

## THEME | Making Cell Culture More Physiological

# Bioprinting stem cells: building physiological tissues one cell at a time

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**Scognamiglio C, Soloperto A, Ruocco G, Cidonio G.** Bioprinting stem cells: building physiological tissues one cell at a time. *Am J Physiol Cell Physiol* 319: C465–C480, 2020. First published July 8, 2020; doi:10.1152/ajpcell.00124.2020.—Bioprinting aims to direct the spatial arrangement in three dimensions of cells, biomaterials, and growth factors. The biofabrication of clinically relevant constructs for the repair or modeling of either diseased or damaged tissues is rapidly advancing, resulting in the ability to three-dimensional (3D) print biomimetic platforms which imitate a large number of tissues in the human body. Primary tissue-specific cells are typically isolated from patients and used for the fabrication of 3D models for drug screening or tissue repair purposes. However, the lack of resilience of these platforms, due to the difficulties in harnessing, processing, and implanting patient-specific cells can limit regeneration ability. The printing of stem cells obviates these hurdles, producing functional *in vitro* models or implantable constructs. Advancements in biomaterial science are helping the development of inks suitable for the encapsulation and the printing of stem cells, promoting their functional growth and differentiation. This review specifically aims to investigate the most recent studies exploring innovative and functional approaches for the printing of 3D constructs to model disease or repair damaged tissues. Key concepts in tissue physiology are highlighted, reporting stem cell applications in biofabrication. Bioprinting technologies and biomaterial inks are listed and analyzed, including recent advancements in biomaterial design for bioprinting applications, commenting on the influence of biomaterial inks on the encapsulated stem cells. Ultimately, most recent successful efforts and clinical potentials for the manufacturing of functional physiological tissue substitutes are reported here, with a major focus on specific tissues, such as vasculature, heart, lung and airways, liver, bone and muscle.

biofabrication; bioink; hydrogels; physiological 3D models; stem cells; 3D bioprinting

## INTRODUCTION

There is an increasing need for clinical alternatives for the regeneration and repair of damaged or diseased tissues. The ability to restore tissue functionality has been pursued for more than two decades (78) via tissue engineering and regenerative medicine (TERM) approaches. Typically, TERM programs can deliver implantable constructs, resulting from the *in vitro* culture and maturation of living cells encapsulated in scaffolding-degradable materials (biomaterials). Terminally differentiated cells can be isolated from patients, but their tissue-specificity, defined function, poor *ex vivo* proliferative ability and most importantly, their rejection to allogenic transplantation have been major limiting factors for their use in TERM. Stem cells are particularly attractive for their ability to self-renew and, most importantly, differentiate into specific cell types when being exposed to specific compounds or included in biomaterials that can influence their ultimate fate (89).

Novel biofabrication technologies that harness the potential of stem cells have come to the fore recently, tackling TERM limitations in 1) providing an architectural and hierarchical arrangement of the cell-biomaterial complex, 2) patterning living cells in three-dimensional (3D), and 3) mimicking the high degree of complexity within tissue organization.

Crucially, bioprinting approaches have shown promise for the generation of functional tissue constructs and the repair of critical size tissue defects. Indeed, 3D printed cell-laden constructs mimicking closely the geometry and the complexity of human tissues (26), can generate a physiological implant for the repair of damaged or diseased tissue.

In this review, we analyze the key biofabrication approaches for 3D printing of physiological tissue constructs as substitutes for native counterparts for regenerative or disease modeling purposes. We illustrate the necessity of bioprinted constructs to follow a similar tissue formation and maturation process as embryogenesis. We introduce different bioprinting approaches and detail biomaterial ink features used for the encapsulation and the printing of stem cells. Crucially, we aim to provide a comprehensive review of the most advanced 3D platforms

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fabricated for the regeneration and modeling of a selected number of human tissue. Thus, we highlight pivotal and novel studies on translational bioprinting approaches for the fabrication of vascular, cardiac, respiratory, hepatic, skeletal and muscle tissue substitutes.

### Tissue Physiology and Stem Cells

A tissue is an organized cellular complex with a specific predetermined function in the body. Cells within a particular tissue, typically share the same embryonic origin and morphological features. The great challenge biologists and engineers have faced over the decades, has been the provision of functional solutions for the repair and replacement of tissue structures whose shape and function are strictly related. The idea (and hope) that damaged tissue—unable to self-repair through intrinsic mechanisms—could be fully renewed or replaced is the premise of TERM. Following damage, a number of human tissues typically activate innate repair mechanisms, which are largely involved in processes that occur during embryogenesis (83). Hence, an understanding of how tissues and organs originate, from a single cell, is essential to imitate these processes and to attempt to successfully engineer new functional implants.

Stem cells are the fundamental building blocks for organ formation and repair. The ability to make identical copies of themselves (self-renew) and to differentiate into multiple types of cells in the body, position them as the perfect candidates to

support TERM technologies (13). The potency of stem cells measures their ability to diversify terminally differentiated cells as key components of multiple tissues. Their location, origin, and potency allow categorization in three major classes.

Embryonic stem cells (ESCs) exhibit unlimited proliferative capacity and are directly responsible for the formation and growth of the embryo, differentiating into any specialized cell of the human body. ESCs derive from the inner cell mass of blastocysts (Fig. 1A) and, during the subsequent gastrulation phases, give rise to three different germ layers to form the outer ectoderm, the inner endoderm, and the interstitial mesoderm (109), which are primarily involved in the generation of any tissue in the human body.

However, because of their restricted availability, safety, and ethical issues, ESCs offer limited use for the printing of functional tissue constructs (15).

Adult stem cells are limited in their ability to differentiate into a selective number of tissues. Adult (somatic) stem cells can grow, maintain, regenerate, and repair the tissue within which they reside (Fig. 1B) (15, 109). Thus, adult stem cells are highly tissue-specific and present a number of advantages compared to ESCs. For instance, mesenchymal stem cells (MSCs), typically reside in the bone marrow and can differentiate into cell types, such as bone and cartilage (20). The ability of adult stem cells to differentiate into specific cell types can facilitate the fabrication of tissue-specific implants since the implanted cells will more likely acquire a similar gene-

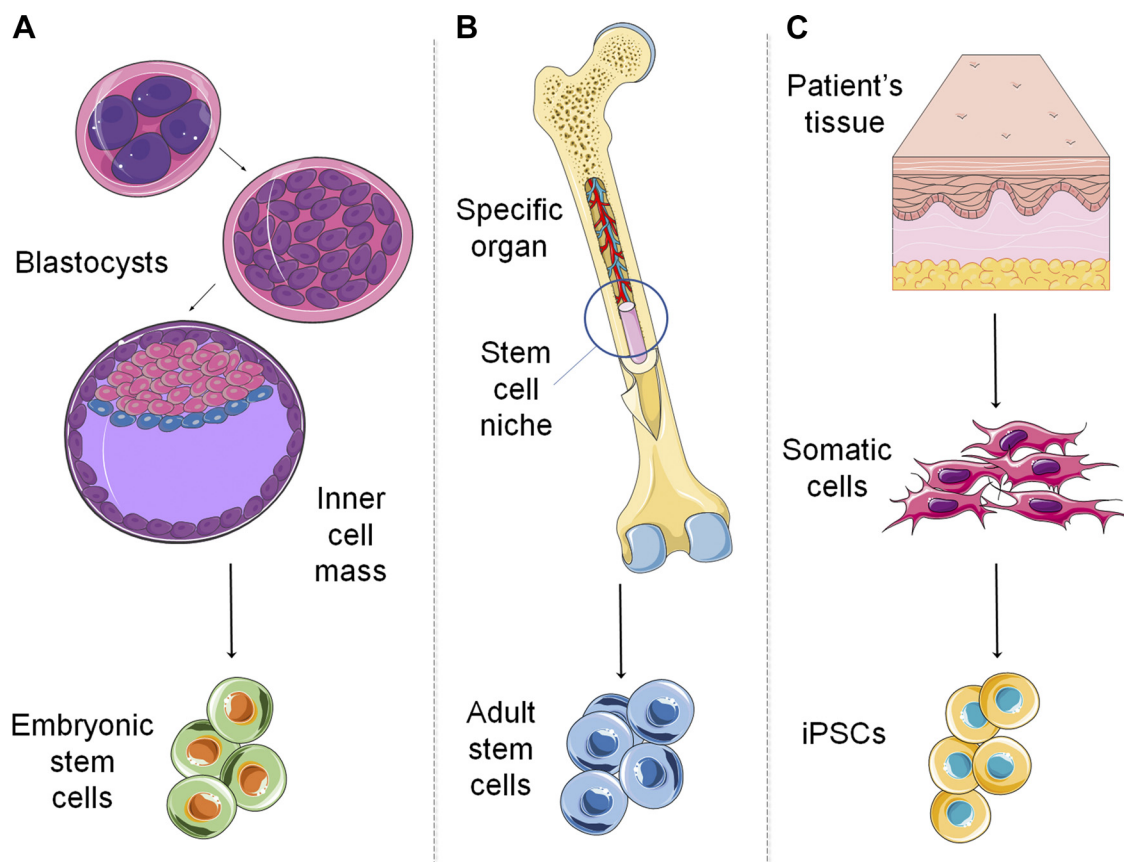


Fig. 1. Major classes of stem cells. Embryonic stem cells (A) originate from the inner cell mass of blastocysts, holding the potential to differentiate in every cell in the body. Adult stem cells (B) reside in a specific tissue, actively participating in the repair of multiple tissues. Induced pluripotent stem cells (iPSCs) (C) can be reprogrammed from somatic cells easily isolated from the patient's tissue with ease of access (e.g., skin).

expression profile to the terminally differentiated cells present in the native tissue (15). However, adult stem cells are sparsely found in tissues and their expansion is impaired or limited by their proliferative ability compared to ESCs.

An alternative solution is represented by induced pluripotent stem cells (iPSCs), which are embryonic-like stem cells generated from somatic cells that have been reverted down the differentiation pathway using the overexpression of specific transcription factors (Fig. 1C). The recent discovery of iPSCs (129) is aiding the 3D printing of complex tissues, overcoming adult stem cell limitations, such as poor availability in terminally differentiated organs and an inability to differentiate into cells of every tissue in the human body. The number of studies involving the printing of iPSCs is on the rise, given the tremendous capacity of iPSCs to generate patient-specific models (47, 63).

To date, stem cells have been widely employed as injectable therapies for the treatment of a plethora of diseased or damaged tissues (87). TERM, and more recently bioprinting, are using stem cells to fabricate functional constructs for the repair or modeling of tissues of interest.

#### *Bioprinting: Tools for Engineering Physiological Tissues for Tissue Repair or Disease Modeling*

TERM aims to harness the potential of stem cells to support and guide the formation or the regeneration of specific tissues (78). The use of biomaterials as supporting matrices has been proven essential for the delivery in vivo of seeded or encapsulated stem cells. Biomaterials must be able to support stem cell viability and growth, promoting or guiding their differentiation toward the desired tissue. The inclusion of cells within these matrices has proved revolutionary in the development of physiologically relevant tissue substitutes compared to classical two dimensional (2D) cell culture approaches.

Indeed, 2D cell cultures have been extensively exploited as in vitro platforms for cell expansion and implantation, and drug screening/discovery, leading in some cases to misleading results (93). It is now well accepted that in vitro 2D assays lack the physiological features related to the spatial organization and biochemical interaction of cell populations in the body. Indeed, the absence of the spatial cell arrangement and interaction in the tissue can impair their functionality, altering physiological structure and function of the human organ. For instance, the hollow cavity of a blood vessel, the long cylindrical fibers of a skeletal muscle, and the layered structure of the skin are but a few structural and geometrical aspects that must be recreated in implantable constructs or tissue-specific models for the preclinical testing of active drugs.

Animal models have been widely employed for the validation of the safety and efficacy of drugs or implants. However, in vivo animal models have been found to poorly recapitulate human pathophysiology due to interspecies differences (10). Thus, the incomplete (albeit fundamental) support of animal models requires the development of novel techniques and culture protocols for the design of more complex implants and in vitro surrogates, where the intrinsic interplay of structure and function observed in nature is preserved.

Biofabrication has recently come to the fore as a versatile platform for the deposition of living cells, growth factors (GFs), and biomaterials in 3D for the fabrication of functional tissue substitutes (Fig. 2). Living adult or reprogrammed stem

cells embedded in biomaterial inks (bioinks) that closely resemble the native microenvironment, can be printed generating a 3D construct that can be further cultured in vitro with the supplement of GFs for stem cells differentiation and implantation into the patient. Alternatively, the printed functional construct can be used to model a specific disease, in vitro, for the testing of safety and efficacy of novel drug compounds.

To date, the use of acellular material inks has been extensively exploited for the printing of implantable constructs that could support damaged tissue regeneration. Nevertheless, the absence of cellular components can impair tissue growth and maturation following implantation. Cell-printing strategies offer promising biofabrication approaches for the manufacturing of biomimetic constructs (26). The deposition of bioinks in 3D can ultimately recapitulate the architecture of a specific tissue, with physiological cell/cell and cell/extracellular matrix (ECM) interactions (94). The bioprinting field is rapidly evolving, currently able to fabricate numerous biological substructures such as peripheral nerve (58), bone (2), kidney (53, 70), muscle (104), heart (101) and vasculature structures (131). Computer-aided design (CAD) supports the versatile ability of bioprinting approaches to manufacture tissue-specific 3D structures. Typically, a syringe dispenser (or a laser) is moved to deposit (cure or remove) material in a layer-by-layer fashion, finally obtaining a 3D structure identical to the blueprint. Currently, three main bioprinting strategies (Fig. 3) are established and widely employed for stem cell deposition: inkjet (5, 72, 82), laser-assisted (66, 125) and extrusion-based (62, 90, 100, 107) bioprinting.

#### *Laser-Assisted Bioprinting*

This technique uses a pulsating laser that interacts with a donor-slide to transfer the material to a co-planar collector slide. The vaporization mediated by the laser on the energy-absorbing layer induces the formation of microbubbles that can expand and, consequently, propel a small (picoliter) droplet of biomaterial ink to the receiver slide. The programmed motion of the laser guides the patterning of droplets and the final printing (Fig. 3A). The droplet size is smaller compared to other bioprinting techniques capable of fabricating 20  $\mu\text{m}$ -resolved objects. Laser-assisted bioprinting platforms typically generate a pulse repetition frequency in the range of 1–100 kHz, resulting in a scanning speed in the range of 2,000 mm/s (65). Laser-assisted bioprinters are nozzle free and therefore suitable for high-viscosity materials (1–300 mPa/s) and particularly useful for reducing cell damage during printing (73). This technology is rapidly evolving toward the physiological printing of functional tissues with different mechanical properties such as corneal (125) and bone (66) tissues.

Despite the high resolution achieved with laser-assisted bioprinting, numerous drawbacks limit its use. The cost of the system is higher compared to other printing methods and, the preparation of the hydrogel system to be housed on the donor slide is complex, especially when multiple cell types need to be deposited with the precise targeting and positioning of cells in need for additional technologies (48).

#### *Inkjet Bioprinting*

Inkjet bioprinting is a low-cost, noncontact, drop-on-demand approach. Initially known as cytoscribing (72), this platform was a modified version of commercial 2D ink print-



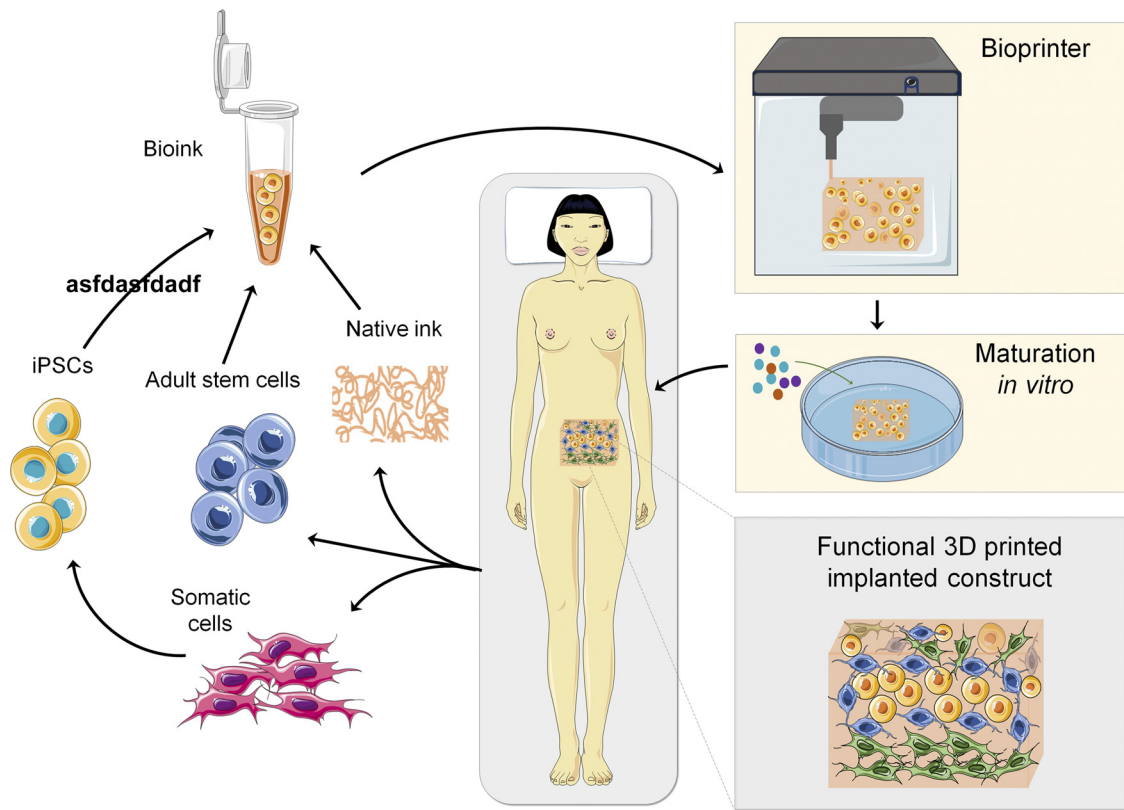


Fig. 2. The rationale behind 3D bioprinting personalized regenerative medicine approaches relies on patient-specific tissue engineering and stem cell culture platforms. Adult stem cells can be extracted from organ-specific niches. Alternatively, somatic cells can be isolated from the patient and directed toward an embryonic-like state exemplified by induced pluripotent stem cells (iPSCs). Native inks can be derived via decellularization of the patient's tissue matrices. A cell-laden ink is then used as a bioink for the 3D printing of a functional construct, cultivated *in vitro* with the addition of growth factors, subsequently implanted in the patient's body after a maturation period.

ers. Recently, inkjet bioprinting has been exploited for numerous biological applications, including the deposition of cell adhesion proteins for cell patterns, fabricating DNA microarrays (102), and printing cell-laden inks on bio-papers (119,

137). Inkjet-based bioprinters commonly deposit small volumes (10–150 pL, 10–70 μm wide) of drop-like material by applying thermal (124) or mechanical (108) force (Fig. 3B). Moreover, such bioprinters can deposit up to 10,000 droplets

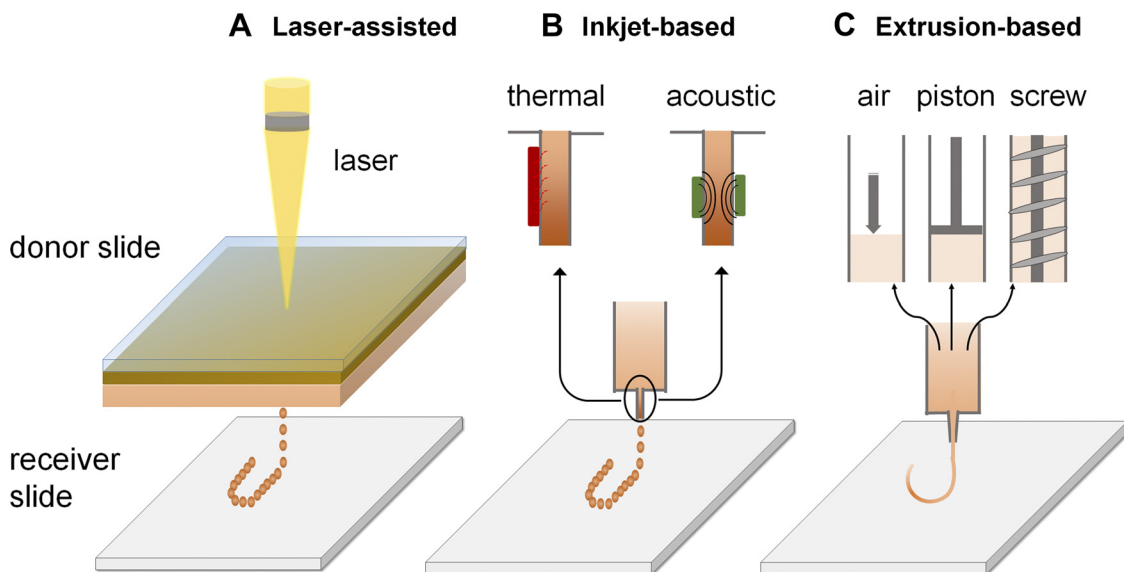


Fig. 3. Current bioprinting approaches. *A*: laser-assisted. A laser transfers a drop-like material from a donor to a receiver slide. *B*: inkjet-based. Droplets of bioink are deposited on the substrate by applying thermal force or acoustic waves at the dispenser's tip. *C*: extrusion-based. A fiber is deposited on the substrate by depositing/extruding the bioink through a fine nozzle with different mechanisms, such as compressed air (pneumatic), or a piston/screw (mechanical).

per second (96), depositing up to  $10^4$  droplets per second (96), thus performing at elevated printing speed (50). Thermal inkjet bioprinting delivers localized heat at the nozzle tip to trigger the collapse of air bubbles causing the rapid pressure-driven deposition of material ink. Even when high temperatures are imposed (200–300°C), the bioink is heated only for a few microseconds (136), resulting in a temperature increase of 4–10°C (42). The mechanical inkjet bioprinting relies on a piezoelectric element positioned at the printhead, which can generate acoustic waves to deposit droplets of bioink whose size is defined by the acoustic parameters, i.e., pulse duration, amplitude, and frequency (33).

The deposition of living cells via inkjet bioprinting has been observed to be functional for the simultaneous printing and stimuli of cells via heat-shock protein overexpression for the modulation of angiogenesis and the following implantation in the host tissue (124).

Despite the high performance in terms of resolution, process speed and cell viability, this technology possesses numerous drawbacks for printing tissue constructs such as uneven droplet distribution, lack of directionality during ejection, and frequent nozzle blockage (96), which limit the printing of functional tissue constructs.

#### *Extrusion-Based Bioprinting*

Extrusion-based bioprinting is an efficient biofabrication strategy, due to its high process speed and flexible printing platform (64). Biomaterial inks loaded in cartridges are physically extruded through a dispenser tip, which continuously deposits the material in 3D (40) (Fig. 3C). The bioink sol-gel transition immediately after deposition can be guided using 1) an ultraviolet or visible light source for photo-cross-linked inks (25), 2) a temperature-controlled deposition bed for materials with temperature-dependent viscosity transition (121), and, 3) a coagulation bath or coaxial needle for ionic crosslinking solutions (30). Extrusion-based bioprinters can fabricate 3D constructs at a slower speed compared to laser and inkjet approaches, in the range of 6–10 mm/s (30, 86). The dispenser orifice size and the extrusion rate greatly influence cell viability and printing resolution ( $>100 \mu\text{m}$  (30, 118)). The print head size and the bioink flow rate are limited by material viscosity: higher viscosity and smaller dispenser tip can induce larger shear stress at the nozzle, greatly impairing cell viability (21). Shear-thinning inks can be engineered to obviate this problem. These materials exhibit a liquid-like behavior under pressure, facilitating flow through the print head and regaining their shape after removal of the shear force (85, 105). Indeed, extrusion-based bioprinting is capable of depositing shear-thinning inks with high precision, being able to fabricate hierarchical constructs made of different types of materials and/or cells closely resembling the physiology of specific tissues (62).

While extrusion-based printing presents crucial advantages over other bioprinting platforms (compatibility with high cell-density and deposition efficiency), the resolution achieved is significantly lower, limiting the ability to generate biomimetic constructs. Moreover, low-viscosity inks are not printable via canonical microextrusion technologies as it results challenging to preserve a continuous filament during deposition. However, high- and medium-viscosity inks have a significant influence

on stem cell viability due to the imposed shear stress. Therefore, the engineering of biomaterial inks capable of supporting stem cell viability and functionality is crucial for the fabrication of clinically relevant tissue substitutes.

#### *Microfluidic Bioprinting*

Recently, novel hybrid bioprinting technologies are coming to the fore to solve numerous disadvantages of common biofabrication platforms and provide a superior printing ability to fabricate more complex and biomimetic 3D constructs.

Microfluidic bioprinting is a step-changing technology, fast apace, which harness the ability of microfluidic chips to assist the control of fiber formation, extrusion, and deposition, allowing the precise patterning of cells and biomaterials in 3D (99, 127). Multihead extrusion bioprinting is used for the printing of multiple materials to produce hierarchical tissue models. However, the use of multiple heads makes the entire process slow and complicated, increasing the time of printing by the number of printheads employed. Microfluidic bioprinting platforms, in less than a decade, have demonstrated excellent potentials for the realization of sophisticated structures where the patterned multideposition of bioinks and/or cell types is performed with high control and precision (30, 55, 77, 127).

A recent study presented a bioprinter able to simultaneously or individually deposit up to 7 different materials with a fast and smooth switching for rapid construction of complex hierarchical tissue models (15 times faster than existing nozzle-based platforms) (86). A single printhead, made from a bundle of seven capillaries, each connected to a bioink reservoir and individually controlled by a pneumatic pressure valve, is employed to 3D print a ring-like scaffold using GelMa and Alginate, recapitulating the gradient of hydroxyapatite (HAp) concentration found in bone.

A wide library of biomimetic construct has been fabricated in the last decade, including pancreatic islet (59), glioblastoma (80) iPSC-derived neurons (132), iPSC-derived cardiomyocytes (90), and cartilage (77). Ultimately, microfluidics offer the intrinsic advantage of scaling down the components used to store and manipulate the bioinks, resulting in higher precision and enhanced efficiency of deposition. Nevertheless, this hybrid platform is still in its infancy and requires further effort in developing better bioinks and improving the ultimate applicable functionality.

#### *Inks for Printing and Sustaining Growth and Differentiation of Stem Cells*

The design of a specific biomaterial able to host and stimulate tissue-specific cells is paramount in printing physiological tissue substitutes. In the human body, cells are embedded in their self-designed material, the ECM, a complex of proteins that provides functional and structural support to facilitate cell signaling, migration, and nutrient diffusion (24). Several ECM components, such as fibronectin, hyaluronic acid, collagen, and laminin, have been identified and are commonly used to culture cells in 2D (tissue culture coating) and 3D (encapsulation) environments (8).

The engineering of novel biomaterial inks ultimately needs to take into consideration functional matrices able to model the physiochemical complexes of the native ECM (6). Particularly,

the mechanical properties of inks are fundamental for the printability and, more importantly, the ability to control the fate of printed stem cells (89). The inclusion of nanoparticles, such as nanoclays (1, 2, 27), the post-printing hardening via light-curing of the printed construct (25, 84), and the use of in situ cross-linking technology (30, 55) are a selected variety of methodological approaches used to enhance the mechanical characteristics of the printable material ink, to directly affect printability and stem cell functionality.

The limitations of biocompatible inks, together with the numerous constraints imparted by the bioprinting technique adopted, narrow the range of suitable materials. Specifically, materials need to be designed to tailor degradation with cell growth, allowing the proliferation of the printed cells and integration within the host tissue, mimicking the complex and dynamic *in vivo* milieu.

Hydrogels are polymeric matrices capable of retaining a large amount of water (19) and thus are often ideal matrices for the encapsulation of cells. Depending on their precursors, hydrogels can form a network undergoing sol-gel transition via physical or chemical cross-linking induced by temperature or pH change (79), light exposure (25), or enzymatic process (115). Thus, hydrogel inks for printing living stem cells can be classified as comprising synthetic, natural, or native materials.

A wide array of novel approaches has been recently exploited to blend multiple types of materials to ultimately synthesize novel composite inks that result increasingly printable and functional for the delivery of stem cells in 3D. Harnessing the properties of synthetic, natural, and native materials, a selected number of composites are increasingly becoming standard candidates for the printing of stem cells, demonstrating superior printability and functionality.

### *Synthetic Inks*

Synthetic hydrogels are central in TERM as scaffolding materials or inks, given their mechanical stability and controllable physicochemical properties. Their viscosity can be tuned to provide optimal printability; however, a cytocompatible environment, and a relatively rapid degradation, upon implantation, must be ensured (26). Synthetic matrices often prevent cell adhesion and proliferation and require further postprocessing after deposition. Nevertheless, synthetic inks can be engineered to better resemble the natural environment. Indeed, chemical modification of synthetic polymers, with functional moieties, allows for the incorporation of binding peptides and enzymatically degradable groups. For instance, Bryant et al. (16) detailed poly(ethylene glycol) diacrylate (PEG-DA) to tune the degree of cross-linking and incorporated arginine-glycine-aspartic acid (RGD) groups to promote interaction with cells, specifically with integrin subunits, significantly enhancing cell attachment and spreading within the hydrogel.

Synthetic hydrogels can be exploited as structural or sacrificial materials. Kang and colleagues (62) 3D printed cell-laden hydrogel and supporting poly( $\epsilon$ -caprolactone) (PCL) together with a sacrificial Pluronic F-127 hydrogel to fabricate large 3D constructs. The fabrication of human-scale mandible bone, ear cartilage, and skeletal muscle showed promising results, exhibiting functional and structural features both *in vitro* and *in vivo*. Despite the important role of synthetic inks for TERM applications, recent efforts have centered on the use of natural

and native hydrogels, which are intrinsically enriched with a plethora of functional groups.

### *Natural Inks*

Natural hydrogels are synthesized from polymeric precursors found in the environment. Typically, natural hydrogels show greater biocompatibility, but lower mechanical properties, than synthetic materials.

Alginate, a polysaccharide made of glucuronic and mannuronic acids obtained from the cell walls of brown algae, can be easily cross-linked upon exposure to divalent ions (e.g. such as  $\text{Ca}^{2+}$  dissociated from  $\text{CaCl}_2$  solution) (106, 113). Alginate is nontoxic, biodegradable, and Food and Drug Administration approved. Sodium alginate conveys a number of crucial advantages (128), such as the ability to undergo instantaneous gelation and reversible physical cross-linking. The robust mechanical properties confer alginate a superior extrusion-based printing ability and fidelity compared with other natural materials (38). Alginate structure can be tuned to enhance physicochemical properties [e.g., improvement of ionic gel strength through covalent crosslinking (97), modification with chemical anchors to promote interaction with cells (36)]. The simultaneous extrusion of alginate and calcium chloride solution through two coaxial needles permits the instantaneous gelation and precise deposition of the fiber (30, 55, 90). Thus, even low-viscosity bioinks can be extruded to give high resolution (30).

Gellan gum (GG), an exopolysaccharide of the extracellular polymeric matrix of *Sphingomonas* bacteria (126), is also widely used. Despite its cytocompatibility, GG does not provide binding sites for cell attachment and thus requires modification for successful cell attachment (37), e.g., blending with multiple polymers or chemical modification. Lozano et al. (88) modified GG with RGD groups and used GG to print primary cortical neurons and glial cells, demonstrating the engineered material ensured cell survival and networking.

While natural hydrogels are extensively used for 3D biological models and often present an enhanced ability to support cell viability and proliferation compared to synthetic alternatives, can only partially simulate the *in vivo* microenvironment, often failing to reproduce the mechanical properties and dynamic biological cues.

### *Native Inks*

Here, we are using the term native inks to indicate a class of hydrogel materials prepared from precursors derived from xenogenic or allogenic sources. The intrinsic properties of native inks are crucial for the functional generation of physiological 3D models as native inks convey the correct features for a favorable biomimetic environment for cell survival, function, and differentiation. Collagen is widely used for bioprinting as it resides in various forms in most soft and hard tissue's ECM and plays a determinant role in maintaining the ECM stability and integrity (22). Collagen can undergo a sol-gel transition above  $15^\circ\text{C}$ , while the presence of RGD motifs that promote cell adhesion makes collagen a cell-interactive material (52). When collagen triple helix degrades upon acidic or alkaline hydrolysis, gelatin forms. The latter is a thermoresponsive hydrogel that physically cross-links when the temperature drops below  $30^\circ\text{C}$  (44). Gelatin is widely used in tissue engineering, also in its modified version, functional-



ized with methacryloyl groups (GelMA) to induce a chemical nonreversible cross-linking (12, 30).

Among the plethora of native material inks, the most promising, given the physiological resemblance with the natural matrix from which it is derived are decellularized extracellular matrix (dECM)-based inks. To date, native dECM inks have been synthesized from several sources, such as adipose (112), heart (56, 112), cartilage (112), liver (49), skin (49, 67), and liver (49, 81). The process of decellularization preserves the structural and functional molecules produced by the resident cells and removes cellular components and antigenic epitopes to reduce the risk of adverse immunologic response by the host tissue (8). To date, because of lack of human organ availability and despite the genetic mismatch, xenogenic sources remain the main source of dECM (24). The dECM composition preserves elevated contents of GFs, sustaining stem cell growth, differentiation, and ultimate functionality (24). However, the mechanisms underlying the interaction between cells and different dECM compositions from each tissue remain unclear and only a few studies have addressed this issue (49). The choice of the appropriate dECM is of pivotal importance as tissue-specific ECM is required to preserve cell phenotypes (122).

#### *Bioprinting for the Engineering of Functional Physiological Tissue Substitutes*

Engineered tissues such as skin, urethra, and blood vessels have been successfully implanted in patients restoring organ functionality (7). Hitherto, the number of 3D-printed implants to reach clinical trials is limited. However, an increasing number of bioprinting approaches, including stem cells and native inks, seek to recapitulate the biological complexity of the targeted tissue, generating functional implantable constructs or biomimetic platforms for in vitro disease modeling. Table 1 reports recent studies on the fabrication of tissue-specific substitutes, listing the main 3D construct fabrication parameters and functionality.

Tissue physiology poses specific constraints on the particular architecture and function of the implant. Clinical approaches are currently aiming to restore the functionality of the damaged tissue. However, numerous limiting factors highlight the need for alternative and effective clinical tools. The following section details relevant bioprinted tissues using stem cells highlighting the studies that succeeded in the generation of physiological tissues (in terms of cells, materials, and structures) significantly contributing to the advancements of clinical therapies of diseased or damaged tissues.

#### *Vasculature*

Blood vessels form a complex tree-like system within the human body with varying size (0.01- to 15-mm diameter), a structure that allows for transportation of blood, nutrients, oxygen, and removal of metabolic waste through the body (131). Vessels are typically composed of three concentric layers: 1) the intima, lined by endothelial cells (ECs) that form a tight barrier separating the bloodstream from the external layers, 2) the tunica media formed by smooth muscle cells that confer elasticity to the vessels, and 3) the adventitia composed of fibroblasts (FBs) that serves as a stable anchorage to the organ (131). The composition of the ECM varies depending on

the layers, but it is predominantly populated by collagen, laminin, fibronectin, elastin, and proteoglycans (120).

Vasculature originates from two main processes, namely, vasculogenesis and angiogenesis. Vasculogenesis occurs during embryogenesis, where embryonic precursor stem cells differentiate toward endothelial progenitor cells that organize themselves in tubular structures. Angiogenesis is responsible for the growth and expansion of the existing vascular network (111). To date, clinical vascular grafting remains efficacious and widely employed. However, the use of vessel sections from cadaveric donors, or the patient's own body, is not desirable and demonstrated the need for TERM alternatives (110).

The majority of studies investigating vascular repair in vivo or modeling in vitro employs primary cells (ECs), given their ease of isolation and culture. Complex vasculature substitutes have been manufactured (75, 76) and have been proven to be functional, particularly in modeling the vascular microenvironment in vitro. Furthermore, bioprinted vascular ECs have been found essential for in vitro and in vivo maturation of tissues (123).

Advancements in the production of vascularized constructs in vitro will consistently aid the development of 3D models suitable for drug screening application. Furthermore, upon implantation in vivo, angiogenesis is typically slow (<10  $\mu\text{m}$  per day), resulting in a poorly oxygenated implant for several weeks (28). Because of the absence of a vascular network, bioprinted systems are still limited in size (<1–2 mm) and can only be kept in culture for up to 2–3 weeks (75). After the successful bioprinting of perfusable vascular channels alone (41, 43, 135), the first important steps toward realizing vascularized tissues have recently been reported for kidney (53), bone (76), liver (117), and heart (90).

Gao et al. (41) reported a functional in vitro vascular model by the direct printing of EC-laden vascular-tissue-derived ECM (VdECM)-based bioink. After 7 days, vessels were found to be functional and capable of responding to shear stress, exhibiting tight cell-cell junctions. The printed vessels showed limited adhesion of platelets following whole blood perfusion due to the printed ECs that populated the wall of the lumen, demonstrating the potential of this vascular model (Fig. 4A). Skylar-Scott et al. (123) proposed a novel in vitro system at high cellular density ( $10^8$  cell/mL) with embedded vasculature. The authors compacted hundreds of thousands of 1) embryoid bodies, 2) cerebral organoids, and 3) cardiac spheroids used as living organ building blocks (OBBs) to create organ-specific functionality. The authors patterned a fugitive ink within the matrix which, upon removal, created a branched perfusable vascular network, providing functional and mature cells for a longer period (123). This study seeking to manufacture larger tissues, embedded with vasculature, paves the way for novel alternative solutions to current unsuccessful clinical treatments. Although, the incorporation of multiscale features and the complex geometry characterizing the vasculature system still represent a major challenge for tissue engineering.

#### *Heart*

The heart is the central organ responsible for pumping blood through the vascular system. The anatomy of the heart comprises four chambers, which temporarily house the blood

Table 1. *Tissue constructs printed with clinically relevant features*

Printed Tissue	Cell Line	Biomaterial Ink	Functionality	In Vitro	In Vivo	Ref.
Vasculature (vascularized tissue)	HUVECs	Pig aortic tissue VdECM and alginate	Physiological: selective permeability antiplatelet/leukocyte adhesion Pathological: increased vascular permeability	×		(41)
	iPSC-derived OBBs/HNDFs	Gelatin-based fugitive ink printed in OBB- loaded ECM-like material (collagen I, transglutaminase and Matrigel)	cell functionality vessel perfusion	×		(123)
Heart	iPSC-CMs/HUVECs	Alginate, PEG-DA, and fibrinogen	CM maturation Vascularization	×	×	(90)
	iPSC-CMs/FBs/EC spheroids	Cell-only	Synchronous beating Formation of rudimental vessels	×	×	(103)
	iPSC-CMs/ iPSC-ECs	Human or pig omentum dECM bioink and gelatin sacrificial ink	Personalized shape and composition Functional activity in vitro and in vivo	×	×	(101)
	iPSC-Cardiac Spheroids/ECs	Silk fibroin, GelMA, PEG-DA	Elevated expression of gap junction proteins Synchronously beating of cardiomyocytes	×		(91)
	CPCs and MSCs	Pig heart dECM ink mixed with: CPCs (bioink no.1) MSCs + VEGF (bioink no.2)	Reduced cardiac hypertrophy and fibrosis Augmented cell migration to the infarct area	×	×	(57)
Lungs and airways	Human lung ECs and FBs	PEG-DA and GelMA	Distal lung subunit capable of withstanding physiological ventilation under perfusion of RBCs	×		(46)
	Chondrocytes, ECs, MSCs	Cell only	Collagen II expressed following in vitro maturation and in vivo transplantation Vascularized construct after 23 days of implantation	×	×	(130)
Bone	HUVECs, MSCs	Fibrinogen and gelatin ink	Early or late angiogenesis depending on fiber distance Enhanced bone regeneration	×	×	(116)
	BMSCs	Nanosilicate (Laponite) and GelMA	Osteogenic differentiation stimulation of angiogenesis	×	×	(25)
	HUVECs, MSCs	GelMA with low-high degree of methacryloyl substitution and containing different concentration of VEGF	ECs lining the core of the construct Perfusion-supported osteogenic differentiation	×		(18)
	MSCs/ hOB/hDPSCs/HUVECs	Alginate, methylcellulose, and human blood plasma bioink printed with supporting CPC	Elevated cell adhesion, viability and proliferation In vitro differentiation toward osteogenic lineage	×		(3)
	iPSCs/ ESCs	Alginate	No significant differences with nonprinted control Albumin secretion delayed in 3D- printed constructs	×		(36)
Liver	Primary hepatocytes, stellate cells, HUVECs	Cell-only or NovoGel 2.0	Glycogen storage and lipid accumulation Drug toxicity in absence of macrophages or lipopolysaccharides	×		(98)
	iPSC-derived hepatocyte-like spheroids and iPSC- derived ECs/MSCs	Alginate-Pluronic F127 ink	Printed spheroids supported prolonged hepatocytes survival, albumin, and urea production	×		(45)

Continued



Table 1.—Continued

Printed Tissue	Cell Line	Biomaterial Ink	Functionality	In Vitro	In Vivo	Ref.
Skeletal muscle	C2C12	Porcine muscle dECM bioink and PCL support	Formation of AChR, Myogenic differentiation and contractility	×		(23)
	hMPCs	Gelatin, fibrinogen, hyaluronic acid, and glycerol bioink mixture printed alongside PCL fiber support	Aligned myofiber-like structure Highly organized multilayered muscle bundle 82% of functional recovery of rodent defect after 8 wk	×	×	(69)
	hMPCs/hNSCs	Gelatin, fibrinogen, hyaluronic acid, and glycerol bioink mixture printed alongside PCL fiber support	hNSCs support long-term survival and myogenic differentiation of hMPCs Rapid innervation and restoration of muscle function in rat model	×	×	(68)

AChR, acetylcholine receptor; BMSCs, bone marrow stromal cells; CM, cardiomyocytes; CPC, calcium phosphate cement; DA, diacrylate; dECM, decellularized extracellular matrix; ECs, endothelial cells; ESCs, embryonic stem cells; FBCs, fibroblast cells; GelMA, gelatin-methacryloyl; hDPSCs, human dental pulp stem cells; hMPCs, human muscle progenitor cells; HNDFs, human neonatal dermal fibroblasts; hNSCs, human neural stem cells; hOB, human preosteoblasts; HUVEC, human umbilical cord endothelial cells; iPSC, induced pluripotent stem cells; MSCs, mesenchymal stem cells; OBBS, organ building blocks; PCL, poly-(ε-caprolactone); PEG-DA, polyethylene glycol diacrylate; RBCs, red blood cells; VdECM, vascular decellularized extracellular matrix; VEGF, vascular endothelial growth factor.

pumped in and out via the contractile activity orchestrated by the sinoatrial node cells. The heart tissue is subdivided into three different layers: epicardium, myocardium, and endocardium (71). The abrupt disruption of myocardium integrity and functionality (myocardial infarction) is the leading cause (80%) of deaths from cardiovascular diseases (4). Heart transplantation remains the sole clinical solution. However, the dramatic disparity between the availability of donors' hearts and patients waiting for organ transplantation and the increased chances of organ rejection identifies an urgent need for alternative strategies (9).

Ong et al. (69) recently proposed a revolutionary approach for the printing of spheroids (103) based on human iPSC-cardiac myocytes (iPSC-CMs), FBs, and ECs to form a cardiac patch. The fusion of cardiospheres formed a compact and intact patch with high viability and functionality (beating and forming rudimentary vessels) both in vitro and in vivo. A recent study of printing multicellular systems (iPSC-CMs and HUVECs) highlighted that cell interaction, and their geometrical organization impacted on CMs maturation and vascularization (90). Indeed, printed iPSC-CMs were found to converge toward a preferential alignment and were supported by a vascular network. Bioprinted constructs implanted in mice led to cell differentiation toward the cardiac phenotype, cell alignment, and formation of large vessels, suggesting a successful model for vascularized cardiac tissue. The manufacturing of biomimetic vascularized cardiac patches was recently explored (101). A novel personalized ink was produced from patients' omental tissue. Computer tomography data were used to build a model fitting the patient-specific heart defect, including the 3D structure and orientation of the major blood vessels. After 7 days posttransplantation within the rat omentum (Fig. 4B), iPSC-CMs and ECs were found to be functional, displaying contractility potential and sufficient vascularization. A mini-heart 3D model was also generated by depositing the same biomaterials in a supporting medium that allowed for printing free-form complex shapes.

Despite the surprising advancements mentioned above, the successful repair of heart tissue, after failure, still represents a daunting challenge. The printed organ needing long-term cultivation necessitates adequate support and hardware development. A large number of cells are demanded for populating the construct and reproducing the high cellular density of the native counterpart that will need an efficient strategy for iPSCs expansion. Finally, more sophisticated technologies for imaging and printing of the entire vasculature will need to be developed for the correct incorporation of blood vessels in the ultimate organ (101).

### Lungs and Airways

The respiratory system is essential for the transfer of oxygen and carbon dioxide between the air and the blood. Lung airways rapidly decrease in size from the trachea (1.5 cm in diameter) to respiratory bronchioles (0.5 mm), which are ultimately wrapped in the parenchyma that is directly responsible for gaseous exchange (54). Alveoli are submillimeter spherical sacs with a wall separating two alveolar spaces ranging from 200 to 400 nm (114).

The multiscale architecture of the respiratory system (from proximal tissues, such as the trachea, and distal, such as alveolar units) and the numerous resident cell types, represent unprecedented challenges for bioprinting. Moreover, their fabrication conveys hurdles related to the printing of hollows, interconnected structures of both vasculature (32) and airways (39).

Toward this, Taniguchi et al. (130) reported the fabrication of a functional tracheal tube using a unique scaffold-free technique. Chondrocytes, ECs and MSCs were allowed to self-assemble into spheroids, consequently used, without the need of biomaterial matrices, as building blocks for the bioprinting of an artificial trachea. The printed trachea was found to be functional in vivo upon implantation in a murine model,

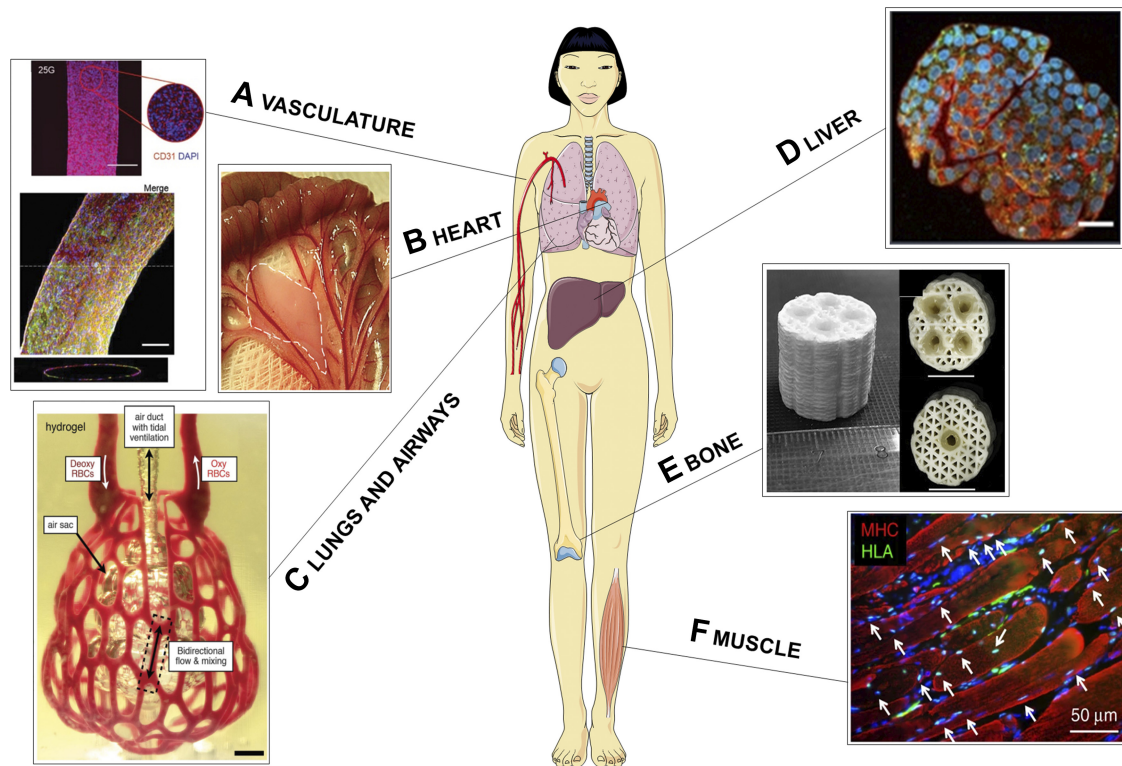


Fig. 4. Expanded view of relevant tissues recently bioprinted and in some cases implanted in animal models for preclinical functionality validation. **A:** vasculature: human umbilical vein endothelial cells staining in the printed vessel show the continuum and confluent endothelial layer 7 days after fabrication. Top image scale bars: 100 and 50  $\mu\text{m}$  in the enlarged graphic; middle image scale bar: 200  $\mu\text{m}$ ; bottom image scale bar: 100  $\mu\text{m}$ . [Adapted from Gao et al. (41) with permission from John Wiley and Sons.] **B:** heart: printed heart patch transplanted in rat omentum, dashed lines indicate the patch borders. [Adapted from Noor et al. (101) under Creative Commons Attribution 4.0 International (CC BY 4.0), <http://creativecommons.org/licenses/by/4.0/>.] **C:** lungs and airways: bioprinted distal lung subunit under red blood cell (RBC) perfusion enclosing the air sac ventilated with oxygen. Scale bar: 1 mm. [Adapted from Grigoryan et al. (46) with permission from AAAS.] **D:** liver: embryonic body stained at *day 18* and used to print spheroid-constructs. Scale bar: 20  $\mu\text{m}$ . [Adapted from Goulart et al. (45) with permission from International Society for Biofabrication.] **E:** bone: bone constructs with perfusable channels. Scale bars: 10 mm. [Reprinted and adapted from Ahlfeld et al. (3) with permission from American Chemical Society.] **F:** muscle: immunofluorescence of injured tibialis anterior muscle in a rat model 8 weeks after implantation of the muscle 3D printed constructs, highlighting muscle fiber formation (white arrows). [Adapted from Kim et al. (68) under Creative Commons Attribution 4.0 International (CC BY 4.0), <http://creativecommons.org/licenses/by/4.0/>.]

capable of bridging a large gap ( $\sim 6$  mm) and restoring airways functionality.

A recent significant step to recreate a more complex lung subunit was attempted by Grigoryan et al. (46), with a complex breathing model, including blood flow and air ventilation. Through the stereolithographic bioprinting of photo-cross-linkable hydrogels, the group sought to mimic the physiology of a distal lung subunit, capable of withstanding ventilation for more than 10,000 cycles at a pressure of 24 kPa and frequency of 0.5 Hz under perfusion of red blood cells (RBCs). Human lung FBs were encapsulated in the bulk of the interstitial space, whereas human epithelial-like cells were cultured in the conducting airways. The complex geometry, captured by the proposed model, and the biomimetic mechanical stimulus applied through the air and blood pumping enabled the generation of a tension similar to that observed in the human bronchioles (Fig. 4C). This model could be used for replicating the pathophysiological conditions of asthma and other diseases involving airway remodeling, such as subepithelial fibrosis (17), smooth muscle hyperplasia and hypertrophy (51), and epithelial cell metaplasia (35, 134), and offers new hope for the development of functional structures that recapitulate the *in vivo* alveoli anatomy and physiology.

However, the fabrication of a functional human-scale lung is still a distant goal since the effort required to print with high accuracy intricate micrometric structures, such as alveoli, necessitates postprinting incorporation into a hierarchical lung structure, which represents an additional issue to solve.

#### Liver

The liver is a large organ carrying out essential functions for the human body, such as blood filtering and protein production. Specifically, the liver is able to process and detoxify numerous metabolites, synthesize proteins, and produce a range of biochemicals. Because of this significant activity of exchange, the liver is equipped with an extensive blood vessel system, delivering substances to/from the organ (60). The microscopic anatomy of the liver is intricate, comprising hexagonal hepatic lobules with hepatocytes irradiating from a central vein, sinusoid ducts that run through hepatocyte plates, and capillaries lined with specialized stellate macrophages (Kupffer cells) constantly breaking down red blood cells (14). Given the complexity of this organ, there has been limited success in the manufacture of liver tissue, leaving the clinical standard (liver

transplant) the sole functional approach to date. Nevertheless, the possibility of fabricating a 3D liver construct is appealing not only for regeneration and disease modeling but also for toxicology studies (98).

Faulkner-Jones et al. (36) ink-jet printed human iPSCs and ESCs that had been differentiated toward hepatocyte-like cells. Cells were printed during the differentiation process in an alginate-based ink, followed by calcium and barium chloride cross-linking. Printed cells were found to be similar in morphology and hepatocyte marker expression to 2D controls, demonstrating the safety of the inkjet technique. Nguyen et al. (98) recently bioprinted compartmentalized 3D human liver tissue using primary human hepatocytes to fill the compartments, while depositing HUVECs and hepatic stellate cells to contour the sections. The hepatic construct exhibited stability and functionality (junction formation, ECM secretion, HUVECs organization in a network, lipid storage, and glycogen storage). Moreover, it demonstrated its superior ability (compared to 2D culture) to model drug-induced liver injury. Hepatic spheroids and endothelial cells derived from iPSCs have been printed with MSCs, in alginate/pluronic blends, to evaluate their potential (in comparison with printed single-cell dispersion) for the development of autologous liver grafts (45). Spheroid-bioprinted constructs showed enhanced viability, prolonged survival, higher albumin secretion, and urea production compared to single-cell dispersion, and displayed a stable phenotype, supporting the idea that spheroids can sustain hepatic function for a prolonged period (Fig. 4D).

Despite its high capacity for regeneration, clinical intervention for liver repair is still a necessity. Numerous persistent inflammations or diseases can ultimately lead to liver function deficit and organ failure. Significant steps have been taken toward liver TERM with the development of liver tissues that can be cultured *in vitro* for extended periods, but to date, the bioprinting of a large portion, if not the whole organ for transplantation, is still impractical.

### Bone

Bone is a dynamic tissue, undergoing continuous renewal and repair (29). Bone tissue is composed of inorganic (calcium and hydroxyapatite) and organic (collagen I, phosphatase, fibronectin, osteopontin, and proteoglycans) phases. Bone structure is central for organ protection and support, movement, and mineral storage (133). In embryogenesis, bone tissue development requires ossification mechanisms that include the agglomeration of bone marrow stromal cells (BMSCs) into a dense structure and their differentiation into osteoblasts to directly form bone tissue or into cartilage with subsequent mineralization in the endochondral bone formation process (11).

Autologous grafts are efficacious for the repair of skeletal defects while failing to support the functional repair for critical size damages. Thus, bioprinting provides a functional platform for the fabrication of physiologically relevant bone implants. Because of innate mechanical properties of bone tissue, stem cells are preferably seeded on 3D printed polymeric scaffolds. However, the printing of MSCs and the patterning of ECs have proven central for the generation of functional bone tissue. Indeed, current clinical solutions for bone defects can fail as a result of insufficient nutrient supply, leading to necrosis and poor interconnection of the surgical graft with the host tissue

(26). An adequate vascularization has been shown to enhance bone repair ability, as vascularization promotes osteoprogenitor cell recruitment, proliferation and differentiation, gas exchange, and bone homeostasis (61).

Toward these goals, Piard et al. (116) investigated the effect of printing distance between MSCs and HUVECs. Results indicated the beneficial presence of HUVECs for bone tissue maturation following endochondral ossification. However, HUVECs that were closer than 200  $\mu\text{m}$  were found to influence vessel maturation, indicating later-stage angiogenesis *in vitro*, while driving the sprouting of a great number of vessels and guiding large regeneration of the calvarial defect *in vivo*. Byambaa et al. (18) generated a 3D platform that was able to closely imitate the bone marrow niche (comprising bone tissue and blood supply) after 7 days of maturation *in vitro*. The unique and controlled deposition in 3D of vascular endothelial growth factor (VEGF)-loaded GelMA facilitated a series of gradients able to direct MSC differentiation, orchestrating HUVEC rearrangement and branching.

Functional implants for bone repair need to possess mechanical properties comparable to the native load-bearing component of the skeletal tissue. Numerous approaches have been developed to reinforce printed scaffolds [e.g. printing of PCL (31) or electrospun fiber support (34)]. A recent approach by Ahlfeld et al. (3) used the patterning of clinically approved calcium phosphate cement (CPC) and MSC-laden in a novel alginate-methylcellulose ink containing plasma from human blood. The multimaterial scaffold could be printed, in clinically relevant dimensions, while the inclusion of plasma supported the adhesion, proliferation, and differentiation of printed MSCs, human preosteoblasts (hOBs), and human dental pulp stem cells (hDPSCs). The printing of HUVECs/MSCs allowed the precise layering of these cells within printed channels in large volumetric constructs, demonstrating the ability for functional repair of critical bone defects (Fig. 4E).

Despite the promising above-mentioned developments in bone biofabrication, the unique mechanical properties of bone and complex static and dynamic loading parameters of bone, as well as the composition gradient/hierarchical architecture, make bone one of the most challenging tissues to reproduce.

### Skeletal Muscle

Muscle is a relatively soft tissue, which contains specific proteins (actin and myosin) enabling it to contract, macroscopically translated in force and motion (95). In skeletal muscles, stimuli are provided by motor neurons, while smooth muscles are stimulated by pacemaker cells. Muscles are involved in the maintenance of posture and movement inside (e.g. peristalsis) and outside (e.g. locomotion) the human body (95).

During the daily activity, 1–2% of muscle tissue disintegrates and is constantly repaired. However, the decrease in muscle mass and function with time (known as sarcopenia) can be detrimental to an aging population. The clinical need for muscle tissue is related to neuromuscular disorders (e.g., Parkinson's disease) and the loss of nervous control of muscles, leading to uncontrolled movement. Bioprinting tools have provided numerous functional approaches for the fabrication of skeletal muscle tissue substitutes (104). Patient-specific 3D constructs can be ideal for the regeneration of a large portion



of damaged or missing skeletal muscle, or as a biomimetic platform for *in vitro* drug screening.

Choi et al. (23) demonstrated the ability to engineer and print a novel native-derived ink to closely imitate muscle microenvironment. A decellularized skeletal muscle ECM (mdECM) that could gel with a temperature shift up to 37°C was synthesized. PCL constraints were printed to guide muscle progenitor cells (MPCs)-laden mdECM bioink deposition and spatial arrangement. The printing of MPCs in mdECM ink was found to sustain the formation of acetylcholine receptors (AChR) and myogenic differentiation. Kim et al. (69) reported a novel study printing patient-derived cells within a fibrin ink in a hierarchically organized fashion, alternating PCL fibers and gelatin gel. 3D printed muscle scaffolds were observed to be functional *in vitro* with organized cross-striated myofibers and laminin matrix, and importantly, in an *in vivo* tibialis anterior muscle defect model, showing significant regenerative capacity and the therapeutic potential for severe muscle injuries. Recently, the same group (68) has successfully engineered skeletal muscle constructs, integrating a neural component for the support of tissue maturation and regeneration *in vivo*. Indeed, human neural stem cells (hNSCS) were found to promote long-term survival of MPCs, and the ultimate generation of a neuromuscular junction (NMJ) in a complex three-dimensional environment. Significantly, this multicellular bioprinted platform was found to fully restore muscle weight in a rat model, significantly accelerating the restoration of function (Fig. 4F).

Future translational approaches will need to involve a multicellular printing platform to improve the fidelity to the physiological skeletal muscle microenvironment with a complex neural and vascular support (68). The use of iPSCs, for their rapid expansion and specific functionality, will be beneficial for the biofabrication of patient-specific skeletal muscle implants and model. Further development of biomimetic native inks will be supporting skeletal muscle printing, facilitating the fabrication of fully functional implants.

### Future Prospects

In the last decade, bioprinting has made significant steps toward the fabrication of physiological tissues. The development of stem cell protocols has defined a new era, giving rise to unique therapeutic approaches, such as personalized medicine and fostering the advancement of regenerative medicine. Currently, adult stem cells are particularly attractive for their potential to differentiate in a selective number of tissues with direct control over their ultimate fate. Harnessing the bioprinting ability to pattern living cells, in 3D, to match tissue-specific architecture and function, has led to research groups focusing on bioprinting approaches using stem cells as building blocks for tissues both *in vitro* and *in vivo* (Table 1). New challenges remain from improvements of the techniques to harvest, reprogram and differentiate stem cells, to ultimately obtain large-scale, reproducible, and clinically applicable cultures for the printing of clinically relevant constructs. Current *in vitro* surrogates contain lower cell densities compared with physiological tissues, raising the need for new approaches, such as organoids, to engineer high cellular density constructs.

The culture and implantation of stem cells can be carried out using 3D matrices that closely mimic the *in vivo* native milieu

of the specific organ (24). Native dECM inks have been derived from several tissues (e.g., liver, corneal, heart, skin) and can be printed in 3D using simple extrusion deposition (49). However, because of their poor mechanical properties, dECM inks require physical [e.g. blending in composite materials, printing alongside PCL strands (112)] or chemical [e.g., genipin cross-linking (74)] support. Native inks are highly promising, but several limitations, including large batch-to-batch variations and xenogenic sources, are hindering further development.

Thus, the generation of a biofabrication tool for functional translational approaches remains a distant goal. Recently, extensive efforts have been spent on the manufacturing of functional tissue substitutes seeking to engineer novel clinically relevant platforms using hybrid bioprinting approaches. Seminal studies on micro-tissue (92) and spheroid (138) printing have demonstrated the potential of printing assembled cell aggregates to promote tissue growth and maturation. Furthermore, technological advancements are required to realize higher resolution and faster bioprinters. This is particularly important for the fabrication of large and multiscale tissues provided with vasculature and innervation. Microfluidic bioprinting has come to the fore as a unique and innovative engineering strategy that can enable the printing of 3D-patterned constructs incorporating the distribution of the cell types and ECM, characteristic of the native counterparts. Recent works on microfluidic bioprinting (55, 90) have demonstrated that the complex microarchitecture of tissues (e.g., gradient of cell/factors) can be reconstructed *in vitro* by integrating (upstream of the extruder) microfluidic platforms capable of controlling deposition, concentration, and distribution of the biomaterials. Alternatively, the combination of different deposition platforms, such as multi-electrospinning writing and bioprinting (34), could help the advancement of the field toward the printing of more complex, structured, and functional tissue constructs.

Although the bioprinting of stem cells for the fabrication of physiologically relevant tissue implants or models is maturing apace, the trial and the routine use of stem cell-laden 3D-printed constructs for the clinical repair and intervention is still distant, however promising and revolutionary.

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### AUTHOR CONTRIBUTIONS

C.S., A.S., and G.C. prepared figures; C.S., A.S., G.R., and G.C. drafted manuscript; C.S., A.S., G.R., and G.C. edited and revised manuscript; C.S., A.S., G.R., and G.C. approved final version of manuscript.

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