Transplantation of Bioprinted Tissues and Organs

Technical and Clinical Challenges and Future Perspectives

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Three-dimensional (3D) bioprinting is a revolutionary technology in building living tissues and organs with precise anatomic control and cellular composition. Despite the great progress in bioprinting research, there has yet to be any clinical translation due to current limitations in building human-scale constructs, which are vascularized and readily implantable. In this article, we review the current limitations and challenges in 3D bioprinting, including in situ techniques, which are one of several clinical translational models to facilitate the application of this technology from bench to bedside. A detailed discussion is made on the technical barriers in the fabrication of scalable constructs that are vascularized, autologous, functional, implantable, cost-effective, and ethically feasible. Clinical considerations for implantable bioprinted tissues are further expounded toward the correction of end-stage organ dysfunction and composite tissue deficits.

Keywords: bioprinting, clinical practices, 3D printing, economic impact, regulatory concerns, stem cells, tissue and organ fabrication, vascularization

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BIOPRINTING: A REVOLUTIONARY TECHNOLOGY FOR ORGAN FABRICATION

Three-dimensional (3D) printing, also known as additive manufacturing, is a process when material is added in layers to create a 3D object while being controlled by a computer. It is used in multiple industries, including biomedical. This technology has been used to create custom implants, anatomic models, prosthetic molds, and surgical guides utilizing titanium, polyether ether ketone, hydroxyapatite, and various resins.[1] In this regard, 3D printing uses acellular, nonbiological material to create the final printed product. When a 3D printer dispenses biological materials, including cells, it is referred to as a bioprinter; however, it still uses additive manufacturing and computer control to generate the printed bioobject. This has recently gained enormous momentum in regenerative medicine as a standalone technology for fabrication of living tissues and organs, which holds great promise for future organ regeneration and transplantation. Bioprinting technology offers greater advantages in fabrication of living tissues due to its ability to pattern biologics (ie, living cells, cytokines, proteins, and drugs) to facilitate appropriate cell to cell and cell to matrix interactions in 3D constructs with anatomically relevant shapes, while generating samples in a high-throughput manner[2] (Fig. 1). In addition, its ability to integrate a vascular network or porous architecture within fabricated constructs facilitates continuous perfusion and oxygenation for long-term cultivation.

Bioprinting technology has been used in various venues including tissue engineering and regenerative medicine, pharmaceutics and drug screening, and cancer research. Various tissue types have been bioprinted; however, they are limited to being thin, hollow, or avascular. With recent achievements in vascular network bioprinting, barriers toward production of larger-scale organs has been diminished.[3] Despite great progress in bioprinting research through the invention of various techniques, printers, and bioinks, clinical applicability still lags. This article prefaces the current state of the art in organ bioprinting for regenerative medicine and discusses the sequential steps required to transplant bioprinted tissues into patients. We specify the current challenges before, during, and after the printing process, while providing the reader with future perspectives on how bioprinting technologies can be translated from bench to bedside.

NEED FOR BIOPRINTED ORGANS

Clinical Implications

Over the past 3 decades, through improvement of surgical procedures and the use of powerful immunosuppressive drugs, cell, and organ transplantations have become the standard of care for end-stage organ dysfunction. More than 120,000 patients in the United States are, however, still waiting for an organ transplant, with the majority either waiting for a kidney or liver.[4] Recent estimates suggest that 22 people die every day waiting for an organ transplant secondary to the scarcity of donors.[4] After transplantation, recipients are maintained on immunosuppression to prevent acute and chronic graft rejection. Previously, steroids and azathioprine were the only agents available for solid-organ transplants and resulted in a 1-year graft survival of approximately 50%.[5] In the 1980s, the 1-year survival improved to 80% with the discovery of cyclosporine. The introduction of several immunosuppressive options (ie, rabbit

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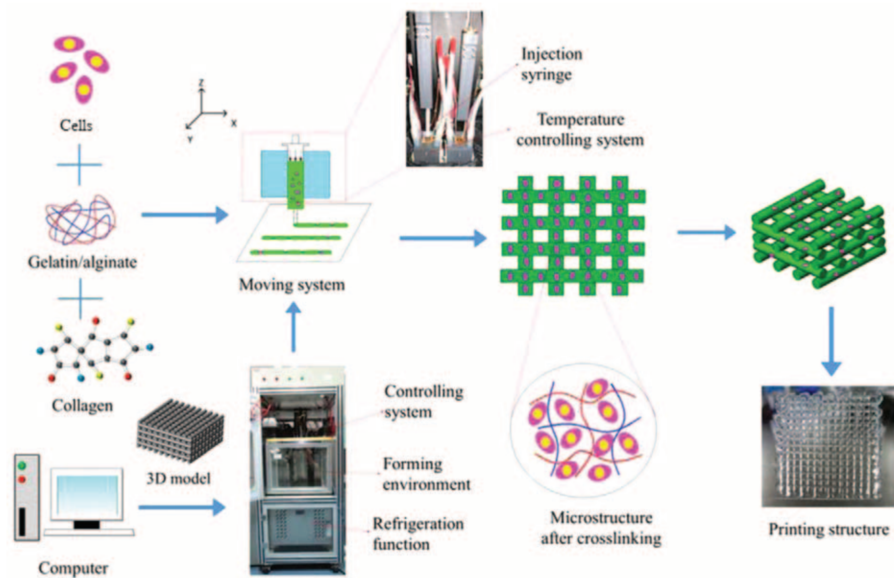

FIGURE 1. Schematic illustration of the 3D bioprinting process and optical images of the printing setup and constructs. (Reproduced and adapted with permission from Wu Z, Su X, Xu W, et al. Bioprinting three-dimensional cell-laden constructs with controllable degradation. *Sci Rep*. 2016;6: 24474. doi 10.1038/srep24474).

antithymocyte globulin, tacrolimus, and mycophenolate mofetil) during the following decade further revolutionized transplantation by improving the 1-year graft survival to more than 90% and reduced acute rejection to less than 10%.[5] With these novel technologies and the advent of reconstructive microsurgery, solid organ transplantation has expanded to vascularized composite tissue allografts (VCA), such as hand[6] and face transplants.[7]

VCAs differ from solid organ transplants in the uniqueness of the graft based on the amount of skin, muscle, and bone it harbors and its potential for continual exposure to the outside environment. Although VCAs may provide the optimal correction of large tissue defects, their life-saving potential is limited; therefore, they are considered more for quality of life and functional indications. Like solid organ transplants, VCA success is reliant on long-term immunosuppression and patients are exposed to all potential side effects, such as malignancies, infection with opportunistic pathogens, and toxicity. Recently, researchers have focused on manipulating the immune response to create a state of tolerance rather than nonspecific immunosuppression[8]; however, this has not been translated into widespread clinical practice in VCA, solid organ, or cell transplantation.

Despite these advances, availability of solid organs or VCAs depends on appropriate donor availability. Recent developments in bioengineering, 3D bioprinting, and regenerative medicine could provide a solid base for the future creation of implantable, bioengineered tissues and organs, obviating the need of immunosuppression and other shortcomings associated with transplants. The ability to produce tissues on demand would eliminate donor shortages, drastically reducing time to therapy while offering the patient a tailored solution.

Current State-of-the-art in Organ Bioprinting

Solid organ bioprinting for human transplantation is still beyond bounds; however, considerable progress has been made on the basic research level recently. Two strategies have emerged for organ bioprinting: scaffold-free and scaffold-based approaches.

In scaffold-free bioprinting, microscale neotissues are fabricated without using exogenous scaffold material, precisely bioprinted according to an anatomic model, and allowed to self-assemble similar to that seen in embryonic development.[9] Using the scaffold-free approach, Norotte and coworkers, bioprinted heterocellular tissue spheroids, producing scaffold-free vascular constructs (Figs. 2A (1–4).[10] Tissue spheroids were printed sequentially using a bottom-up approach in a 3D printed agarose mold. After bioprinting, the spheroids fused and resulted in mature tissue with a well-defined lumen. Subsequently, the sacrificial agarose mold was removed to further culture the blood vessels in a perfusion bioreactor. Employing a similar approach, multicellular constructs have been fabricated using various cell types such as human umbilical vein endothelial cells (HUVEC) and skin fibroblasts.[10] A similar study was conducted for nerve conduit generation by depositing cell pellets between strands of agarose hydrogel. Agarose is a naturally derived polymer, which is inert to cell adhesion and facilitates aggregation of cell pellets in 3D. In most studies, an inert support, referred to as a mold, is required to the aggregation of cells and, limits ultimate tissue size. In order to overcome size restriction, Yu et al[11] demonstrated the use of free-standing tissue strands as building blocks for scaled-up articular cartilage patches. The 8 cm long cartilage strands were loaded into a nozzle and bioprinted in close proximity to one another, leading to a scaled-up cartilage patch after tissue fusion (Figs. 2B1–B4). Because cartilage is an avascular tissue, large patches could potentially be used for joint repair. In addition, Owens et al[12] printed a fully cellular nerve graft using a scaffold-free approach.

Another fabrication approach for scaled-up tissue creation is a scaffold-based method which uses bioink. The development of bioink hydrogels is a crucial part of attaining a biochemically and mechanically relevant environment for cell proliferation. Furth et al[13] described a wide range of biomaterials specific for tissue engineering and regenerative medicine; however, they need to be adjusted for the bioprinting process. The ideal bioink needs to be printable, nontoxic, promotes cell proliferation, insoluble in vivo and in culture medium, and mechanically stabile while degrading at a rate and time appropriate for specific tissue regeneration. Cells can be blended with a natural or synthetic hydrogel, which mimics the exogenous extracellular matrix (ECM) for bioink generation. Following deposition, cells in the scaffold proliferate and mature in 3D. Although natural hydrogels are more cell friendly,[14] they are characterized by weak

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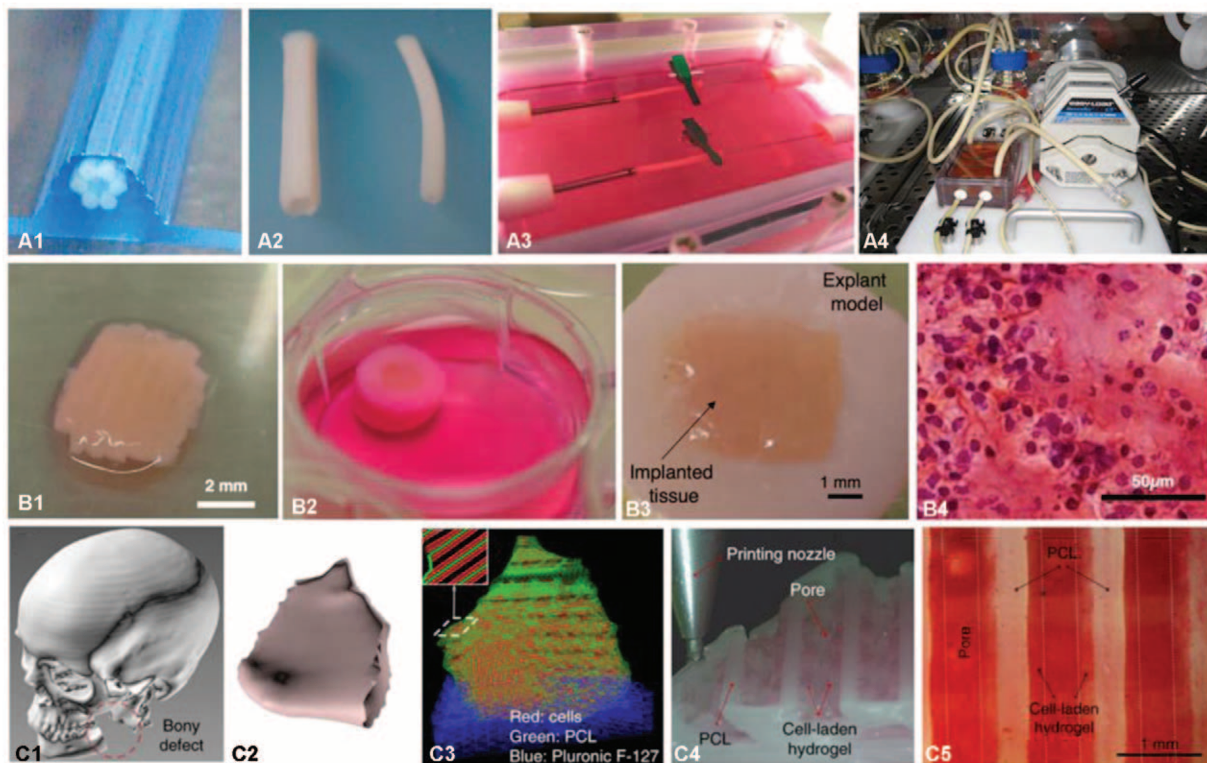

FIGURE 2. Examples of tissue printing: (A1) Scaffold-free bioprinting of a cell pellet within a 3D printed agarose mold support (stained in blue) facilitated aggregation of pig smooth muscle cells in 3 days (A2) followed by removal of agarose support. (A3–A4) Bioprinted blood vessels were then cultured in a bioreactor for further tissue maturation and deposition of collagen and elastin proteins [Reprinted from Norette C, Marqa FS, Niklason LE, et al. Scaffold-free vascular tissue engineering using bioprinting. *Biomaterials*, 2009;30(30):5910–5917 with permission from Elsevier. http://dx.doi.org/10.1016/j.biomaterials.2009.06.034]. (B1) Multilayer bioprinting of free-standing cartilage strands facilitated complete fusion in 1 week followed by (B2–B3) implantation on a bovine osteochondral explant with a 4×4 mm defect. (B4) Further cultivation of the bioprinted patch resulted in cartilage that was histologically close to native bovine cartilage [Reprinted with permission from Macmillan Publishers Ltd (Scientific Reports) Yu Y, Moncal KK, Li J, et al. Three-dimensional bioprinting using self-assembling scalable scaffold-free ''tissue strands'' as a new bioink. *Scientific Rep*. 2016:6. doi: 10.1038/srep28714]. (C1–C2) A computer-aided design (CAD) model of a human mandible generated for a defect captured using computed tomography (CT) images. (C3) A toolpath plan was generated for the cell-laden bioink, PCL, and sacrificial Pluronic F-127 and (C4) 3D printing was performed accordingly. (C5) Alizarin Red staining demonstrated osteogenic differentiation of human amniotic fluid derived stem cells (hAFSCs) in a long-term cultured mandible construct [Reprinted by permission from Macmillan Publishers Ltd. Kang H-W, Lee SJ, Ko IK, et al. A 3D bioprinting system to produce human-scale tissue constructs with structural integrity, *Nature Biotechnol*. 2016;34(3). doi: 10.1038/nbt.3413].

mechanical properties. Common natural hydrogels include agarose, alginate, chitosan, collagen type I, fibrin, gelatin, hyaluronic acid, and Matrigel. Synthetic hydrogels are more adaptable to the bioprinting process with chemical modifications allowing adjustments to mechanical and structural properties. In addition, they can be modified with specific amino acid sequences, such as RGD (Arg-Gly-Asp), to assist in cell attachment. Polycaprolactone (PCL), a polyester-based polymeric compound, has been used as a thermoplastic frame and 3D printed in conjunction with cell-laden bioink and sacrificial Pluronic ink, to generate porosity. The mechanically strong constructs exhibited successful osteogenic differentiation of human amniotic fluid-derived stem cells (Figs. 2C1–C5), and has further been applied to implantable cartilage and muscle, in murine models. Pati et al[15] used decellularized components of various tissues and applied a similar concept to fabricate tissues using bioprinted PCL fibers as a structural frame. Although PCL excels in mechanical integrity, its slow degradation rate restricts its potential in solid organ bioprinting. Bone matrices capable of producing alkaline phosphatase have also been printed using a gelatin methacrylate hydrogel.[16] Researchers have also printed functional neural tissue which is GABAergic and calcium responsive,[17] sheets of cardiac cells,[18] and hepatocytes.[19] Most of these studies have only been performed in animals.

Although large-scale clinical translation is still lacking, there have been successes in tissue engineering and reports of printed acellular scaffolds implanted into patients. Several tissue engineered skin substitutes are Food and Drug Administration (FDA) approved providing an additional wound healing option for patients. For example, Apligraf, consisting of human keratinocytes and fibroblasts, is approved for ulcers secondary to venous insufficiency and diabetic neuropathy. Other approved skin substitutes include Integra (bovine collagen/glycosaminoglycan) and Oasis Wound Matrix (xenogenic collagen scaffold from porcine small intestine).[20] Tissue engineered vascular grafts (TEVGs) were first described in the 1980s

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and are still undergoing limited clinical trials. Most reports indicate the utilization of a biodegradable scaffold with autologous cell seeding for hemodialysis access. TEVGs, however, continue to be commercially unavailable with uncertain costs.[21] In 2013, an acellular 3D printed resorbable tracheal splint was placed into an infant, who suffered from severe tracheobronchomalacia, under emergency FDA approval,[22] illustrating the utility of bioprinting to create a complex individualized replacement strategy with specific dimensions. The manufacture of intricate geometries, including branch points, and cellular incorporation are distinct features of bioprinting in tissue engineering applications.

CHALLENGES IN ORGAN BIOPRINTING

Organ printing is a computer-aided process in which cells and/or cell-laden biomaterials are placed according to a blueprint model and serve as building blocks that are further assembled into 3D constructs and matured toward functional organ formation. An automated approach offers a pathway for scalable and reproducible mass production of engineered organs, in which multiple cell types can be positioned to mimic their natural counterparts. To successfully employ organ printing at the clinical level, robust automated protocols and procedures should be established. Figure 3 illustrates the

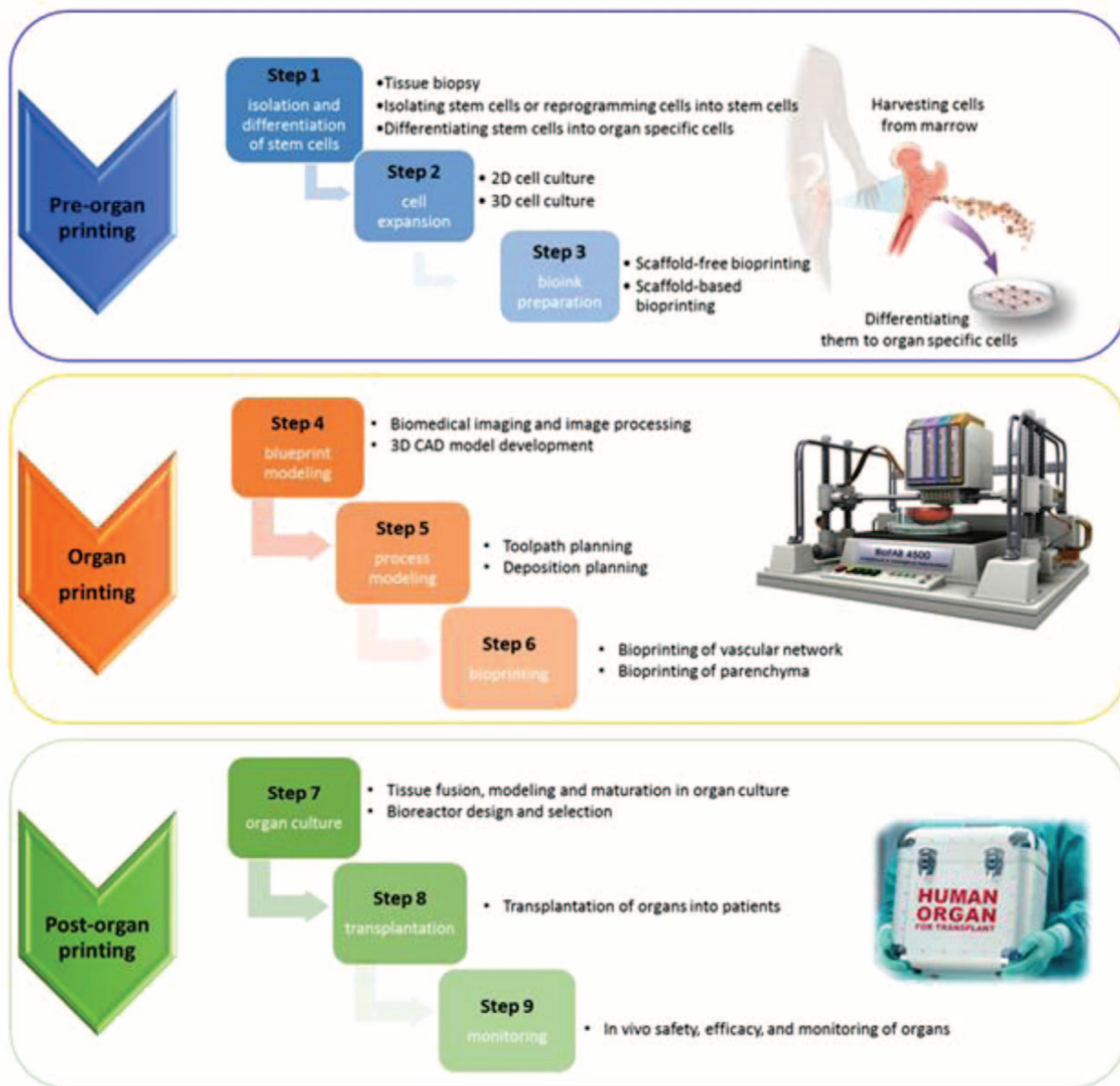

FIGURE 3. Roadmap of bioprinting: In the preorgan printing stage, stem cells undergo isolation, differentiation, and expansion, and bioink is prepared. In the organ printing stage, a blueprint is created, the toolpath and deposition is planned, and the organ is printed. Finally, in the postorgan printing stage, the organ is allowed to mature in culture then transplanted. After transplantation, the organ is monitored for safety and efficacy. [Image courtesy of Elsevier for stem cell isolation (Ozbolat I *3D Bioprinting*, 2016), image courtesy of Christopher Barnatt for bioprinter (www.explainingthefuture.com), image courtesy of Elsevier for organ transplant (Ozbolat I *3D Bioprinting*, 2016)].

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roadmap to organ printing, which is composed of 3 major steps including preorgan printing stage, organ printing stage, and post-organ printing stage.

Preorgan Printing Stage

In this stage, the required raw materials for bioprinting are assembled and consist of biomaterials, growth factors, cytokines, and most importantly cells. Cells should be both patient and organ specific and can be derived from primary and stem cell lines. Autologous primary cells are fully differentiated cells that can be harvested and expanded in vitro. For example, mature adipocytes could be harvested from an individual, expanded in vitro, and suspended in a methacrylated gelatin bioink that would be suitable for 3D printing.[23] This printed tissue could then be reimplanted back into the same individual to treat a soft tissue defect such as seen in Parry-Romberg syndrome. Autologous primary cells may, however, prove more difficult to retrieve in individuals suffering from visceral dysfunction such as heart, lung, or liver failure and may not be possible in diseases such as type 1 diabetes, where there are no functioning β-cells remaining. Stem cells have the remarkable potential to develop into many different cell types and may offer the ability to generate replacement cells and tissues in vitro.

Although human embryonic stem cells are pluripotent and can give rise to any tissue lineage, their availability has been marred by ethical concerns. Induced pluripotent stem cells (iPSCs) offer the benefits of pluripotency, have been extensively studied, and are able to differentiate into adult cells from all 3 germ layers in vitro, and hence providing substantial use in regenerative medicine applications. Depending on the methods used, reprogramming of adult cells to obtain iPSCs may, however, pose significant risks, which may limit their ultimate clinical translation. Over the past decade, it has been recognized that fat is not only an energy reservoir but also a rich source of multipotent stem cells. Human adipose-derived stem cells (ADSCs) offer an abundant, easily accessible, and rich source of adult stem cells, which have potential for tissue engineering applications.[24] ADSC harvest is devoid of ethical concerns, and allows for patient specificity while still maintaining pluripotency.[25] Subcutaneous adipose deposits are ubiquitous and easily accessible in large quantities with safe, minimally invasive procedures, which can be done in an outpatient setting. Other potential stem cells sources include dental pulp, amniotic fluid, and bone marrow; however, all sources have pros and cons associated with their harvest and ultimate clinical application. Cellular material can be maintained in either 2D or 3D cultures with growth factors and cytokines to optimize differentiation and expansion to create bioink preparations that mimic the cell variety and density seen in the desired tissue.

Organ Printing Stage

The organ printing stage commences with the acquisition of precise anatomic models, which serve as the basis for master blueprints. Currently, several noninvasive medical imaging techniques exist, such as magnetic resonance imaging and computerized tomography, to capture the 3D geometry of human organs. Then, the blueprint model can be prepared using one of the appropriate computer-aided design (CAD) techniques and imported into software to control the bioprinter. At this step, the bioprinter needs 3 major pieces of information including what, where, and when to print. During printing, parenchyma and stromal tissue should be deposited in tandem with supporting structures. At this step of fabrication, constructs can be printed using any combination of existing modalities including droplet-, extrusion-, and laser-based bioprinting.[3,26] Droplet-based technology uses thermal-, piezo-, or acoustic-driven mechanisms to deposit droplets of cell suspension in a high-throughput manner, whereas extrusion-based devices use mechanical or pneumatic-driven systems to deposit cells into larger-scale constructs. Laser-based bioprinting, on the contrary, uses laser energy to deposit cells in high resolution. It has the highest resolution with a droplet size of 20 μm, whereas inkjet- and extrusion-based printers have droplet sizes of 50 to 300 μm and $100\ \mu m^{-1} \cdot mm$, respectively.[27] Laser-based bioprinters enable cells to be deposited within 5 μm of the initial pattern.[28] In bioprinting, all steps must be performed in a sterile environment such as a biological safety cabinet, whereas in non-biologic printing the products may be sterilized postprinting utilizing either radiation or ethylene oxide gas.[29]

Bioprinting Vascular Network

In order to print at clinically relevant volumes, robust technologies and protocols should be developed to enable printing of vascular constructs in multiple scales ranging from arteries and veins down to capillaries. Because it is difficult to print submicron scale capillaries using current bioprinting technologies, 1 alternative strategy can be to print a macrovasculature network and rely on capillary network formation through angiogenesis.[30] In this regard, 2 approaches have been described. Indirect bioprinting uses a sacrificial ink[31] instead of directly printing a vascular network in tandem with the rest of the construct.[30]

During indirect bioprinting, cell-laden hydrogels are used as the base material, whereas the vasculature is created by printing a sacrificial material (ie, Pluronic F-127, agarose, gelatin) followed by removal after complete gelation. The integrated and perfusable vascular network results in increased cell viability compared to slab tissue constructs in which regions near the macro-vasculature exhibit significant differences in cell viability compared with regions away from the channels.[32] The majority of researchers have attempted to create vascular networks in macroscale by generating an endothelial lined lumen via colonization of endothelial cells through perfusion. Lee et al[32] took one step forward and successfully achieved angiogenesis by sprouting endothelial cells within a fibrin network loaded with other supporting cells (Figs. 4A1–A2). Recent research has shown the capability of endothelial cells to control the shape and position of vascular formations using arbitral-assembling techniques in 3D engineered tissues.[33] Arbitral-assembling techniques allow creation of circular or triangular shapes to be formed by scaffold shrinkage. Another report combined 3D bioprinting platforms and immobilized bioactive elements and cells into hierarchical constructs for multicellular tissue regeneration while facilitating the formation of a flexible vascular network around a hard scaffold mimicking bone.[34] Despite the great flexibility in bioprinting perfusable channels and the ability to facilitate angiogenesis, this technology still faces several challenges for clinical transplantation. Although endothelialization with tight junctions can be obtained, native blood vessels have other components, such as smooth muscle and adventitia providing sufficient mechanical strength. Without developing these components, mechanical stimulation can easily induce structural deformation in cell-laden perfusable hydrogels, possibly causing occlusion. Without possessing sufficient strength, the macrovasculature created by indirect printing cannot be sutured and anastomosed to recipient vascular pedicles.

In addition to efforts using temporary sacrificial materials, direct vascular network bioprinting has been demonstrated. For example, scaffold-free printing of vascular networks has been performed using tissue spheroids as building blocks (Fig. 4B1–B2).[10] Six days after deposition, tissue spheroids made of human skin fibroblasts (HSFs) completely fused and matured into vascular tissue with branches, demonstrating their ability to self-assemble. In addition to the scaffold-free approach, scaffold-based approaches have been extensively studied through the use of a coaxial nozzle apparatus (Fig. 4C), which allows direct bioprinting of vasculature with immediate crosslinking of sodium alginate bioink, generating a

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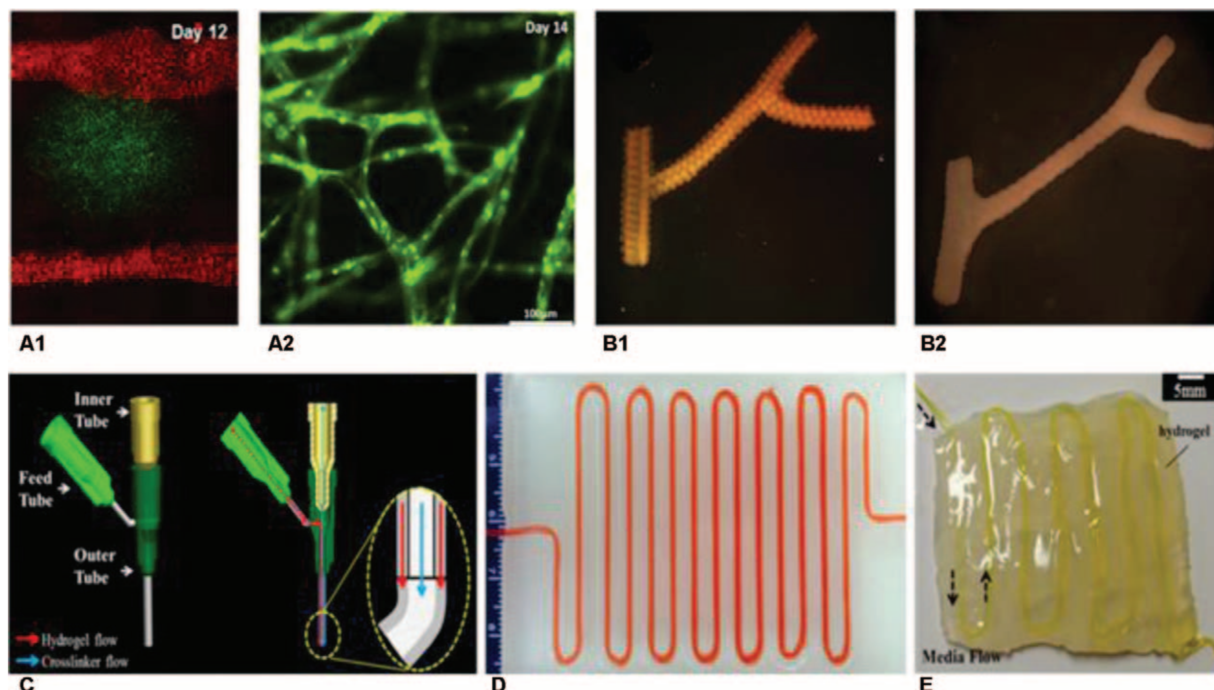

FIGURE 4. Examples of vascular network bioprinting: (A1) Fibrin scaffold with 2 vascular channels. RFP-HUVEC cells (red) in the channel during perfusion and GFP-HUVEC cells embedded within fibrin (green). (A2) Sprouting capillaries within fibrin scaffold. [Reprinted with permission of Springer from Lee VK, Lanzi AM, Haygan N, et al. Generation of a multi-scale vascular network system within 3D hydrogel using 3D bio-printing technology. *Cell Mol Bioeng*. 2014;7(3) with original copyright as given in publication in which original material was published. doi: 10.1007/s12195-014-0340-0]. (B1) Multicellular spheroids assembled into a branched vascular network before and (B2) after fusion (Reprinted with permission from Norette et al as reference above in 2A). (C) Schematic structure of coaxial nozzle apparatus composed of 3 parts: outer, inner, and feed tube (reproduced and adapted from Zhang Y, Yu Y, Chen H, et al. Characterization of printable cellular micro-fluidic channels for tissue engineering. *Biofabrication*, 2013;5(2):1–23. doi:10.1088/1758-5082/5/2/025004.). (D) Printed vasculature in a chamber under perfusion [Reprinted with permission from ASME Yu Y, Zhang Y, Ozbolat IT. A hybrid bioprinting approach for scale-up tissue fabrication. *J Manuf Sci Eng* 2014;136(6)] or (E) embedded within a multilayer hydrogel (reproduced and adapted from Zhang et al as referenced above in C).

smooth and continuous lumen of any desired length.[35] The anatomy can be determined by controlling the printing parameters. The shape of the vascular network can be mediated by loading fibroblasts and smooth muscle cells; and culturing in a perfusion chamber for a prolonged period (Fig. 4D).[36] Complex patterns have been printed with the vasculature easily integrated into larger-scale constructs while demonstrating greater than 95% cell viability over a week long culture (Fig. 4E).[37]

Postorgan Printing Stage

After the printing process, bioprinted constructs are highly fragile and not structurally coherent or integrated at a sufficient level to facilitate transplantation. Therefore, the postorgan printing stage is critical to obtain functional, mechanically stable, and innervated organs for transplantation. This cultivation period necessitates proper bioreactor technologies to enable mechanical and chemical stimulation, while allowing complex signaling to regulate organ remodeling and growth. Upon sufficient maturation and testing, fabricated organs can be transplanted into the patient, while monitoring functionality and in vivo safety parameters.

Organ Remodeling and Maturation

After the printing process, fabricated constructs need to be transferred into a bioreactor for long-term culture to facilitate cell growth, proliferation, vascularization, organ remodeling, and maturation. Maturation of printed tissue spheroids from a fluid-like state to a solid-like organ state is an essential step in the postprinting stage. It usually takes several months of bioreactor culture and conditioning to achieve a native-like state. Longer incubation times have demonstrated increased levels of adhesion molecules, ECM molecules, and collagen.[38] The kinetics of tissue fusion are determined by specific agents of maturation. Measuring the level of tissue overlap, via envelopment assays, can be used to test for tissue fusion, suggestive of maturation.[38] Complex organ systems such as vascularized constructs require perfusion culture,[39] which provides appropriate physical stimulation, continuous supply of nutrients and biochemical factors, and removal of by-products from cellular metabolism. Perfusion-based maturation assessment is, however, intensive and requires specialized equipment. Bioreactors may also facilitate sufficient electrical and mechanical stimuli to induce innervation and mechanotransduction, respectively. Bioprinted constructs mature with diverse biological and morphological changes, which are dependent on printing strategy and ink. For example, organs made using a scaffold-free approach mature differently than cells printed in hydrogels. Different cell aggregate-based bioinks experience a unique series of events during organogenesis.[3] Cells in pellet form when confined in a mold, adhere to each other to minimize free energy, facilitate cell to cell interactions, and form

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connections through cadherin-mediated adhesion.[40] Over time, cells deposit their own ECM, which promotes cell adhesion and generates contractile forces forming intact neotissues that are smaller than their original size. Cells continue to deposit ECM components such as elastin and collagen, which allows for an increase in tissue cohesion and mechanical properties. Following maturation, tissues nearly attain native morphology and physiology. If other cell aggregate-based bioinks are used, such as tissue spheroids and strands, fusion starts immediately through cross-migration of cells and ECM deposition into spaces between aggregates. In order to minimize the configurational energy during fusion, aggregates assume a more rounded geometry followed by ECM deposition and maturation toward a native-like morphology. On the contrary, cells printed in hydrogel are exposed to a different environment and exhibit a distinct chain of events during organogenesis. Cells attach to the scaffold matrix, proliferate, and deposit their own ECM. Meanwhile, they express proteinases including matrix metalloproteinases, which degrade the bioink material. As cells proliferate and the matrix around them degrades, changes are observed in the morphology and physiology of the organ. The intricate understanding of in vitro engineered tissue maturation is, however, still in its infancy.

Tissue engineering strategies seek to reinvent the complex organogenesis that initiates in utero. Internal organs develop from the ectoderm, endoderm, and mesoderm within the 3rd to 8th weeks in utero. This multifactorial process includes numerous signaling pathways,[41] soluble factors,[42] and differentiation guides[43] with germ layers differing by folds, splits, and condensation. For example, in lung development early asymmetrical bud formation orients tissues fusion by providing a scaffold for maturation,[44] whereas in the liver a diverticulum differentiates from a monolayer to a multilayer structure creating a bud.[45] Maturation of in vitro bioprinted cells to engineered tissues and organs is not trivial and is an ongoing area of intense research. It is likely to be affected by starting cell type and combination, culture environment, soluble factors, and the printing process itself.

Transplantation of Bioprinted Tissues and Organs

Surgical Challenges

Bioprinted organs will present challenges similar to allogenic organ transplants. Cellular ischemia limits cadaveric organ harvest necessitating an expedited time to implantation, which requires transport on ice and utilization of various flush solutions to prevent cellular edema, delay cell destruction, and maximize organ function after perfusion is reestablished.[46] Depending on the source of origin, the printed organ will suffer some degree of ischemia during transport. Ischemia, however, should be minimized as the transplant can be performed in an elective fashion for the printed organ and is usually performed urgently in the cadaveric setting. Once the organ has arrived to the operating room, for optimal surgical reanastomosis, the vascular pedicle must be uninjured and of an appropriate size match in both the recipient and printed construct. Bioprinted organs must offer parenchymal perfusability in line with a large caliber vascular pedicle, allowing for immediate reperfusion after vascular anastomosis. Likewise, the printed organ must be an appropriate size match for the perspective recipient. Preoperative imaging can be obtained to detail anatomic measurements so a precise bioprinted organ could be contrived regardless of sex and age. With the emergence of CAD technologies, an exact visceral dimensional match is possible as are supporting vascular, ductal, and airway structures. Bioprinted VCA transplants will require unique planning strategies to optimize composite tissue construct dimensions in line with aesthetic proportions, as required in face transplantation. Cooperation will be required between the surgeon and engineering team as CAD/Computer Aided Manufacturing software requires expertise that most clinicians may not possess, and will need to be formulated into preoperative planning stages.

Tissue Engraftment and Innervation

Bioinks must be able to integrate with the ECM of native cells without having negative interactions and allow for vascular ingrowth. The maximum nutrient diffusion distance for cell survival without vasculature is 100 to 200 μm.[26] Recently Kang et al[47] produced larger cartilage structures, which showed adequate tissue formation without necrosis in vivo using microchannels to extend this diffusion limit. Larger constructs require more robust vascular structures[26] for immediate parenchymal perfusion; however, for the transplant to become fully engrafted it will require angiogenic integration with the recipient microcirculation. Benjamin et al[48] designed highly interconnected 3D microvascular channels for bone scaffolds that facilitated microvascular integration upon implantation and exhibited bone-like physical properties. Although it is currently infeasible to print capillaries due to their scale, it may be feasible to print macrovascular channels (~100 μm) while relying on angiogenesis to create the finest connections.[2] Recruitment of circulating stem cells into ischemic sites is achieved by targeted activation of concerted pathways or cell seeding into constructs. In this respect, Tasso et al[49] reported that mesenchymal stem cells seeded into porous cubes resulted in homing of pericyte-like cells or circulating endothelial progenitor cells (EPCs) after implantation in vivo. Homing can also be performed using growth factors such as platelet-derived growth factor. This allows EPCs to further differentiate into an endothelial cell lineage followed by sprouting of capillaries from the host to the construct. Additional angiogenic factors include vascular endothelial growth factor, fibroblast growth factor, and transforming growth factor.[50,51] Many vascular network forming cell sources like HUVECs are used in tissue engineering research, although they are not available to the patient. Thus, alternative sources such as autologous circulating EPCs, iPSCs, and postnatal stem cells are being continually experimented with to produce stable and mature 3D capillary networks.[51] Ischemic resilient organs such as kidney may be more amenable to this strategy than liver and pancreas.

Hollow channels in tissue constructs may also serve as conduits for nerve regeneration. Currently, during transplants, the organ is denervated; however, it does recover some nerve function over time. Histologic staining of transplanted kidneys shows evidence of reinnervation starting early after transplant and continuing for years. In orthotopic heart transplants, it has been shown that partial sympathetic reinnervation increases with time and was seen in 40% of patients 1 year postoperatively.[52] Therefore, the potential for reinnervation can be designed into the bioprinted construct to at least replicate and possibly augment what is seen with current allogenic transplants. Recently, cardiac bioimplants in swine showed new nerve fibers and neovessel formation in the scaffold when studied by magnetic resonance imaging.[53] Monocytes and their derivatives are critical for supporting tissue integration and regeneration of implanted tissues. Monocytes differentiate into macrophages upon leaving the bloodstream and entering the parenchyma. Treatments preventing macrophage infiltration into implanted materials demonstrated significantly reduced neovessel formation. This may prove to be a critical interaction for bioengineered tissues.[54]

Macrophages can have both positive and negative roles in tissue remodeling depending on their polarization toward an M1 or M2 phenotype. Studies suggest that M2 is the anti-inflammatory phenotype.[55] A positive correlation has been observed between the proportion of M2-polarized macrophages and positive remodeling outcomes such as vascularity, tissue organization, cellular infiltration, and degree of encapsulation.[54]

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Immunosurveillance

3D bioprinted organs have the ability of using either autologous primary or stem cells, which may avoid some of the negative immune response after implantation; however, biomaterials can acquire a layer of host proteins, which modulate the immune response by interaction with inflammatory cells. Macrophages mediate the adhesion of foreign body giant cells to the surface and both cell phenotypes can release mediators of degradation. Studies have shown the importance of antioxidants to inhibit the foreign body reaction leading to degradation of the implant.[56,57] Testing of cytokines may be the initial evaluation of biocompatibility as macrophages on biomaterials that do not promote integration secrete higher levels of proinflammatory cytokines including interleukin-1 beta (IL-1β) and IL-6.[56,57] Degradable polar hydrophobic ionic polyurethane (D-PHI) attenuates pro-inflammatory cytokine release of tumor necrosis factor alpha (TNF-α) and IL-1β, and promotes release of the anti-inflammatory cytokine IL-10. Battiston et al[58] cultured monocytes on D-PHI, which demonstrated increased cell growth and ECM deposition. D-PHI limits the exposure of the fragment antigen-binding region of the immunoglobulin G molecule.[58] An understanding of how biomaterials interact with proteins is essential for monitoring cellular response and bioimplantation success.

Postoperative Monitoring

Traditionally, transplant patients are placed in the intensive care unit postoperatively to manage fluid shifts and electrolytes. Immunosuppressive agents are started early and adjusted based on blood levels and functional status of the transplanted organ; however, 3D bioprinted organs utilizing autologous cells may not require immunosuppression. Although infections are commonly noted in allogeneic transplants secondary to the effects of immunosuppression, they may prove to be troublesome in printed tissues as well. Nosocomial infections are the fourth leading cause of morbidity and mortality and greater than 60% are associated with biomedical devices or implants.[59] Staphylococcus epidermidis is the most prevalent organism, which is in part attributed to its formation of biofilms. During the in vitro tissue fabrication process, there may be multiple instances in which either the biomaterial or cellular component can be contaminated, leading to a unique type of nosocomial infection. This would mandate aggressive postoperative monitoring for infection, such as fever, increased white blood cell count, and organ failure. Antimicrobial therapy would need to be initiated promptly. Overall, organ function can be assessed by established laboratory testing. For example, International Normalized Ratio and bilirubin can be used to assess liver function and blood urea nitrogen and creatinine to assess kidney function. Any suggestion of inappropriate function would need to be evaluated by additional imaging or diagnostic modalities. Because of many uncertainties, this novel technology would require the initial patient cohort to undergo close monitoring and follow-up similar to or more stringent than traditional transplant patients.

FUTURE PERSPECTIVE

In Situ Bioprinting

Bioprinting holds significant prospect for incorporation into clinical practice, which can be enabled through implementation of safe and sterile processes. In situ bioprinting, defined as direct printing of living tissue constructs into the defect site in an operative setting,[2] holds tremendous clinical promise to repair body parts directly. The process entails bringing a bioprinter into the desired surgical field in a well-coordinated fashion to prevent a breach in the sterile process. Sterilization and safety of the printer must be rigorously tested before being implemented into clinical scenarios. Its major advantage is that it provides fundamentally, highly advanced robotic systems that can print different cell types in tandem while positioning them precisely in predefined anatomic locations. Autologous cells can be obtained intraoperatively and used to prepare a bioink for immediate treatment. The printing system can be integrated with a 3D scanner to scan the defect area, acquire images, and generate a printing plan for robotic movement and deposition.

Successful application of bioprinters into clinical practice will require a product that is simple, easy to use, and seamlessly integrates into the operative process. The operative field is a complex environment that incorporates several functioning elements, which have to work in unison. The process has to be safe, efficient, and capable of adjusting in real time. Several variables need to be accounted for including minor changes in positioning, tight surgical quarters, and the ability to adjust for changes in the printing field, such as clearing fluid accumulation.

In situ bioprinting has several advantages. It is an efficient process in that the scanned defect can be repaired rapidly while minimizing surgeon manipulation. Manual interventions, such as implanting prefabricated scaffolds can alter the shape due to swelling, contraction, or deformation. In contrast, in situ bioprinting enables precise positioning of cells, genes, or cytokines. This technique offers multiple applications such as craniofacial reconstruction, soft tissue repair, and composite tissue printing. Figure 5 presents the concept of in situ bioprinting for cranial bone regeneration. A multiarm bioprinter can be used to deposit an ink solution, including cells, biomaterials, and biofactors (ie, plasmid DNA) directly into cranial defects. This technology is at its infancy and further research is needed before routine clinical implementation can be achieved.

Clinical Translation Practice

Ethical Dilemmas

Bioprinting and in vitro replication of cell lines are quickly evolving the field of regenerative medicine as demonstrated by recent achievements in bladder engineering,[60] airway regeneration,[61] and multilayered skin fabrication.[62] Although clinically exciting, these advances stress established ethical and legal standards. Bioprinting typically commences by obtaining a digital scan (eg, a CAD model) of the patient with the data converted for use in computer-aided manufacturing. This provides the practitioner and engineer extensive medical information, which the patient may be unaware of. The large reservoir of digital data shared among many participants has the potential for loss of control and breaches in confidentiality.[63] Furthermore, patient-specific digital data may be used as a template for unrelated individuals without proper permission and leads to the inquiry of who owns it. Cellular materials also represent areas of ethical and legal concerns.[64] Because the immortalization of the HeLa cell line taken without consent[65] from Henrietta Lacks in the 1950s, the ethical integrity of in vitro expansion has been questioned, especially when commercial development from discoveries is entertained. Stem cells have the potential for endless self-expansion and differentiation, and therefore represent an ideal raw material for organ fabrication. This, however, allows for loss of possession and control of the cell line, relegating the donor to nothing more than a repository. In vitro expansion also provides an opportunity for loss of cellular genetic integrity, and the potential for unconsented genetic sampling through next-generation sequencing technologies.[63] Because most endeavors are currently taking place in academic settings, ultimate progression to commercialization and financial incentives will further complicate the ethical scenario. Patients, physicians, universities, and biotechnology companies each derive

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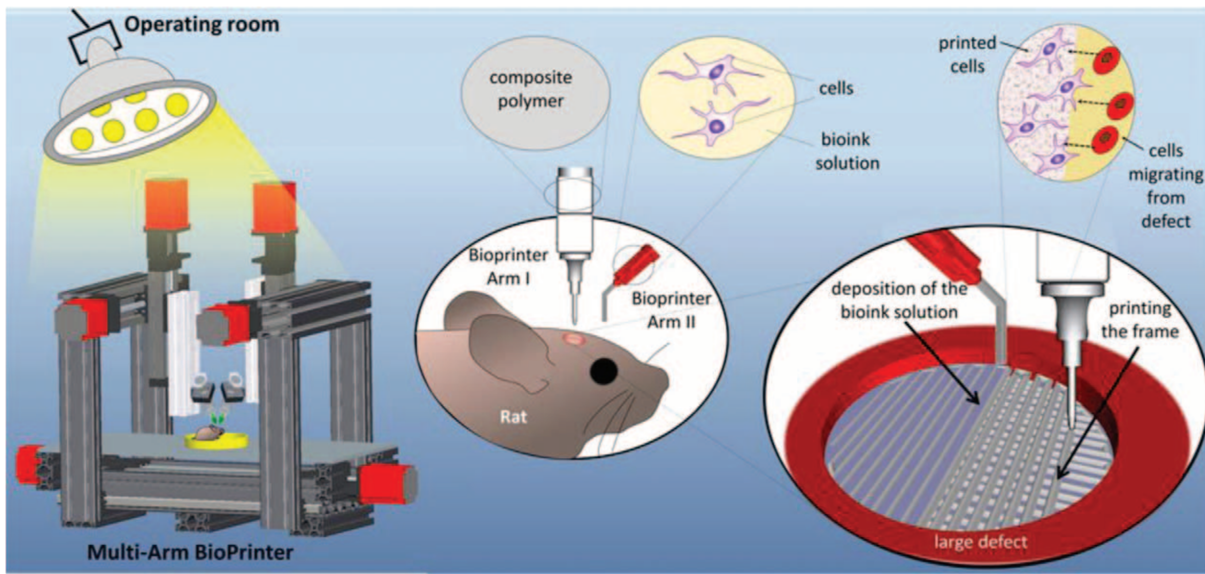

FIGURE 5. In situ bioprinting directly into the defect site: tissue constructs printed directly into a rat cranial defect model. Cells with bioink solution are printed in combination with a composite polymer from 2 separate arms. Over time, native cells also migrate to help fill in the defect.

different benefits from the fabrication process and the ethical roadmap has yet to be developed.

Regulatory Issues

Tissue and organ printing relies on biomaterials, cellular matter, and fabrication platforms for success. Because of its versatility, 3D bioprinting applications may require FDA regulation of devices, biologics, and/or drugs. Biomaterials are substances that can be implanted into a patient without rejection and include hydrogels, polymers, and ceramics. These materials need to be nontoxic, strong, and amenable to vascularization and ultimate tissue integration. Starting materials need to be adapted for the desired bioengineered construct and appropriateness for human implantation. Standalone biomaterials have commonly been regulated as devices by the FDA as demonstrated by the approval of injectable poly-L-lactic acid (Sculptra, Sanofi-Aventis) in 2009. Human stem cells (ie, iPSCs, ADSCs) and desired differentiated lines will likely need to be harvested and nurtured in vitro before implantation for medical treatment. In 2004, the FDA published "Current Good Tissue Practice for Human Cell, Tissue, and Cellular and Tissue-Based Product (HCT/P) Establishments; Inspection and Enforcement" (69 FR 68612). This rule requires HCT/P establishments to follow current good tissue practice, which governs the methods used in, and facilities and controls used for manufacture of HCT/Ps; recordkeeping; and establishment of a quality program. Under this, rules were also established for certain "core current good tissue practice requirements" [21 CFR 1271.150(b)] that are directly related to preventing the introduction, transmission, or spread of communicable diseases.[66] Antimicrobials and other pharmacologics are likely to be used in bioprinting and thus represent another potential area of FDA oversight. A number of fairly simple 3D printed medical devices have received the FDAs 510(k) approval[67]; however, this may not be identical to utilizing the technology for regenerative medicine applications, which will likely be subjected to more demanding regulatory requirements. Regenerative products focus on the repair, replacement, or regeneration of damaged tissues and organs, which distinguishes them from other medical products. A unanimously accepted concept of medical products derived from tissue engineering and regenerative medicine has yet to be formulated. Several terms have been proposed including "tissue-engineered medical product (TEMP)" and several definitions have been ascribed. The term TEMP has been defined in a standard document of the American Society for Testing and Materials.[68] TEMP is defined as "a medical product that repairs, modifies, or regenerates the recipient's cells, tissues, and organs or their structure and function, or both." This terminology has been included in the FDA-recognized consensus standards database. In this definition, what comprises TEMPs is not clarified as they derive their therapeutic potential from various components used alone or in combination.[69] If a TEMP is only composed of cells, biomaterials, or chemicals alone, it might be correspondingly thought of as a biological product, medical device, or drug. Regenerative medical applications are likely to be an amalgamation, which, however, may be composed of any combination of drugs, devices, and biological products. TEMPs will ultimately need randomized controlled trials to demonstrate risks and benefits, which could present a barrier to wide-scale applicability. In addition, manufacturing regulations and state legal requirements could impose additional obstacles. The persistent ambiguous nature of the technology and evolving field has spawned the development of FDA working groups to assess technical and regulatory considerations regarding 3D bioprinting. Currently there are, however, no manufacturing standards for the bioprinting process. The current lack of regulatory oversight needs to be rectified before utilization on a clinically relevant scale and has led to speculation that the process may be banned in the future secondary to fierce ethical debates.[70]

Cost Aspect

One major concern about the development of personalized regenerative medicine is the uncertainty of cost. Commercial bioprinters currently cost \$5 to \$350k and additional obligate devices for tissue fabrication are also expensive.[71] The complexity of the desired tissue or organ will, however, ultimately determine final manufacturing cost. Requisite fees associated with cell acquisition

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TABLE 1. Medicare Transplant Costs (2012)

Organ	Medicare Part A	Medicare Part B	Percentage of Total Expenditure (%)
Kidney	1,927,726	860,208	63
Pancreas	156,928	67,072	5
Liver	509,566	159,385	15
Lung	231,328	53,901	6
Heart	345,151	104,428	10
Intestine	36,611	12,305	<1

Total cost in $1000 units for all patients alive with graft function in 2012 and percentage of total expenditure of transplant recipients. Costs incurred after transplant failure are excluded (adapted from Schnitzler MA, Skeans MA, Axelrod DA, et al. *OPTN/SRTR 2013 Annual Data Report: Economics*).

and processing, scaffold manufacturing, bioreactor maturation, surgical implantation, and postoperative care will be among the major expenditures. As with any new scientific advances, the cost will likely decrease as the technology evolves and becomes more prevalent. It is, however, quite likely that the process could actually be used to help reduce the cost of organ transplantation and its mandatory immunosuppression in the long term. The overall Medicare cost on recipients of solid organ transplants was approximately $4.5 billion in 2012, where Part A and Part B Medicare expenditures are seen in Table 1.[72] These numbers underestimate the true costs as private insurers and out of pocket expenses are omitted. When factoring in pretransplant life-saving therapies such as dialysis and ventricular assist devices, the overall cost of end-stage organ failure is much greater. For example, per-person per-year dialysis-related costs in the United States were on average $87,272 in 2011 with Medicare-related total end-stage renal disease expenditures (including Part D) of $34.4 billion.[73] These numbers omit individuals awaiting VCA transplants, a therapy which is expected to substantially increase in the future and does not take into account lives lost waiting for a transplant. Although it is currently impossible to determine the exact economic effect of this technology, it will likely alter the financial landscape of organ and tissue replacement significantly.

CONCLUSIONS

This article presents clinical translation practices of emerging bioprinting technology, which has made a substantial leap in the last decade. Advances have been made in demonstration of scaled-up tissue and organ constructs, integration of vascularization, and potential for operative translation. Despite progress, clinical implementation has been curtailed due to technical challenges in fabrication of human-scale, vascularized, and physiologically relevant constructs along with clinical, economical, and ethical obstacles. The authors, however, envision that, with increasing clinical demand and the current momentum in bioprinting, translation into clinical practice can be expected in the near future.

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