and sensory tissues, opening a new branch of translational medicine, the military medical research (8).

By the late '90s, the advent of stem cell research had sparked a new direction in the field of regenerative medicine. In addition to cell replacement therapy, using stem cells to treat cancers of the blood or for bone marrow reconstitution after high-dose chemotherapy (9), recent work has shown the feasibility of creating three-dimensional (3D) tissue cultures. Indeed, thanks to their ability to self-renew and to give rise to differentiated progeny, stem cells spontaneously organize into 3D structures containing a cellular hierarchy. These structures constitute a promising starting point for the development of functional, transplantable whole organs, hopefully in the near future. Our review will discuss breakthroughs and challenges in this rapidly evolving field. Traditional and novel organogenesis assays including organoid culture, organs-in-a-dish, organs-on-chips, 3D bioprinting, programmed synthesis of 3D tissues, and blastocyst complementation will be considered. Finally, we will discuss our lymph node-based in vivo platform, which models the structure and function of different organs.

From organotypic culture to self-assembling miniature organs

Although the ancestors of present-day organoids were first described in the early 20th century, research involving organoid cultures did not begin to accelerate significantly until recently, when tissue-specific stem or progenitor cells in epithelial organs were identified and isolated.

The term *organoid culture* should not be confused with *organotypic culture*. These terms, which are often used interchangeably, actually refer to two distinct culture approaches. Organotypic culture implies the interaction of two or more cell types from a complex tissue or organ (10). Organotypic cultures, including but not limited to whole-organ, organ-slices, and reaggregate cell cultures, go back to the beginning of the last century. The *watch-glass technique* is one of the earliest organotypic culture preparations. Introduced in 1929 by Fell and Robinson, a clot consisting of chick plasma and chick embryo extract and contained within a watch-glass was used to grow embryonic organ rudiments (11). Chen later introduced a modification. His *floating lens paper technique* permitted the growth of either whole-organs or organ-slices (12). Reaggregate organotypic cultures have also been widely used to develop engineered tissues. Some cells, like neural stem cells, possess the ability to spontaneously aggregate into free-floating well-rounded 3D structures, so-called *spheres* (13) (Figure 1A). The finding that a floating culture might help cells acquire or maintain stemness properties (14) has led to the use of sphere-forming assays to investigate stem cells

and progenitors derived from organs other than the brain in both normal and cancerous tissue development. However, there are several limitations surrounding the sphere-forming assay (15). First, spheres may simply result from an aggregation of cells. Free-floating spheres may themselves aggregate and fuse, due to intrinsic locomotion. Therefore, clonality is only guaranteed by single cell plating. However, even clonogenic spheres consist of heterogeneous cell populations including multipotent stem cells and unipotent progenitors in different states of differentiation (16). Within a purified population, cells may also be in different stages of the cell cycle. This intraclonal heterogeneity can affect data reproducibility when spheres are used, for example, as an in vitro model to test drugs. The most common method used to obtain spheroids consists in plating cells onto ultra-low attachment plates, where cell attachment is inhibited by a covalently bound hydrophilic and neutrally charged hydrogel layer. Spheroids can also be obtained by reaggregating enzymatically-dissociated cells in a rotation culture, a technique introduced more than 4 decades ago (17), or by using the hanging drop technique, or magnetic cell levitation technology (18–20).

Organoid culture refers to a collection of organ-specific cell types that develops from stem cells or organ progenitors and self-organizes through cell sorting and spatially restricted lineage commitment in a manner similar to in vivo (21). Since the late 2000s, there has been a tremendous interest in organoid research. Mouse mammary gland epithelial structures (22) and intestinal crypt-villus units (23) were first established in vitro from single stem cells. This culture platform was later applied to other types of epithelial tissue, including tissues of human origin (24). Our lab has recently described organoid assays for both human intestine (25) (Figure 1B–C) and mouse esophagus (26) (Figure 1D). Organoids have proven to be valuable modeling systems for the study of organ development (27) as well as clinical applications including disease modeling and gene therapy (28), screening and validation of novel drugs (29), and regenerative medicine (30).

Selecting an appropriate stem cell source is a critical step for the generation of functional organoids to be used for therapy. Embryonic stem cell (ESCs), which are derived from in vitro culture of the blastocyst inner cell mass (31), would be ideal candidates for organoid generation. However, ethical quandaries hamper their use worldwide spurring efforts to isolate adult stem cells. Intensive research came up with the idea that most tissues and organs contain rare adult stem cells, which help maintain the tissue. Yet, scalable, efficient expansion of these cells for clinical purposes remains a challenge.

Recently, the development of induced pluripotent stem cell (iPSC) technology has enabled the induction of pluripotency in mature somatic cells by treatment with defined factors (32). This Nobel Prize-winning discovery has generated unprecedented opportunities to create patient-specific surrogate organs and has been met with tremendous enthusiasm in the scientific community. We are witnessing a boom in the generation of miniature organs from iPSCs (33). Cerebral organoids displaying discrete brain regions (34), vascularized and functional liver organoids (35), gastric organoids (36), kidney organoids containing nephrons associated with a collecting duct network surrounded by renal interstitium and endothelial cells (37), and insulin producing pancreatic organoids (38), are only some examples of miniature organs generated on a dish from human iPSCs.

Microfluidic culture devices: organs-on-chips

Organoids are usually grown in a Matrigel bead, a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix (ECM) proteins such as laminin, collagen IV, heparin sulfate proteoglycans, entactin/nidogen, and a number of growth factors. Although this natural ECM-based hydrogel helps tissue organization, it does not replicate the physical and mechanical forces cells experience within the body. Organs-on-a-chip promises to better replicate the mechanical forces and dynamic physical microenvironment that is an essential part of living organs. A multifunctional microdevice that reproduces key structural, functional, and mechanical properties of the human alveolar-capillary interface, the fundamental functional unit of a living lung, was recently developed (39). The fabricated device consisted of a microfluidic system composed of two closely apposed microchannels separated by a thin, porous, flexible membrane made of polydimethylsiloxane (PDMS). The intervening membrane was coated with ECM. Human alveolar epithelial cells and human pulmonary microvascular endothelial cells were cultured on opposite sides of the membrane. Once the cells were grown to confluence, air was introduced into the epithelial compartment to produce a cyclic stretching of the tissue-tissue interface, mimicking the physiological movement of breathing. This microdevice enabled the analysis of the mechanical forces visited upon various physiological and pathological lung functions, including interactions with immune cells and pathogens, epithelial and endothelial barrier functions, and toxicity and absorption of nanoparticulates across the critical alveolar-capillary interface.

More recently, the Radisic lab developed a human cardiac micro-tissue, termed *biological wire*, which captures some of the architectural complexity of the native myocardium and enables maturation of cardiomyocytes derived from human pluripotent stem cells following electrical stimulation (40). To demonstrate the feasibility of this platform for pharmaceutical testing, the same group later added a polytetrafluoroethylene (PTFE) tubing template to the biowire bioreactor (41). Nitric oxide (NO) was then released from perfused sodium nitroprusside (SNP) solution and diffused through the tubing. The NO treatment slowed spontaneous beating of the cardiac biowires as well as degraded the cardiomyocyte myofibrillar cytoskeleton within the biowires. Electrical stimulation was incorporated into the biowires using carbon rod electrodes, further improving the cardiomyocyte phenotype, as indicated by an organized contractile apparatus, higher Young's modulus, and improved electrical properties. Thus, this pulsating heart-on-a-chip provides a unique opportunity to uncover the effects of NO on cardiomyocytes at the tissue level.

Scaffold-based tissue engineering

Stem cells isolated from rapidly proliferating organs such as the intestine seem to contain all the instructions necessary to self-organize in vitro as an organoid which faithfully recreates the complex tissue architecture from which the stem cells are derived. However, other tissue-specific stem cells might need structural support to achieve the spatiotemporal architecture and physiological function of the native tissue. The ECM plays an essential role during tissue development and morphogenesis (42). Not only does the ECM structurally support the cells, but it also contributes to the mechanical properties of tissues, provides bioactive cues

for cells to respond to their microenvironment, serves as a reservoir of growth factors and potentiates their actions, and provides a degradable physical environment to allow neovascularization and remodeling in response to tissue dynamic processes such as wound healing (43). Consequently, the best scaffold for an engineered tissue should be the ECM of the target tissue in its native state. Decellularization of cadaveric organs has the potential to overcome the need to artificially recreate the conditions for ECM deposition. However, the majority of described decellularization protocols show limitations in preserving the ECM native structure, mostly due to the harsh conditions required to remove cellular material (44). At the moment, this technique only works with the heart in humans (45), and not with the other major organs. Alternatively, supportive scaffolds can be produced from biomaterials including natural or synthetic polymers (46). One of the best examples of human organs generated using synthetic materials is the bladder. Bladders have been reconstructed by seeding autologous cells on collagen-polyglycolic acid (PGA) composite matrices. These reconstructed bladders were then implanted in patients with end-stage bladder disease requiring cystoplasty, improving urinary continence (47). Besides decellularization and pre-made porous scaffolds for cell seeding, other scaffolding approaches include cell sheets with self-secreted ECM, and cell encapsulation in self-assembled hydrogel matrix (43). Cell sheet engineering approach is excellent for epithelium, endothelium and cell-dense tissues, while encapsulating cells in biodegradable hydrogels has found its main application in cartilage and bone tissue engineering (48).

Organ bioprinting: towards scalable solutions

The combination of (stem) cells and scaffolds may be achieved by exploiting the new 3D bioprinting technology. In 3D bioprinting, layer-by-layer exact positioning of living cells, biomaterials, and biochemicals, with the desired spatial and temporal control, is used to fabricate 3D structures (49). There are several approaches to 3D bioprinting, including biomimicry, autonomous self-assembly and mini-tissue building blocks.

Biomimicry is a creative and innovative way seeking solutions to human problems by emulating nature's patterns, strategies, and models. Biomimicry has contributed significantly to advances in architecture and civil engineering and more recently, has also inspired biomedical research to reproduce the cellular and extracellular components of a tissue or organ. Different marine biomimetic approaches have been envisaged. Marine biopolymers like collagens, gelatins, keratins, and elastins have been investigated during the last decade as potentially manufacturable, renewable biological sources (50, 51). Repairing bone tissue with the use of coral and marine sponges represents only an example of biomimetic application (52).

Another approach to replicating biological tissues is to use embryonic organ development as a guide to create the bioprinted tissue. The third approach of bioprinting, mini-tissue building blocks, combines both the biomimicry and self-assembly approaches, and it has been used to self-assemble branched vascular networks (49).

In order to bioprint human organs it is necessary to develop a technology for the scalable, accurate and repeatable deposition of biologically active materials. Fabrication of large-scale

tissues can be obtained using an integrated tissue-organ printer (ITOP). This technique allowed human-scale mandible bone, ear-shaped cartilage and organized skeletal muscle (53). The bioprinted bone, ear and muscle constructs generated from human amniotic fluid-derived stem cells, rabbit ear chondrocytes and mouse myoblasts, respectively, showed promising structural and functional characteristics in vitro and in vivo. Indeed, when implanted in vivo, all three constructs showed evidence of vascularization without necrosis, and the muscle constructs showed the presence of neuromuscular junctions.

Overall, this technology has the potential to produce whole functional organs in the future, benefitting patients with end-stage chronic organ failure. However, more research is needed before such 3D printed tissues could be tested in human patients.

3D bioprinting of vascularized organs-on-a-chip: a new frontier of drug discovery and regenerative therapies

Constructing the microvascular niche is essential to mimic in vivo organ functions. The first organ-on-chip systems described lacked a fully integrated 3D microvasculature. To overcome this limitation, the Radisic lab very recently fabricated a scaffold, called AngioChip, which supports the assembly of parenchymal cells on a mechanically tunable matrix surrounding a perfusable, branched, 3D microchannel network coated with endothelial cells (54). Specifically, the AngioChip scaffolds were constructed using a biodegradable elastomer, poly(octamethylene maleate (anhydride) citrate) (POMaC). 3D stamping enabled patterning of POMaC into various intricate structures mimicking a vascular bed. The synthetic built-in vascular walls were thin and flexible, yet strong enough to mechanically support a perfusable vasculature. Nanopores and micro-holes were incorporated into the vascular walls. This combination of thin channel walls with nanopores and micro-holes were key features permitting effective molecular exchange, cell extravasation in a vascularized 3D tissue model, and physiologically relevant modes for delivery of test drugs by convection–diffusion. By establishing a stable, permeable, vessel network within AngioChips, the authors were liberated from material constraints, enabling the use of ECMs with cells in the parenchymal space and tissue remodelling. The AngioChip was therefore used to generate both in vitro cardiac and hepatic tissue models with defined vasculature. Importantly, AngioChip cardiac tissues implanted with direct surgical anastomosis to the femoral vessels of rat hindlimbs immediately established blood perfusion (54).

Therefore, by combining different approaches, AngioChip allows mimicking human organ physiology for drug screening and regenerative medicine.

Programmed synthesis of 3D tissues

A useful material for bioprinting should be biocompatible, easily manipulated by the bioprinter technology for deposition to complex 3D structures, and must maintain cellular viability and function, thereby providing structural and mechanical support to the developing tissue (49). Many published studies use a limited range of materials, including collagen, hyaluronic acid, alginate, modified copolymers and photocured acrylates (49). It seems

unlikely that any single material will have all the properties required to recapitulate tissue function (49). One interesting approach is the development of functionally adaptive materials that reprogram their shape, properties or functionality on demand, based on external stimuli (49). Recently, a method to reconstitute the multicellular organization of organoid-like tissues having a programmed size, shape, composition and spatial heterogeneity has been developed. This method, called DNA-programmed assembly of cells (DPAC), uses a DNA-patterned substrate as a template and temporary DNA-based cellular adhesions as synthetic linkages between cellular building blocks (55). Briefly, cells bearing complementary cell-surface oligonucleotides are introduced above the patterned substrate at high concentration and at a controlled flow rate using a flow cell. Cells adhere to the appropriate DNA spot, and excess cells are removed by gentle washing. Iteration of this process assembles cells into 3D microtissues. Addition of liquid ECM incorporating DNase releases the assembled microtissues from the template where they are trapped in the embedding ECM as it gels. The gel is then peeled off the glass, releasing the tissues. Underlay of the gel with additional ECM results in a fully embedded 3D culture. DPAC is uniquely capable of reconstituting cell-dense microtissues with a tailored spatial heterogeneity. Unlike a printer, DPAC defines cell position by cell-cell connectivity rather than the co-ordinates in a 3D Euclidean space. Therefore, the templating DNA pattern, and the order of addition of different DNA-functionalized cell populations determine the cell-cell connectivity of the assembled microtissue.

Exogenic organs: the blastocyst complementation technique

Despite success, concerns over the in vitro creation of surrogate organs exist. Indeed, the in vitro culture system may represent an adverse environment for self-organization and morphogenesis. The possibility exists that iPSCs require transplantation into live animals to fully mature and generate a functional organ (56).

As an alternative to generating organs from iPSCs in vitro, providing iPSCs with an empty developmental niche for the specific organ might be beneficial. Indeed, according to the *organ niche* theory, developmental failure in organogenesis caused by defective genes, can be compensated by xenogeneic pluripotent stem cell transplantation into genetically affected blastocysts. First described in 1993, this *blastocyst complementation* technique generates an organ almost entirely composed of cells derived from the donor pluripotent stem cells (57). The best example is the generation of a functional pig pancreas using somatic cell cloning technology in pancreatogenesis-disabled porcine embryos (58). The pancreata derived from exogenic pluripotent cells were normal in their configuration and functions.

Blastocyst complementation using cloned porcine embryos may permit the generation of functional organs from xenogenic pluripotent stem cells, including human iPSCs, with the xenogenic barrier a key challenge. Pigs are estimated to have diverged from humans approximately 100 million years ago, the fear being power and ambition may eventually lead scientists down a *slippery-slope* to the use of incapacitated humans as incubators (59).

The lymph node bioreactor

Our studies indicate that the lymph node can serve as an *in vivo* factory to generate complex organ structures, including liver, pancreas, thymus, and kidney (60–64). This *in vivo* bioreactor might offer unprecedented opportunities to study stem cell maturation and function, as well as provide a unique organogenesis site for therapeutic purposes. There are over 500 lymph nodes in the human body, many of which are easily accessible. Although a single lymph node structurally limits the number of donor cells that can be transplanted, it is technically feasible to transplant more than one lymph node to gain sufficient organ or tissue function from the transplanted cells. The potential loss of function in a few lymph nodes does not seem to compromise the overall function of the lymphatic system (65). Finally, lymph nodes have ready access to the bloodstream, nurturing the transplanted cells with nutrients as well as hormones and signaling agents needed for growth. Importantly, new angiogenesis occurs quickly enough in this site to sustain cell survival and engraftment. We hypothesize that what makes the lymph node so special is the rich vascularization and the presence of non-hematopoietic lymph node stromal cells (LNSCs). These cells are essential to the structure and function of the lymph node. Indeed, when receiving external signals, LNSCs express specific features for creating tissue structure and supporting immune reactions (66). LNSCs also provide a microenvironment that facilitates the establishment of cancer metastases (67). We speculate that LNSCs similarly provide the necessary regulatory cues to cells and tissues transplanted into the lymph node. Our future work will therefore aim toward an integrated tissue engineering approach that combines a scaffold-free LNSC-based system implanted with human stem/progenitor cells to regenerate functional organs.

Conclusions

Since their discovery, stem cells have held great promise for their application in tissue regenerative medicine. *In vivo*, stem cells reside in a highly specialized 3D structure, the so-called niche (68). Reproducing this dynamic and complex microenvironment in culture is challenging, either because the mechanisms that control stem cell fate *in vivo* have not yet been fully elucidated, or because of ethical and technical issues. Conventional two-dimensional cell cultures poorly mimic the physiological environment. Key events like cell-to-cell and cell-ECM interactions, cell polarization, adhesion, and migration, necessary to generate precise tissue structure-function relationships, cannot be fully recreated in a cell monolayer. Considerable effort is presently being invested in establishing methods for engineering more physiologically relevant organs, where the chemical, physical and mechanical microenvironment of the stem cell niche can be replicated. In a 3D self-organizing culture, progenitors induced by differentiation conditions undergo multiple cellular interactions (69). These local interactions start to build up emergent collective behaviors leading to self-organization of complex structures as a whole and are patterned in space and time (69). Therefore, the long-term goal is to rebuild tissue and organ function in 3D space over time, i.e. in four dimensions. In other words, the aim is to modulate dynamic interactions, artificially allowing tissues to self-organize at one's command (69).

Although numerous tissue types have been successfully engineered *in vitro*, clinical translation has been achieved only for thin tissues or those with a low metabolic demand (for

example, skin, cartilage and bladder) (54). Indeed, vascularization for large solid tissues or organs with higher metabolic requirements still remains a challenge (70). A breakthrough in the tissue engineering field came very recently from the development of a fully 3D structure complete with internal blood vessels (54). This microfluidics-based device presents several advantages, such as the ability to control fluid flow, cell patterning, and mechanical forcing regimens (71). Although still in its infancy, such organ-on-a-chip technology is opening up unprecedented opportunities to study basic mechanisms of organ physiology and disease. However, given the complexities of organ function, it is unlikely that organs-on-chips will replace animal testing anytime soon (71). Indeed, their scalability is questionable. In the future, the goal will be to create an unlimited supply of transplantable organs by combining all protagonists in the tissue engineering scene: cells, biomaterials, chemicals, and chips.

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Figure 1.

A, Appearance of a 5-day floating primary mouse neurosphere and immunofluorescence staining for Nestin (neural stem cell marker), Sox2 (neural stem cell marker), or Ki-67 (proliferation marker) (*red*). Nuclei were counterstained using Hoechst (*blue*) (Scale bar = 100µm). **B and C**, Phase contrast microscopy and hematoxylin and eosin stain images of small (**B, top**) and large (**C, top**) intestine organoids, and immunofluorescence staining for Epcam (*green*, epithelial marker), Mucin 2 (Muc2, *red*, goblet cell marker) or Chromogranin A (ChgA, *red*, enteroendocrine marker) on both small (**B, bottom**) and large (**C, bottom**) intestine organoids. Nuclei were counterstained using Hoechst (*blue*) (Scale bar = 50µm). **D**, Phase contrast microscopy, hematoxylin and eosin stain, and immunofluorescence staining images of an esophageal organoid expanding over the course of 9 days (Scale bar = 50µm). Organoid was stained for cytokeratin 14 (CK14, basal epithelial cell marker), p63 (basal epithelial cell marker/progenitor cell marker), EdU (proliferation marker), and cytokeratin 13 (CK13, suprabasal epithelial cell marker) (*red*). Nuclei were counterstained using Hoechst (*blue*) (Scale bar = 50µm).