Rethinking Regenerative Medicine from a Transplant Perspective (and Vice Versa)

Rethinking regenerative medicine from a transplant perspective (and vice versa)

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Abstract

No field in health sciences has more interest than organ transplantation in fostering progress in RM because the future of no other field more than the future of organ transplantation will be forged by progress occurring in RM. In fact, the most urgent needs of modern transplant medicine - namely, more organs to satisfy the skyrocketing demand and immunosuppression-free transplantation -, cannot be met in full with current technologies and are at risk to remain elusive goals. Instead, in the past few decades, groundbreaking progress in regenerative medicine (RM) is suggesting a different approach to the problem. New, RM-inspired technologies among which decellularization, 3D printing and interspecies blastocyst complementation, promise organoids manufactured from patients' own cells and bear potential to render the use of currently used allografts obsolete. Transplantation, a field that has traditionally been immunology-based, is therefore destined to become a RM-based discipline.

However, the contours of RM remain unclear, mainly due to the lack of a universally accepted definition, the lack of clarity of its potential modalities of application and the unjustified and misleading hype that often follows the reports of clinical application of RM technologies. All this generates excessive and unmet expectations and an erroneous perception of what RM really is and can offer.

In this manuscript, we will reason on these aspects of RM and transplant medicine, will propose a definition of RM and will illustrate the state of the art of the most promising RM-based technologies of transplant interest.

Introduction

Regenerative medicine (RM) has shown an immense potential to profoundly impact transplant medicine (TM) by meeting its two most urgent needs: a new and potentially inexhaustible source of organs and the achievement of an immunosuppression-free status[1]. Through the development of technologies that will make organ fabrication possible using patient-derived biomaterials – cells and supporting scaffolding materials – RM promises to enable *organ-on-demand* whereby patients will receive organs that will not be rejected and in a timely fashion. This will make registration in the waiting list and anti-rejection medications unnecessary and, as the new organs will be implanted immediately after fabrication, ischemia-reperfusion injury secondary to organ preservation will not be a problem anymore.

However, the contours of RM remain unclear, mainly due to the lack of a universally accepted definition, the lack of clarity of its potential modalities of application and the unjustified and misleading hype that often follows the reports of clinical application of RM technologies. All this generates excessive (and unmet) expectations and an erroneous perception of what RM really is and can offer.

With the present manuscript, we intend to address these concerns, propose a definition of RM pertinent to TM and elucidate the RM technologies that may be applied to and serve the mission of TM. We will also briefly discuss the most relevant product development challenges and the immunological implications of the biomaterials currently under development.

Definition

"Regenerative medicine" is an umbrella term of still unclear significance. For example: in 2006 the United Nations Educational, Scientific and Cultural Organization (UNESCO) defined RM as a super-discipline whose contours are still being defined (http://unesdoc.unesco.org/images/0014/001454/145409e.pdf). In the document, it was stated that the definition of RM "*can be either narrow or very wide*" and that the field "*is generally about replacement, repair and regeneration to address deficient organ function resulting from congenital defects, disease, trauma or wear and tear*". From this definition, it may be inferred that RM and TM share the same interests and pursue the same goal, namely the replacement of terminally diseased organs with new functioning organs. However, while the term *replacement* is intimate to organ transplantation, *repair* and *regeneration* are not, unless we consider the case of auxiliary heterotopic liver transplantation, performed to allow the native liver devastated by an acute damage to regenerate and resume normal function[2].

More recently, the term RM has been used to define – more succinctly – a field in the health sciences that aims to replace or regenerate human cells, tissues, or organs to restore or establish normal function[3]. The process of regenerating body parts can occur *in vivo* or *ex vivo* and may require cells, natural or artificial scaffolding materials, growth factors, gene manipulation, or combinations of all the four elements. However, RM is commonly used as synonymous to "tissue engineering", but it has been noted that "tissue engineering" is "*narrower in scope and strictly defined as manufacturing body parts ex vivo, by seeding cells on or into a supporting scaffold*"[4]. According to NIH, "*tissue engineering evolved from the field of biomaterials development and refers to the practice of combining scaffolds, cells, and biologically active molecules into functional tissues*", with the ultimate goal of assembling "*functional constructs that restore, maintain, or improve damaged tissues or whole organs*" (https://www.nibib.nih.gov/science-education/science-topics/tissue-engineering-and-regenerative-medicine).

The NIH also defines RM as "*the process of creating living, functional tissues to repair or replace tissue or organ function lost due to age, disease, damage, or congenital defects. This field holds the promise of regenerating damaged tissues and organs in the body by stimulating previously irreparable organs to heal themselves; […] empowers scientists to grow tissues and organs in the laboratory and safely implant them when the body cannot heal itself. Importantly, regenerative medicine has the potential to solve the problem of the shortage of organs available through donation compared to the number of patients that require life-saving organ transplantation*". (https://report.nih.gov/nihfactsheets/viewfactsheet.aspx?csid=62). This definition is quite comprehensive but neglects two critical aspects of modern TM, namely immunosuppression-free transplantation and ischemia-reperfusion damage deriving from organ preservation and storage. In fact, RM research aims at building autologous tissues and organs from patient's own cells with the ultimate goal of bypassing the need for lifelong anti-rejection therapy, with consequent obvious clinical and financial benefits. Moreover, by enabling physicians to implant bioengineered organs immediately after maturation, ischemia-reperfusion damage is prevented.

Last, to the best of our knowledge, none of the societies that may claim parenthood to the field of RM has ever released an official definition of the term. Therefore, we herein propose the following definition:

RM is a field in the health sciences that aims to regenerate, repair or replace functionally impaired tissues and organs in order to restore normal function. The process of regenerating body parts can occur in vivo or ex vivo and may require cells, natural or artificial scaffolding materials, growth factors, genetic manipulation, or combinations of them. RM promises to address the longest standing limitations of organ transplantation, namely the identification of an inexhaustible source of transplantable organs, immunosuppression-free transplantation and organ-on-demand, whereby a patient in need and deemed suitable for transplantation is enabled to promptly receive an organ that

will be bioengineered from his/her own cells. This will rule out the need for lifelong immunosuppression and, by allowing implantation of the graft immediately after production, will likely prevent the damage resulting from organ preservation, storage and ischemia-reperfusion. As a corollary, RM will eliminate the waiting list.

RM technologies applied to organ transplantation

Decellularization. The term decellularization refers to a process whereby the cellular compartment of living tissues is removed by chemical or physical means[5]. The end product of this process is an acellular scaffold consisting of the innate extracellular matrix (ECM) of the original tissue that is being used for different purposes. For example, acellular scaffolds may serve as template for the *ex vivo* bioengineering of implantable organs[6], or to promote functional tissue restoration after implantation *in vivo*[7]. In the first case, the scaffold will be recellularized with progenitor or adult cells first, and either allowed to mature in bioreactors before implantation or implanted directly in patients thereafter [8]. In the second case, the acellular scaffold is implanted in the diseased tissue to orchestrate a constructive remodeling *in situ*[7]. The rationale behind these applications is that the ECM is the fundamental 3D network that not only provides structural support to cells, but by interacting with cell surface molecules and serving as a reservoir for growth factors, also plays a critical role in tissue and organ development, homeostasis and regeneration after damage[9]. Although the decellularization process does produce some damage to the innate ECM, the scaffolds produced with current technology retain most of the biochemical complexity, nanostructure, and bioinductive properties of the native matrix that are essential for cells to attach, migrate, proliferate and function, and have been shown to promote the creation of site-specific, functional tissue *in vivo*[10]. Moreover, as the framework of the innate vasculature is preserved, patent, and able to sustain the physiological blood pressure[11], acellular ECM scaffolds (aECMs) seem ideal for the bioengineering of transplantable organs. AECMs may also be a source of hydrogel and used as such as cell delivery tool. Notably, more than 80 ECM based products are currently available in the market for a variety of clinical applications[12].

From a TM perspective, the use of aECMs as a template for whole organ bioengineering undoubtedly holds immense potential. Since the milestone report on the bioengineering of a functional heart in 2008[13], more than two hundreds papers have proven that aECMs may be produced from virtually all transplantable organs from clinically relevant animal [11, 14-16] and human donors [16-24], including the human hand, face and face subunits [25-29]. As one of the major objectives of RM is to identify an inexhaustible source of organs, animals may be considered an ideal source of aECMs for intra-abdominal and intra-thoracic bioengineering, whereas human donors should provide organs for limb, face and face subunit bioengineering for obvious reasons. Interestingly, the term *semi-*

xenotransplantation has been proposed to indicate the bioengineering of implantable organs whereby aECMs of animal origin are seeded with human cells [30].

Despite the fact that viable and functioning bioengineered tissues and/or organs supplied with their own vascular pedicle have never previously been described in animal models, the literature reports a few anecdotal cases [8, 24, 31-34] or small studies in humans [35] where relatively complex tissues were transplanted without any vascular pedicle, and therefore lacked an immediate connection to the recipient's vasculature. Although some short-term success has been obtained, and to some extent, it can be claimed that the proof of concept has been provided, the reported morbidity and mortality are extremely high [36]. This probably reflects our incomplete understanding of the biology of organ regeneration and underestimation of the true anatomical and physiological complexity of the organs in question[37]. As recently stigmatized by Badylak in an illuminating editorial, the big mistake that has been reiterated by tissue engineers in the past decade is the erroneous belief that organs can be manufactured without the critical elements required to maintain the viability and function of all living tissues; namely, adequate lymphatic and innervation networks, and – more importantly – vascularization [38]. Moreover, most clinical and experimental studies report that after cells are seeded on aECMs, the so-obtained construct is allowed to mature in bioreactors for conventional periods of one or few weeks, which is probably insufficient as this is much shorter than the time needed for any given organ to develop *in utero*.

3D printing technology. 3D bioprinting promises to have a disruptive impact in TM and represents a significant technological advancement in the manufacturing processes used for tissue and organ engineering. Where the conventional manufacturing approach requires the manual fabrication by a skilled technician, 3D bioprinting is automating this process, with subsequent improvements in standardization, reproducibility, resolution and accuracy. These advances have arisen from the adoption of design and manufacturing techniques used in the non-biological manufacturing sector, such as the use of imaging and design software, and the increased availability, and reduced cost of 3D printing hardware. 3D printed medical devices have already been transplanted into patients [39, 40], and simple bioprinted tissues such as cartilage and bone have been successfully transplanted in preclinical animal studies [41-43]. However, just like above, current 3D bioprinted tissues lack essential functional elements such as vasculature, innervation, lymphatics and the number and diversity of functional and supporting cell types required for more complex or larger tissues and organs.

In health care, 3D printing has been applied for the manufacturing of surgical guides, anatomical models and prosthetics [39], and more recently, for custom implants [44]. Medical uses of 3D printing have usually been confined to static, non-living constructs, including patient-specific craniofacial implants and hip and mandibular prostheses [40]. In 2013, the clinical application of 3D printing was

expanded, with the implantation of a 3D printed, bioresorbable external airway splint into an infant with tracheobronchomalacia, which was followed up with a 2015 report of a further 3 infants recei

ving patient-matched 3D printed splints [45]. These constructs, while non-living, were designed to prevent external airway compression over a predetermined time period before bioresorption to accommodate airway growth [46]. While these advances have demonstrated the promise of 3D printing technology for medical applications, the progression from non-living constructs to 3D printed living cellular constructs has not been as rapid. Significant challenges surrounding the formation of complex, heterogeneous tissues, with sufficient vasculature, innervation, and function, means that we are currently years away before even simple constructs make their way into clinical use. It is likely that the first advances in the clinical transplantation of 3D printed living tissues will be made in relatively simple tissues before advancing to tissues with more complex geometries, cell types and functions.

In contrast to many other tissues, cartilaginous tissues are avascular and aneural structures containing a relatively low density of cells, potentially minimizing three of the most difficult hurdles in the field. For this reason, cartilaginous tissues are likely to be one of the first types of 3D printed tissues to progress to clinical transplantation, and multiple examples of 3D printed cartilage tissue have been described at the pre-clinical stage of development. Cui and colleagues have applied inkjet 3D printing technology to repair human articular cartilage [41], achieving a tissue construct with a compressive modulus in the same order of magnitude as hyaline cartilage [47].

Another novel approach involves the fabrication of tissue constructs using self-assembling spheroids of chondrocytes to form cartilage strands, significantly increasing cellular density and improving post-transplantation maturation and function [48]. Recently, the biofabrication and implantation of human-sized 3D printed cartilage tissues has been reported, with tissue constructs possessing histological and mechanical characteristics of human auricles after animal implantation *in vivo* [49].

Bone has been well studied by the materials engineering community due to its unique structure and mechanical properties. Biomaterial scaffolds that exploit the inherent properties of nanoparticles have been developed that meet the physicomechanical requirements of bone regeneration, formulated to control the mechanical properties and degradability of scaffolds upon transplantation [50]. In one example, Inzana and coworkers fabricated a calcium phosphate, collagen composite bone scaffold using a modified inkjet-based 3D printer. The implants were confirmed to be osteoconductive and biodegradable in a critical sized murine femoral defect [43]. However, to date, many 3D printing approaches rely only on hard scaffolds to reproduce the appropriate mechanical properties for cortical bone, but fail to fully recapitulate the cellular, spongy component of cancellous bone. One approach to overcome this limitation includes incorporating bone marrow-derived mesenchymal stromal cells into osteoconductive hydrogel bioinks. These soft bioinks are then supported by a network of reinforcing poly(ε-caprolactone) (PCL) microfibers to enable the fabrication of mechanically

reinforced constructs with decoupled biological and mechanical functionality. These 3D printed constructs mimic the geometry and bulk mechanical properties of trabecular-like endochondral bone with a supporting marrow structure, and undergo endochondral ossification over time following implantation [51]. Using a similar approach, human-scale mandible and calvarial structures have been 3D printed, with size and shape similar to what would be needed for facial reconstruction after traumatic injury. Implantation of 3D printed bone constructs into animal defect models resulted in the formation of mature, vascularized bone tissue in implants retrieved up to 5 months later [49].

The application of 3D printing technology to fabricate relatively simple tissues such as cartilage and bone has been facilitated by the development of new biomaterials and 3D printing technology that can accurately and reproducibly deposit these materials. 3D bioprinting techniques can be broadly classified by their mechanism of cell deposition into inkjet [52], microextrusion [49, 53-55], or laser-assisted bioprinting [56-58]. The basic technologies and their applications have been extensively reviewed [59, 60]. Recent advances in bioprinter technology have facilitated the patterning of multi-component constructs containing both synthetic and natural materials capable of resolution down to 2μm for biomaterials alone and down to 50 μm for encapsulated cells [49]. Further progress in the field will require the ability to deposit an even wider range of material types concurrently with increases in print resolution and speed. Some progress has been made in this area, such as the use of microfluidic switching nozzles that swap between two different inks on demand [61], as well as mixing nozzles that can be used to print materials at the microscale with tunable gradients of differing material properties [62]. Additionally there has been remarkable achievements in the high-resolution patterning of matrix materials using light-based free-form fabrication. One example of this is two-photon lithography, where transparent photoresist materials are photopolymerized with multiphoton absorption events with highly controllable focal volumes and print speed [63].

The combination of materials that provide mechanical strength and those that are compatible with cell function has resulted in the successful fabrication of human scale, cellular tissues that have shown long-term function post-transplantation. Biomaterials commonly used for bioprinting are predominantly based on either naturally derived polymers (such as tissue-based extracellular matrix proteins including alginate, gelatin, collagen, chitosan, fibrin and hyaluronic acid) or synthetic molecules (polyethylene glycol; PEG). Often, the synthetic materials provide physical integrity at the at the macro level, while softer materials, such as hydrogels, provide an appropriate environment for cell encapsulation and placement. However, synthetic materials often fail to provide physiological interactions with the cellular component. On the other hand, the weak mechanical properties of hydrogels is a considerable limitation for their contribution to the physical properties of the tissue. Further advances in the development of biological materials are needed to improve control of the structural, mechanical, and biological properties of constructs to replicate tissue structure and function [64, 65]. One approach towards overcoming this challenge include chemical modification of the hydrogels to enable the materials to cross-link with other materials, therefore controlling its

mechanical strength or other parameters such as degradation times. Synthetic hydrogels like PEG-based hydrogels have been modified to covalently tether ECM-derived biomolecules [66]. Similarly, there is a need for the continued development of 3D printers that are specifically designed for these biological materials, combined with the decreased cost of these technologies.

However, before we can expect to see successful 3D printing of larger, or more complex tissue types, several significant limitations and obstacles need to be overcome. For larger tissues, the incorporation of intact vasculature will be essential for the survival and function of the implanted tissues. One potential approach to overcome this bottleneck is the utilization of light-based 3D printing technology, capable of photopolymerizing a wide range of biological materials, with significantly improved speed and resolution. For example, microscale continuous optical bioprinting (μCOB) has been used to create prevascularized tissue constructs within a soft hydrogel network [67]. The ability to pattern increasingly complex cellular structures with increased resolution would provide many opportunities to incorporate other functional tissue components and architectures such as vascular, neural and lymphatic networks and potentially lumens, tubules and ducts. Another limitation to the fabrication of larger, more complex tissues is the requirement for increased quantity and diversity of cell types. Many studies have utilized either primary cells or tissue-derived multipotent stem cells, but the limited expansion and differentiation capacity of these cell types may limit their application for larger or more complex tissues. Potential approaches to overcome this problem include involve viral transfection [68] or use of small molecules to induce cell proliferation or differentiation [69, 70].

Stem cell technology. Cells within the inner cell mass of blastocyst-stage embryos give rise to all adult cell types and are thus termed 'pluripotent'. In 1981, it was discovered that these 'embryonic stem cells' (ESC) could be isolated from mouse embryos and expanded in culture without losing their pluripotency [71]. Following the isolation of the first human ESC lines in 1998 [72], there was huge optimism that these cells could not only replace cells lost in degenerative diseases such as Parkinson's disease, but could also be combined with natural or bioengineered scaffolds to generate replacement tissues and organs [73]. However, apart from the ethical issues surrounding the use of human embryos, several challenges facing the development and application of ESC-based therapies were soon identified, including (i) their tumorigenic risk; (ii) the need for reliable culture conditions to direct their differentiation to fully functional specialized cells; (iii) strategies to prevent immune-rejection.

Much progress has been made; for instance, methods to identify and remove undifferentiated ESCs from administered cell populations have now been developed [74], reducing the risk of tumor formation; and although some ESC derivatives remain functionally immature [75, 76], others, such as ESC-derived retinal pigment epithelial cells, display the typical characteristics of their adult counterparts [77] and have already been applied in clinical trials [78]. Some ESC-based therapies can

involve the transplantation of progenitor cells which then further differentiate *in vivo* to generate functionally mature cell types; for example, ESC-derived dopaminergic neuron progenitors can undergo maturation in rats with chemically-induced Parkinson's disease and can ameliorate motor deficits [79]. The problems with immune-rejection, however, still remain, because ESC are non-autologous. Therefore, unless ESC-based therapies are applied to immune-privileged sites like the retina and brain, immune-suppressant therapies or other strategies to prevent immune-rejection are required.

Reports that pluripotent stem cells could be isolated from bone marrow[80] appeared to circumvent the aforementioned ethical issues as well as the problems with immune-rejection, as these cells can be self-derived. Although it is now clear that the bone marrow does not harbor *pluripotent* stem cells, there is good evidence that *multipotent* mesenchymal stromal cells (MSC) isolated from various sources, including bone marrow, adipose tissue and umbilical cord, have the potential to generate bone-, cartilage- and adipocyte-like cells following in vitro culture under specific conditions [81]. This has led to much enthusiasm regarding the use of autologous MSC-derived cells in combination with biomaterial scaffolds to generate replacement tissues for transplantation, an example being the use of MSC-derived chondrocytes to regenerate cartilage in the upper airway [24]. However, although such constructs have been used in human patients under 'compassionate use', data from animal studies indicate that MSC-derived chondrocytes fail to engraft and there is no evidence of cartilage regeneration [82], which might partly explain the high mortality rates observed in the clinic [83]. Most studies now show that while MSC and other somatic cell-based regenerative medicine therapies can have significant beneficial effects, these are mediated by paracrine factors that either directly or indirectly stimulate endogenous repair [84-86]. Thus, while MSC could be useful for promoting the repair and regeneration of transplanted tissues and organs, it is unlikely that they will be able to directly replace damaged tissues [87]. For instance, liver MSC-derived exosomes administered in an ex vivo normothermic liver perfusion system displayed regenerative functions and promoted in vivo repair [88].

The seminal work of Yamanaka, who showed that somatic cells could be reprogrammed to generate induced pluripotent stem cells (iPSC) that have the same plasticity as ESC [89, 90], addressed some of the problems encountered with ESC and MSC; for instance, iPSC generation does not require human embryos, they can be patient-derived, and unlike MSC, they are pluripotent. The plasticity of iPSC raises the possibility that they could be used as a source of specialized cells types for the recellularization of tissue and organ scaffolds for transplantation. Indeed, iPSC appear to represent a potentially unlimited supply of pluripotent cells that could overcome cellular challenges related to quantity and specificity of cell sources for recellularization [91]. Improvement in pluripotent stem cell differentiation techniques are continuously in development [92]. Further optimization has to be determined exploiting the local cues and the functional stimuli occurring in the in vivo setting to acquire functional maturation. While some progress has been made with de- and

recellularization of kidney [93], heart [21], pancreas [94] and liver scaffolds [95], at present an adequate kidney scaffold recellularization *in vivo* appears challenging and available infusion protocols inadequate [96].

Overall, several additional key points need to be clarified to make stem cell research more realistic and practical. The extent and quality of vascularization required by tissue-engineered constructs for their in vivo stabilization and maintenance still need to be determined [97]. RM would benefit of methods to allow a constant in vivo tracking of cell viability and functions. Magnetic resonance imaging and optical imaging appear the more suitable approaches for high spatial resolution and high sensitivity, respectively. A fist approach to track endothelial after seeding in a trachea scaffold has been recently reported using bioluminescence technology cells [98].

Organoids and blastocyst complementation. Apart from the potential of using iPSC in combination with scaffolds for tissue replacement, recent progress has been made towards generating 3-dimensional iPSC-derived organoids *in vitro* representative of several different organ systems, including renal, liver and heart organoids [99]. Exciting breakthroughs have been made with renal organoid development in particular, where it has been shown that iPSC-derived renal progenitor cells can generate organoids comprising all key renal cell types [100]. While organoids could potentially open the door to the development of bioengineered tissues and organs for transplantation in the future, many problems first need to be overcome, including appropriate vascularization. This is actually a major challenge because in the developing embryo, the major organ systems develop together with their capillary network and main feed arteries, ensuring that blood is supplied at the correct pressure. This problem is exemplified by a previous study showing that fetal rat kidneys do not mature beyond a neonatal stage following transplantation into adult rats, likely due to their abnormal vasculature and failure to develop a renal artery [101].

A potentially more promising iPSC-based technology for generating autologous tissues and organs for transplantation is interspecies blastocyst complementation (IBC). In this approach, genetic manipulation of the host precludes the development of an organ which is then compensated by stem cells from a donor that produce the missing organ. Proof of principle for this approach was demonstrated in 1993 to generate T and B lymphocyte lineages by implanting murine ESC into the blastocysts of $Rag2^{-/-}$ mice [102]. Using host blastocysts derived from *Pdx1*-/- mice that display pancreatic agenesis, Melton's group showed that complementation with wild-type mouse ESCs resulted in the pancreatic epithelium being derived from the donor *Pdx1*+/+ cells [103]. A later study showed that complementation of *Pdx1-/-* mouse blastocysts with rat iPSC resulted in the development of functional rat pancreases within the adult mice hosts, thus demonstrating interspecies complementation [104]. These groundbreaking studies raise the possibility that, by genetically modifying pig blastocysts so that they are unable to generate specific organs, and then complementing with patient-derived human iPSC, it could be possible to generate autologous organs for

transplantation within the host pig. A key advantage of this approach is that apart from being autologous, functional and of the correct size, the organs could be transplanted with their own vascular pedicle. However, a number of challenges need to be addressed. For instance, although rat iPSC could generate pancreata within mouse hosts, they were unable to generate kidneys in *Sal1*-/- mice that display renal agenesis [105], suggesting that for some organs, additional modification of the donor iPSC might be required to enable them to interact appropriately with the developing host embryo. Furthermore, previous attempts to undertake interspecies complementation using human pluripotent stem cells and mouse blastocysts have had limited success [106, 107]. Nevertheless, using a 'primed' pluripotent state, Belmonte's group has shown that human pluripotent stem cells could contribute to developing mouse embryos following grafting into gastrulating mouse embryos [108], thus providing proof of principle for interspecies blastocyst complementation using human iPSC. Importantly, while decellularization and 3D printing rely on bioreactors for the maturation of the bioengineered constructs, with this technology, the organs develop *in utero*, which presents the most convenient and physiologically appropriate conditions.

However, some issues related to the generation of interspecies blastocyst complementation derived organs need to be faced. The purity of the generated organs, in terms of cell composition, need to be addressed. In fact, endothelial cells or other cell types derived from the host could contaminate the donor-derived organ. In addition to the technical problems, ethical concerns has been emphasized in relation to the possibility that human cells could contribution to the formation of non-targeted organs, such as brain or germ cells, generating chimeric brains or fetuses [109].

Expanding the donor pool by the application of regenerative medicine strategies. The narrowest concept of RM presumes the creation of neotissues from a cell source. This presumptive approach entails addressing barriers that may take decades to overcome including those related to manufacturing practicality, safety, regulation and cost/reimbursement. However, established solid organ transplantation may be considered to already encompass a truly RM approach best illustrated by the successful transplantation of kidneys with severe acute kidney injury and most recently, donation after circulatory death (DCD) heart transplants. These clinical successes have allowed organs historically considered unusable to be successfully transplanted but the approach in each case relies on firstly predictable in-vivo regeneration but in the latter case, actively managed ex-vivo muscle cell regeneration in the context of normothermic reperfusion.

Given the exceptionally successful results of solid organ transplants and the global "mantra" that this excellent selection of treatments is limited only by organ availability, it is possible to reason that managed regenerative treatment of the many thousands of deceased donor organs currently declined for transplant worldwide may represent a rapid route for clinical translation of the variety of regenerative therapies currently being developed. This diverges from the assumption that the generation of functioning neotissue is essential for patient benefit and instead uses regenerative cells

or alternative therapies to protect the intrinsic regenerative capability of the solid organs from damage and promote its augmented activation, during and after the multifaceted phase of peri-transplant graft injury.

This indication is evidently a major arena of clinical need. Candidate regenerative interventions – with the potential for multiple mechanisms of action – may be more effective and are already in phase 1 studies with particular promise for the application of MSC or pleomorphic regenerative cell populations such as those derived from adipose tissue. The former have been widely administered for immunoregulatory purposes but increasingly the focus of such therapies is more regenerative with a recent study in ex-vivo perfused human livers confirming up to 50 x 10^6 cells can be delivered safely via the hepatic artery. The latter have been safely administered intra-arterially or intra-portally, without vascular complications in animal models of kidney [110], lung and liver as well as directly into porcine and human coronary arteries [111-113]. These interventions have the added advantage of ex-vivo applicability in the context of organ storage or normothermic machine perfusion. This latter scenario offers the opportunity for regenerative therapy in the context of optimized biodistribution and pre-implantation efficacy/safety assessment.

An alternative RM-based approach may be molecule-based, in spite of the fact that multiple small molecule approaches for the indication that we are herein discussing have already failed to show clinical benefit. While on one hand this failure is likely to reflect the extensive redundancy in mechanisms of peri-transplant injury, on the other hand failure may simply tell us that we have not picked the right drug(s)! Ideally, we should consider molecules possessing high regenerative potential rather than molecules that target this or that pathway of the inflammatory response complicating ischemia-reperfusion. For instance, recent groundbreaking work from MDI Biological Laboratories identified MSI-1436 as a first-in-class regenerative medicine drug candidate [114]. In fact, in adult zebrafish, administration of MSI-1436 stimulated the rate of regeneration of caudal fin tissue and heart muscle by 2–3-fold without apparent tissue overgrowth or malformation. Moreover, administration of the drug to adult mice for 4 weeks beginning 24 h after inducing cardiac ischemia increased survival, improved heart function, reduced infarct size, reduced ventricular wall thinning and increased cellular proliferation in the infarct border zone. In a Phase 1 and 1b clinical trials attesting the potential of MSI-1436 for treating obesity and diabetes, good tolerability was demonstrated, and it was found that doses effective at stimulating regeneration were 5–50-times lower than the maximum well tolerated human dose; hence, this molecule shows great promise for applications in multiple TM scenarios.

Given the major financial effects associated with delayed graft function of solid organ transplants, regenerative therapies in this context may also find a role in reducing peri-transplant injury and augmenting post implantation regeneration even in those solid organs currently utilized for transplant.

Product development challenges for cell-based therapies

a. *Scaling-up production*. The scaling up of cell production is not a trivial process as the physical environment where the bioengineered tissue will be implanted will exert a number of known and unknown effects on the physiologic and phenotypic characteristic of the final product. The type, dimension and material where cells are grown is known to impact their characteristic including cell proliferation rate and differentiation potential. Using standardized modular unit in parallel is usually the simplest and safest approach to scaling up from laboratory to industrial production for products dedicated to single individuals. However, this approach result also in an increase of cost, labor and risk of failure for single units (http://www.bioprocessintl.com/manufacturing/cell-therapies/streamlining-cell-therapy-manufacture-328083/).

As illustrated above, the integration in a tridimensional structure of different cell types can be obtained using ECM as an instructing guide. However, the timing, composition and degree of differentiation of the cell populations used to regenerate the cellular compartment of these structure remains unclear, as well as the stimuli needed to obtain a complete differentiation before (or after) implantation. Due to the complexity of the function of complex modular organs like the kidney or the heart [115], the issue of scaling out is depending on the ability to replicate the essential manufacturing characteristics in different physical location and/or time. This in turns depends on the ability to identify the key factors regulating the consistency of the manufacturing process and control them [116](https://nam.edu/manufacturing-cell-therapies-the-paradigm-shift-in-health-care-of-this-century/). Single use modular apparatus are likely to be the simplest answer to this particular need.

Assessing a complex construct requires the understanding of the specific characteristic desired for any given clinical application and the technologies to measure them. Both non-invasive approaches and surrogate biomarkers will have to be developed in order to perform the identity qualification of the final product, both in terms of functionality and expected half-life after transplantation. However, while it is possible to standardize production, it may be difficult to apply the principle of "one-size-fits-all" to the recipient due to the intrinsic inter-individual variability but also to environmental effects. Adaptation of the process to a finite number of possible recipients is likely to be a necessary step.

b. *Key attributes of proposed RM interventions/products that demonstrate their readiness to be advanced into clinical trials*.

1) Critical quality attributes

In order to progress to application in humans, any new RM product should have a clear indication in terms of expected functions *in vivo*, the definition of surrogate biomarkers for the estimation of the efficacy and an imaging technology to assess the integration and biodistribution. The expected half-life *in vivo* should be clear, and remedial approaches in case of failure should be well defined. The

choice of parameters defining the products (i.e. identity) should be justified by a risk assessment and the intended use (https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm081670.pdf and http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500003987.pdf).

2) Critical process parameters

The critical process parameters are strictly depending on the cell type and need to cover basic safety and efficacy *in vivo* after transplantation. Both donor and recipient characteristics should be included in the evaluation of these parameters. Usually these parameters encompass at least cell number, viability and proliferative index, which per se do not exclude the necessity of more specific biomarker testing.

3) Material attributes

As the cells respond in a complex – yet, still not fully understood – manner to the materials present in the culture media and containers used during the manufacturing process, any substitution or alteration has to be carefully assessed against the panel of final desired characteristics. Depending on their complexity, such assurance could be obtained by either testing the materials before accepting them for the manufacturing process, or by the full qualification of the supply chain. A strict definition of the materials sourcing should be established early in the development process.

c. What are the key regulatory science questions that should be addressed in the next five years?

The understanding of the expansion limit of the different stem cell lines with potential for clinical applications is the first knowledge gap to be filled. Stem cells should be expanded and harvested without incurring in genomic alterations that would obviously undermine safety. Such limit is now defined in a conservative way for MSC [117] but remains unclear for ESC and iPS. However, the necessity to obtain and qualify new donor cell population regularly constitute a strong limitation to the application on a large scale of RM.

Information about the stability of the transplanted organ/tissue and its response to environmental stimuli *in vivo* is fundamental for progress in the field but will be obtained only after a more substantial number of applications will be done. Currently, it is not possible to predict the fate of an artificial tissue *in vivo*, which could engraft permanently while exerting (some) function, but may also fail in time, or be colonized and replaced by endogenous cell. The long-term stability and the ability to exert (to some extent) physiological function(s) after implantation will therefore have to be determined. In this scenario, it will be critical for authors to disclose with honesty and integrity not

only short- or mid-term results, but also the long-term results. On their side, journals should require authors to provide outcome updates on a regular basis.

As discussed above, so far it has been common practice to implant bioengineered tissues without the reconnection to the blood stream of the recipient or the nervous system. For solid organs or vascularized composite allografts, this is not an option. Therefore, research should devise strategies to allow the integration of the vascular and nervous system of the host with the bioengineered tissue.

Regenerative immunology

One of the most critical questions to answer is how the immune system could react against a bioengineered cellular construct and if it would be possible to modulate this response [118]. A bioengineered construct consists of two components, the cellular compartment and the cell-supporting system, namely the ECM. While a fully developed lab-grown organ consisting in well differentiated cells deriving from a genetically different donor will certainly be subjected to the same well codified immune response as an allograft, it was initially speculated that tissues derived from allogeneic pluripotent stem cells (PSCs)- were not immunogenic and could therefore evade allorecognition [119]. This hypothesis was based on the observation that primordial cells like PSC present low MHC expression and immunogenicity, and that lab-engineered biological constructs lack dendritic cells and a lymphatic system that are primary drivers of alloimmune response. However, a growing body of literature has clearly shown that PSC are not immune privileged and that even tissues derived from autologous iPS may elicit an inflammatory reaction and succumb to rejection [119]. Therefore, strategies to promote local or systemic tolerance or immunomodulation are currently under investigations. One approach to solve the problem of graft immunogenicity is the cloaking of lab-grown (allo- or auto-) grafts in immune-neutral substances, such as nanofilms [120-123]. Alternatively, researchers are assessing whether the constitutive secretion of immune-modulating cytokines, including TGF-beta, by tissues differentiated from PSC promotes polarization of infiltrating T cells toward a regulatory T cell (Treg), immune modulatory phenotype [119, 124-126]. Interestingly enough, natural ECM-based scaffolds obtained from human organs that are being used as supporting scaffolding material for bioengineered tissues, have been reported to contain significant amount of TGF-beta [127-130] and to be able to induce T-cell apoptosis and promote conversion of naïve $CD4^+$ T cells into $CD4^+CD25^+Foxp3^+$ Treg [118, 129]. This observation is consistent with the evidence showing that the ECM possesses strong immunomodulatory properties. Studies in rats showed that rabbit acellular decellularized muscle scaffolds down-regulated T cell xenogeneic responses and T_H1 effector function compared to fresh tissue by inducing a state of peripheral T cell hyporesponsiveness [131]. Moreover, ECM obtained from human normal or diseased organs, promote a protolerogenic macrophage polarization similar to the one that is observed in the adaptive regenerative healing response whereby a phenotypic transition from the pro-inflammatory M1 to the

immune-modulating M2 phenotype occurs [128, 132, 133]. Therefore, combining the intrinsic ability of PSC-differentiated tissue to release TGF-beta with the immunomodulatory properties of ECM-based scaffolds, may represent a valuable strategy to reduce immunogenicity of bioengeneered organs.

Conclusions

The transplant era began in 1902 in Vienna, with the successful autotransplantation of a kidney in the neck of a dog performed by Hungary born surgeon Emerich Ullman who however did not succeed in performing any transplant in humans. It took fifty-two years before the first successful renal transplant could be executed in humans, and more than seven decades before transplantation became standard of care for a myriad of clinical settings requiring replacement surgery. In the past few decades, a new field of health science referred to as RM has shown potential to deliver to the bedside organs manufactured from patient's own cells thus bypassing allorecognition and ultimately rendering anti-rejection medication unnecessary. In doing so, RM promises to meet the more urgent needs of our field, proposes a new Holy Grail for modern TM[1] and identifies a field of investigation of immense interest to transplantation (Figure 1).

Few patients have truly benefited from the successful implantation of bioengineered organs, while in the majority of others the graft did not work. Moreover, the anatomical and physiological complexities of modular organs like the liver, the kidney etc., has not been replicated yet and a lot of work remains to be done before organ bioengineering will approach the bedside and so change the paradigm that has dominated transplant medicine for more than a century using lab-grown organs rather than organs procured from deceased or living donors. However, the proof of concept has been provided and researchers are now studying how to fully develop its potential and allow translation. Despite the road to the ultimate objective appears long, winding and difficult (Figure 2), the different RM technologies are still immature and several questions will have to be answered before translation may occur (Table 1), the days when success will be the usual outcome are ahead of us and closer are the days when TM, a discipline that traditionally has been immunology-based [134, 135], will realize that RM should become its major research core. If we agree on this, then TM should allocate more funds to RM-inspired research, transplant societies should twin with RM societies and established RM community of practices and committees, transplant journals and conferences should grant the due consideration and visibility to RM manuscripts. The good news is that most of this is already being done.

To the authors of the present manuscript, it is clear that no field in health sciences has more interest than organ transplantation in fostering progress in RM simply because the future of no other field more than the future of TM will be determined and forged by progress occurring in RM.

Figure 1. In the history of organ transplantation, we identify three phases or eras. The first can be referred to as the surgery phase and spans from the early days to the advent of cyclosporine. The introduction of this potent immunosuppressant allowed transplantation to become a lifesaving procedure for a myriad of clinical scenarios characterized by irreversible organ failure. The second phase (immunology) spans from the advent of cyclosporine to nowadays. During that phase, we have learned how to manage anti-rejection medications and their impact on patient's quality of life. Importantly, given the burden of side effects that comes with lifelong immunosuppression, we have realized that we should devise strategies to minimize the immunosuppression if not withdrawing it completely sometime after the transplant. Unfortunately, immunosuppression-free transplantation remains unrealistic, despite intense research and multiple attempts to translate promising laboratory findings into the clinic[136, 137]. The third phase has just begun and can be referred to as the regenerative medicine phase. RM promises to meet the most urgent needs of modern transplantation, namely, the identification of a new potentially inexhaustible source of organs and immunosuppression-free transplantation (adapted from Salvatori et al. Xenotransplantation 2015 and Orlando G. Transplantation 2017, with permission).

Figure 2. Roadmap for ex vivo solid organ bioengineering using decellularization and 3D printing technologies. The figure briefly summarizes the milestones to reach on the path towards the Holy Grail. However, the cartoon does not contemplate interspecies blastocyst complementation, which – to the authors – bears the greatest potential for the field because all steps of organ ontogenesis occur in vivo and are strictly regulated by the surrogate animal, without any need for any intervention from the outside. Instead, based on current views, in the case of decellularization and 3D printing, cells and supporting scaffolding materials need to go through a maturation phase whose duration, dynamic and physiology remain largely unknown.

Table 1. State of the art, perspective and hurdles to overcome in the major RM technologies of transplant interest.

Legend: ECMs extracellular matrix scaffolds; aECMs acellular ECMs; iPSC induced pluripotent stem cells; IBC interspecies blastocyst complementation; GLP good laboratory practice

	State of the art	Perspective and hurdles to overcome
DECELLULARIZATION	1. Virtually all organs from all clinically relevant mammalian species including humans can be decellularized to obtain acellular ECMs 2. aECMs preserves most yet not all molecular and physical characteristics of the innate ECM, as the decellularization process damages the ECM to an extent that depends on the method and the organ 3. Partial regeneration of the endothelial and parenchymal compartments has been reported, yet results are inconsistent and difficult to reproduce 4. The maturation phase reported in the literature for the different organs was always far inferior to the time needed in utero to develop the organs in questions 5. The implantation in vivo of a viable and functioning bioengineered organ has never been reported	1. In-depth understanding of the mechanisms underlying organ development, regeneration and homeostasis 2. In-depth understanding of the mechanisms of ECM-cell interactions 3. Cell selection for recellularization 4. Harmonious harnessing of lymphatic, nervous and vascular components 5. Improving the design of *ad hoc* bioreactors to support maturation 6. Strategies to achieve adequate recellularization
3D	1. Successful isolation and expansion of many functional and supportive cell types 2. Replication of mechanical and biophysical properties	1. Production of an adequate number of regeneration-competent cells that do not elicit an immune repose following

	of simple tissues at the macro-level 3. Bioprinting of cells with natural and synthetic biomaterials with high resolution 4. Implantation and *in vivo* maturation of small avascular tissues	transplantation 2. ECM-based materials that provide much stronger mechanical strength while maintaining the cell-supportive environment 3. Improvements in speed, resolution, material flexibility and scalability of bioprinters 4. Bioprinting of multi-scale vascular networks within instructive bioink that promotes angiogenic sprouting and neovascularization
iPSCs	1. Generation of various types of complex organoids in vitro (e.g., renal, liver, heart, pancreas) from human iPSCs 2. Generation of human pancreatic tissue in vivo following transplantation of iPSC-derived organoids in mice 3. iPSCs can be generated from individual patients, circumventing the need for immunosuppressants following transplantation into patients	1. iPSC-derived organoids typically resemble foetal tissues/organs and are unlikely to mature into functioning adult organs 2. iPSC-derived organoids generated in vitro do not have the blood vessels, lymphatics and neuronal innervation required for them to function in vivo
IBC	1. Development of functional rat pancreata following IBC of Pdx1-/- mouse blastocysts 2. Generation of a biallelic knockout in pigs using nuclease-based genome editing shows it could be possible to generate pig embryos for IBC that lack specific organs	1. To improve the efficiency of generating human-pig chimeric embryos, we need a greater understanding of how the status of human iPSCs (ie, whether they are ‘naïve’, ‘primed’ or ‘intermediate’) affects

	3. Development of mouse-human and pig-human chimeric embryos using 'primed' human iPSCs	their ability to integrate into post-implantation pig embryos 2. The contribution of human iPSCs to developing pig embryos is limited and it has not yet been possible to generate human organs using IBC 3. Even if the above challenges were addressed, a further problem is that human organs developed using IBC would have pig blood vessels, lymphatics and neuronal innervation, which would probably lead to immune-rejection.
RM for IR	1. Multiple candidate cell populations showing efficacy beyond previous small molecule alternatives. 2. Emerging evidence of favourable biodistribution avoiding off-site effects 3. Natural organ architecture available in transplant context. 4. Complementary benefits with normothermic, ex-vivo perfusion.	1 Obtaining adequate numbers of point of care derived autologous cells. 2. Obtaining adequate numbers of efficacious, non-immunogenic GLP manufactured allogeneic cells. 3. Reassurance regarding potential vascular/microvascular complications

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