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Bioprinting: A Further Step to Effective Regenerative Medicine and Tissue Engineering

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Editorial

Regenerative medicine is a multidisciplinary field that aims to replace or regenerate human cells, tissues, or organs in order to restore or establish normal function. In this broad sense, this operational definition should include the ultimate goal of tissue (bio) engineering, i.e. 'the manufacture of living functional tissues and organs suitable for transplantation in reasonable time scales' [1]. The process of regenerating body parts can occur in vivo or ex vivo, and may require stem cells, natural or synthetic cell-supporting scaffold materials, bioactive molecules such as for example trophic factors, genetic manipulation, or combinations of all of the above [2]. The interest in embryonic stem cells has increasingly faded away when the possibility of obtaining pluripotent cells by reprogramming adult somatic cells was achieved. Induced Pluripotent Stem Cells (iPSCs) represents nowadays the most interesting source to be used in regenerative medicine, as, besides pluripotency, they are obtained from the very same patient whom they will administer to and should thus not give any immune reaction [3]. Regenerative medicine and tissue engineering have broad interest as to the application to different fields of general surgery, among which skin restoration, heart repair, bioengineering of vessels, kidney, gastroenteric and upper respiratory tracts [4]. The medical application in this field started up in 2006 when Atala and colleagues implanted in patients who need cystoplasty bladders engineered ex vivo from the seeding of autologous cells (urothelium and muscle cells) on collagen-polyglycolic acid scaffolds as artificial supporting biomaterial [5]. Another milestone was the manufacture of a trachea from human components. Macchiarini and colleagues transplanted the first tissue-engineered trachea, utilizing the patient's own stem cells, into a 30-year old woman with end-stage bronchomalacia, with positive results about respiratory functional tests following the transplantation [6]. The trachea was denuded and re-seeded with cells from the recipient, i.e. chondrocytes differentiated from hematopoietic stem/progenitor cells on the outer surface and epithelial cells obtained from the right bronchus on the inner surface. A 5-year follow-up reported the safety and efficacy of this procedure highlighting the function of the tissue-engineered trachea and, importantly, the well-being of the patient [7].

In regenerative medicine, however, the engineering of complex vascularised organs is presently the major challenge to be overcome to guarantee transplantation of organs which are very limited in supply from other individuals [8]. The use of autologous cellular components with an internal vascular bed will theoretically overcome the two major hurdles in transplantation, namely the shortage of organs and the toxicity deriving from lifelong immunosuppression. Thus, major progress in regenerative therapies will require cell-based products made of many cell types to recapitulate organ metabolic function, and structure to support mechanical function. Recently, this has been partially accomplished by the generation of functional Liver Buds (LBs) from iPSCs [9]. Hepatic endoderm cells from human iPSCs (iPSC-HEs) were cultivated with stromal cell populations, Human Umbilical Vein Endothelial Cells (HUVECs) and human Mesenchymal Stem Cells (MSCs), self-organizing in three-dimensional clusters in vitro and forming iPSC-LBs. In vivo studies demonstrated that: i) transplants became functional by connecting to the host vessels within 48 hours; ii) the formation of functional vasculatures stimulated the maturation of iPSC-LBs into tissue resembling the adult liver; and iii) mesenteric transplantation of iPSC-LBs rescued the drug-induced lethal liver failure model.

Three-dimensional (3D) cell bio-printing is a relatively new engineering tool being used to design 3D cell constructs (rather than cell suspensions) for transplantation therapies. A definition of bio-printing has been given by Guillemot, Mironov and Nakamura in 2010: 'the use of computer-aided transfer processes for patterning and assembling living and non-living materials with a prescribed 2D or 3D organization in order to produce bio-engineered structures serving in regenerative medicine, pharmacokinetic and basic cell biology studies' [10]. An outline of the steps involved in 3D bio-printing is given in Figure 1.

This technological platform has taken advantage of the one based on 2D inkjet printing. Bio-printing with inkjet technology allows to spray extracellular matrix proteins for providing a defined substrate for cells, elaborate complex cell structures, or to deliver gene and enzymes to cells [11-13]. In its simplest version, 3D bio-printing is aimed at print one layer of cells atop the layer of other cells or scaffold biomaterials. On the other hand, 3D bio-printing would be a platform that facilitates construction of complex, multicellular tissues or organs in architectures appropriate for function, and, in one version, it is based on 3D cellular building blocks, instead of liquid inks [12].

Since the beginning of the XXI century, in labs around the world, bioengineers have begun to print first bacteria, mammalian cells and then prototype organs: heart valves, ears, artificial bone joints, menisci, blood vessels and skin grafts [10,12,14-21]. Three factors are leading the evolution of 3D bio-printing: more sophisticated printer, advances in cell therapy and regenerative medicine, and refined Computer-Assisted Design/Computer-Assisted Manufacturing (CAD/CAM) software. Moreover, the team may use Computed Tomography (CT) or Magnetic Resonance Imaging (MRI) scans to create a CAD file of the wound or organs like meniscus.

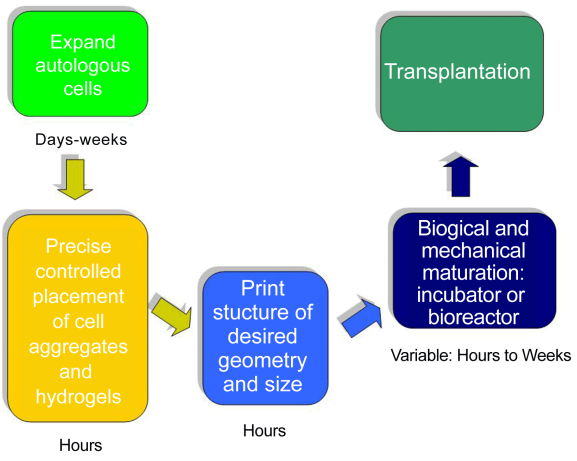

Figure 1: Schematic flow of the main steps involved in 3D bio-printing.

The advantage of a 3D bio-printer is that a tissue or an organ can be created layer by layer to achieve accurate anatomical geometries. 3D bio-printing can be obtained by either Laser-Assisted Bio-printing (LaBP) or Inkjet Printing (IBP). Other bio-printing techniques, such as acoustic/ultrasonic method and pneumatic based syringe extrusion method are also used for single cell printing or polymer deposition [12]. LaBP uses a pulsed laser source to induce vaporization of a metal film coated with cells or biological material on a ribbon of glass or quartz, and offers the possibility to place cells of a specific type wherever in the tissue they are needed. Importantly, cells survive the transfer without damage and alteration of their phenotype [15]. Inkjet printing has been used to print a 3D composite construct containing muscle cells, endothelial cells and stem cells [19]. The major disadvantage of inkjet bio-printing, however, is the high shear force of the nozzle, leading to severe cell impairment [22]. Using a modified printer, the viability of mammalian cells printed by thermal inkjet varied from 85% to 95% depending on the cell concentration [23]. Moreover, the pores developed during printing could be utilized for gene transfection and drug delivery when depositing cells. In the following paragraphs, I will briefly review the most recent medical application of 3D bio-printing.

The current cartilage tissue engineering strategies still cannot fabricate new tissue that is indistinguishable from native cartilage with respect to zonal organization, Extracellular Matrix (ECM) composition, and mechanical properties. Cui and co-workers printed by the thermal inkjet technique human articular chondrocytes and Poly(Ethylene) Glycol Dimethacrylate (PEGDMA) layer-by-layer into a cartilage defect within an osteochondral plug (called also 3D bio-paper) [24]. An even distribution of printed human chondrocytes was obtained in the 3D PEGDMA hydrogel with simultaneous polymerization during printing. Importantly, printed cartilage implant attached firmly with surrounding tissue, indicating the importance of direct cartilage repair and promising anatomic cartilage engineering using 3D bio-printing technology. However, optimal cell densities from cartilage tissue engineering cannot be achieved using actual bio-printing approaches [22]. This is why the role of growth factors in chondrocyte proliferation and ECM production was studied in bio-printed cell-laden 3D hydrogels. Cui and colleagues treated the cartilage bio-printed samples with Fibroblast Growth Factor (FGF)-2 and Transforming Growth Factor (TGF)-1 and increased cell proliferation by 40% as compared with (TGF)-1 only treated samples was obtained. The synergistic treatment made the initial 8×10^6 cells/mL seeding density equivalent to a seeding density of over 11×10^6 cells/mL [25], which is well within the range of cell seeding density for optimal ECM deposition for cartilage tissue engineering.

Inkjet bio-printing was also used to create a hybrid cellular construct which may have clinical implications for building vascularized bone tissues. Three different cell types, including Human Amniotic Fluid-Derived Stem Cells (hAFSCs), which have been demonstrated to differentiate into osteogenic lineage, canine smooth muscle cells (dSMCs), and Bovine Aortic Endothelial Cells (bECs) were combined with the cross-linker (CaCl2) and printed into sodium alginate-collage composites using a modified thermal inkjet printer to form a pie-shaped multi-cell heterogeneous tissue construct [19,26]. Each cell type within the printed construct retained viability, proliferation ability, phenotypic characteristics, and basic physiological properties and functions after the printing process in vitro and in vivo. The bioprinted constructs which were implanted subcutaneously into the backs of athymic nude mice showed a vascular network integrated with the existing vasculature of the recipient mouse, and de novo bone formation.

Part of the body	Team	Components	Applications	References
Ear	Cornell University, NY	Collagen type I hydrogels seeded with bovine auricular chondrocytes	Microtia	[17]
Kidney	Wake Forest Institute for Regenerative Medicine, NC	Kidney cells cultivated from a biopsy	Kidney transplantation	[16]
Blood vessels	University of Pennsylvania and MIT	Sugar filaments and polymer obtained from corn	Angiogenesis in tissues	[16]
Blood vessels	University of Iowa, IA	Cartilage precursor cells encapsulated in alginate	Angiogenesis in tissues	[20]
Blood vessels	Organovo, CA	Layers of hydrogel rods and a bio-ink made of spheres or cylinders that contain thousands of human cells	Angiogenesis in tissues	[10,12]
Skin grafts	Wake Forest Institute for Regenerative Medicine, NC	A layer of fibrin, a layer human fibroblasts, and a layer of keratinocytes	Wounds and ulcers. Directly printed on the site of the wound.	[16]

Skin grafts	Hannover Medical School, Germany	Laser-assisted bio-printing of fibroblasts and keratinocytes on topo of stabilizing matrix	Burn injuries	[21]
Bone	Washington State University	Scaffold made of ceramic powder, which is baked, and covered with human bone cells	Fractures	[16]
Bone	Wake Forest Institute for Regenerative Medicine, NC	hAFSCs, dSMCs, and bECs combined with the cross-linker (CaCl2) and printed into sodium alginate-collage composites	Vascularized bone	[19]
Cartilage	The Scripps Research Institute, La Jolla, CA.	Layers of human articular chondrocytes and PEGMA	Cartilage defects	[24]
Liver	Digestive Disease Institute, Cleveland Clinic, OH	Transparent materials	Live donor liver transplantation	[30]

Table 1: Recent investigations in 3D bio-printing and medical applications

The reconstruction of the ear in subjects with microtia and other auricular structural abnormalities relies on autologous techniques, in which costal cartilage is harvested, sculpted to recreate the three-dimensional structure of the auricle, and implanted under the periauricular skin. To overcome suboptimal aesthetic outcomes and morbidity at the costal cartilage donor site, normal pediatric ears were digitized and synthetic ears were fabricated by the tissue injecton molding technique from collagen type I hydrogels which were then seeded with bovine auricular chondrocytes [17]. Upon implantation subcutaneously in the dorsa of nude rats, cellular implants largely mimicked the native auricle both mechanically and histologically, even after 3 months post-implantation. This strategy hold immense potential for tissue-engineered auricular reconstructions in the future, although construct evolution over a longer implantation interval (i.e., at least 6-12 months) and ultimately, use of patient-specific chondrocytes and/or pluripotent MSCs must be evaluated prior to translation of this platform to the clinical setting.

Using a cybernetic concept, Mannoor and colleagues generated a bionic ear via extrusion-based 3D printing of a chondrocyte-seeded alginate hydrogel matrix with an electrically conductive silver nanoparticle coil antenna, connected to cochlea-shaped electrodes supported on silicone [18]. The printed ear was cultured up to 10 weeks showing a uniform distribution of chondrocytes in the construct and accumulation of hydroxyproline, as a marker of collagen content, and glycosaminoglycans, as a marker of proteoglycans. Nanoindentation measurements allowed determining that hardness was relatively uniform at various anatomical sites of the auricle. Finally, the printed ear exhibited auditory sensing beyond normal audible signal frequencies.

Autologous split-thickness skin grafts and keratinocytes are used for wound coverage but their availability is limited. Although several skin substitutes like Integra® and Matriderm® are already employed in the clinical application [27,28], full success in burn wound regeneration has not been reached yet. Michael and colleagues have reported successful creation of a multi-layered fully cellularized skin equivalent for the future treatment of burn patients [21]. Laser-assisted bioprinting was used to print 20 layers of fibroblast-containing collagen and 20 layers of keratinocyte-containing collagen subsequently onto a sheet of Matriderm®, used as a stabilization matrix. The skin constructs were placed into full-thickness skin wounds created in the dorsal skin of nude mice and were fully connected to the surrounding tissue when explanted after 11 days. At the histological level, the printed cells formed a tissue which is quite similar to the native skin, including collagen producing fibroblasts and keratinocytes with beginning differentiation, forming a dense epidermis. Since very important for the take of a grafted skin or skin substitute is its fast vascularisation, it was of interest that blood vessels could be found to start growing into the Matriderm® from the wound bed and the wound edge mostly in the direction of the transplanted cells.

NovoGen MMX™ Bioprinter (Organovo, San Diego, CA) is a system made up of a printer which is used to place cellular building blocks in precise orientations or geometries, such that maturation of the final tissue construct occurs in an architecture that resembles that of the normal tissue or organ [12]. Scaffold-free bioprinted constructs can be generated in a number of distinct geometries, manily cylinder or spheres, according to the structures to be created, i.e. Tubes or tissue sheets. For the construction of small diameter vessels [12], vascular grafts were printed using cellular cylinders made from a pre-determined ratio of primary Human Adult Aortic Smooth Muscle Cells (hASMCs), endothelial cells (hAECs), and Dermal Fibroblasts (hDFs). First, a base of proprietary agarose-based rods was printed, on which the cellular cylinders were extruded in a predetermined geometry. After incubation in tissue culture medium and fusion of adjoining cell cylinders, supportive hydrogel cylinders were removed and the nascent blood vessel was transferred into a perfusion bioreactor. The multi-layered, fully biological, ECM-rich vascular conduits could be perfused for at least 28 days, with progressive increases in mechanical integrity. More recently, Bertassoni and coworkers used a 3D micomolding technique to fabricate a multitude of interconnected tiny fibres inside hydrogels made up of different polymers, among which Methacrylated Gelatine (GelMa) [29]. They then covered the GelMa-based structure with a cell-rich protein-based material, which was solidified by applying light to it. Lastly they removed the bio-printed fibres to leave behind a network of microchannels coated with human endothelial cells, which self organised to form stable blood capillaries in less than a week.

Finally, the first human livers were printed and validated against the actual native liver at the time of surgery. Indeed, Zein et al. [30] have now used 3D printing in the setting of adult-to-adult Living Donor Liver Transplantation (LDLT), by creating semi-transparent hepatic prototypes containing visible hepatic vessels and bile ducts. These models were shown to have a very high accuracy, with a mean dimensional error of less than 4 mm for the entire model and less than 1.3 mm for the vascular diameter. A 3D-printed model of the liver could help to reduce the risk of small-for-size syndrome (when pretransplant volumetry overstimates the volume of a procured liver), or that of large-for-size syndrome (when a transplanted graft cannot be placed in a small abdominal cavity) in the setting of pediatric LDLT [31].

3D bio-printing will be fully successful when complex organs such as lung and kidney will be obtained from this technological platform. It seems that the major advantage brought by 3D bio-printing will be to allow the presence of conduits resembling arteries and veins for oxygen and nutrient delivery and waste removal, respectively. The achievement of this important goal will require more biologically sophisticated software, increased automation, more involved tissue design, and need for a controlled environment during printing. Once all these issue will be resolved, 3D bio-printing will expand the applications of cell-based therapies, in the regenerative medicine and tissue engineering fields.

Author Disclosure Statement

No competing financial interests exist.

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