EDITOR'S PICK

The choice of editor's pick for this edition comes courtesy of Henrik Lauridsen. A stimulating consideration of the nature of regeneration and the impact of humans' low regenerative potential is given throughout this paper. The author provides some excellent examples from the natural world and, in discussing the current technological state of affairs, highlights how far this field has developed. It is certainly thought-provoking to contemplate the potential of artificial tissue regeneration.

A REGENERATIVE BIOLOGY VIEW ON ARTIFICIAL TISSUE CONSTRUCTION AND THREE-DIMENSIONAL BIOPRINTING: WHAT MAY WE LEARN FROM NATURAL REGENERATIVE PHENOMENA?

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ABSTRACT

The implications of the low tissue regenerative potential in humans are severe and widespread. Several of our major diseases are direct results of this deficiency that leaves us vulnerable to events of tissue damage. This is opposed to some animal groups, such as the urodele amphibians (salamanders), that display distinct tissue regeneration after injury. An important goal of biomedical engineering is the construction of artificial tissue that can ultimately be transplanted into patients, however, such constructs are still in their infancy for more complex structures. Approaches of constructing artificial organ structures by decellularisation/recellularisation procedures and recently with three-dimensional (3D) bioprinting show promising results in obtaining anatomically accurate constructs, however, the function of these artificial tissues is still lacking compared to natural tissues. This review will highlight how the relatively mature fields of regenerative biology and medicine can have potential usage in the younger bioengineering field of artificial tissue construction by drawing on the knowledge of how intrinsic tissue regeneration takes place in nature.

Keywords: Regeneration, three-dimensional (3D) bioprinting, artificial tissue.

THE RECONSTRUCTION OF TISSUE

Tissue regeneration is the process of replacing tissue lost from damage, disease, age, or injury with new tissue. This process is fundamental for life and all animal species to some degree possess mechanisms that can maintain homeostasis and the functional integrity of cells and tissue. Richard J. Goss,[1] one of the pioneers of regenerative research, summed up the relationship between life, death, and regeneration with the words: "If there were no regeneration there could be no life. If everything regenerated there could be no death. All organisms exist between these two extremes. Other things being equal, they tend toward the latter end of the spectrum, never quite achieving immortality because this would be incompatible with reproduction". However true this statement is, regenerative potential varies quite

a bit between different organisms. Humans and mammals, in general, have not been blessed with extensive regenerative potential and tend to resolve tissue damage events by fibrosis and scar formation.[2] This fibrotic situation results from an inflammatory response to injury that generates fibroblastic granulation tissue that is ultimately modelled into an acellular collagenous scar. This maintains the overall integrity of the damaged organ but usually reduces function. On the other hand, 'lower' vertebrate species (e.g. urodele amphibians) do exist with an unsurpassed ability to recapitulate embryonic development and regenerate tissue and even whole organs and appendages to perfection without any scar formation.[3-5] This perplexing conundrum of why mammals have lost this apparently ancestral and seemingly highly beneficial trait has been a driving force in the history of regenerative research. It may be that evolution of warm-blooded mammals susceptible to infections has favoured the individuals with the fastest response to injury, with this being a swift immunological response and subsequent fibrosis to 'seal off' damaged parts rather than regeneration.[6,7] After all, although regeneration of extremities and organs is desirable, it is not essential for reproduction.

The low regenerative potential of humans has far-reaching consequences in medicine. Heart, liver, and renal failure; disorders of the nervous system (e.g. amyotrophic lateral sclerosis, multiple sclerosis, and Parkinson's, Huntington's, and Alzheimer's diseases); and burns and traumatic injuries (to skin, bones muscles joints, ligaments, and tendons) are all examples of diseases and conditions resulting from poor regenerative potential. Other than the personal consequences for patients suffering from these ailments, the cost to society is enormous. In the USA alone the annual cost was estimated to be >$400 billion.[3] Naturally, this has inspired great interest with the field of regenerative medicine seeking to develop and apply future regenerative therapies for human patients. However, it is highly unlikely that regenerative medicine can fulfil this task without a thorough understanding of the basic mechanisms involved in regenerative events. Therefore, advancements in the field of regenerative biology and the understanding of basic molecular and cellular processes during tissue regeneration are a prerequisite for regenerative medicine to develop its full potential.

THE ENGINEERING SOLUTION TO LOW REGENERATIVE POTENTIAL

The lesson from nature is that intrinsic *in situ* regeneration of even highly complex tissues is possible in basic vertebrate models. Most human tissues do in fact replenish themselves to some degree over life; with the strategy to stimulate innate tissue regeneration being one of the most desirable approaches for future regenerative therapies. However, as the key to unlock this intrinsic regenerative potential in humans is yet to be found it is worth considering alternative strategies for rebuilding damaged organs.

A fundamentally different approach of constructing organs or tissue components has arisen in the last decade, namely the *de novo* construction of organs *in vitro* that can later be transplanted into patients. An instructive example is the construction of heart transplants. Since Dr Barnard's successful heart transplantation in the mid-twentieth century the heart transplantation procedure has become a well-established lifesaving treatment to extend and dramatically improve the quality of life for recipients. A patient's heart can either be fully replaced with a donor heart (orthotopic procedure) or supported by an extra heart (heterotopic procedure). Unfortunately, suitable donor hearts, as well as other organs, are a scarce commodity and as the demand is increasing, so is the need for alternative strategies. Two novel engineering approaches to alleviate the problem of scarce donor organs have received much attention in recent years, namely the construction of bioartificial organs either using a decellularisation/repopulation procedure of extracellular matrix scaffolds or bottom-up constructions of artificial organs using three-dimensional (3D) bioprinting.

ARTIFICIAL ORGANS BY DECELLULARISATION/ RECELLULARISATION

Bioengineering laboratories around the world have long sought to construct artificial biocompatible scaffolds for cell seeding and transplantable tissue generation. This has led to several successful procedures and especially in orthopaedics multiple useful constructs have been designed.[8] Similar approaches of scaffold construction have been applied for soft tissue construction; however, it is hard to replicate the complex cellular environment found in most organs in simple scaffolds moulded

in the lab. One way to circumvent this limitation is to generate extracellular scaffolds made by decellularisation of true organs that may be too old or do not match the receiver type and hence cannot be used directly for transplantation. This procedure can be a complicated washing process in which all living cells are removed from the organ leaving behind only the supporting extracellular matrix as intact and unmodified as possible.[9] The resulting tissue scaffold is then reseeded with living cells either by immersion in a cell containing medium or more efficiently by perfusion via the skeletonised vasculature of the decellularised organ. As a cell source, either differentiated cells that have previously been dissociated from their host tissue into a single cell state or patient-specific stem cells that have been cultured prior to perfusion, can be applied. The underlying assumption of the decellularisation/recellularisation procedure is that perfused cells migrate through the extracellular matrix scaffold. Differentiated cells should ultimately settle when they reach a suitable environment, whereas stem cells may in fact start to differentiate in a manner defined by cues in the surrounding matrix components.

Following a maturation period of cell proliferation, the final result is a 3D multi-cell type culture that can be thought of as a 'breath of life' into a once dead and skeletonised organ. However, the decellularisation/recellularisation technique does not come without important limitations. In 2008 Ott et al.[10] applied the procedure to recellularise rat hearts using neonatal cardiomyocytes, fibrocytes, endothelial cells, and smooth muscle cells and subsequently transplanted the constructed hearts into host rats. Remarkably, this study demonstrated how contractile and drug responsive hearts could be constructed, however the function of the generated constructs were only ~2% of that of an adult rat heart. In a similar fashion, Lu et al.[11] in 2013 were successful in repopulating decellularised mouse hearts with induced pluripotent stem cell-derived cardiovascular progenitor cells from humans. This attempt also yielded heart constructs that exhibited spontaneous contractions, generated mechanical force, and were responsive to drugs. However, in similar fashion to the attempt by Ott et al.,[10] these constructs only showed minute pump function and were overall unable to propel blood.

The attempts described above to rebuild the heart, as well as other organs,[12] by decellularisation/recellularisation of already existing organs, are interesting because they suggest that this type of organ construction that can be viewed as a very engineering-based mindset (breakdown then build up) and not a biologically inspired way of regenerating organs. However, for now the procedure is insufficient in producing fully functional organs. To overcome this obstacle, repopulation technologies need to consider how organs are constructed during development and in natural regeneration in competent species.

THREE-DIMENSIONAL BIOPRINTING: A POTENTIAL LOOPHOLE FOR TISSUE RECONSTRUCTION

A fundamentally different approach of producing complex organs to that of cell repopulation of scaffolds is 3D bioprinting. The prospects of this technology have been glorified in recent years in TED talks and other quasi-scientific fora; however, the method has in fact shown promising results in terms of producing *de novo* tissue constructs.

As the name suggests, 3D bioprinting originates from 3D printing (also known as rapid prototyping or more precisely as additive manufacturing) of prototypes and models in a large variety of materials such as plastic, wood, ceramics, glass, and metals in industry. 3D printing is additive in nature, i.e. it starts from nothing and ends with a structure after adding layer upon layer. This is opposed to computer numerical control drilling, which performs what can be described as negative manufacturing by carving out a structure from a solid object. From an historical perspective, it is interesting that 3D printing has existed for several decades,[13] but primarily due to proprietary issues gridlocking technological development and competition the technology has only flourished within the last decade with the expiration of early patents. This has resulted in modern day 3D printers becoming better and more affordable at an astonishing pace. 3D printing has been applied in medical and life sciences to create organ models from computed tomography and magnetic resonance imaging (MRI) information and implantable constructs.[14-18] Some major 3D printer manufactures now offer dedicated software and hardware for this field of modelling i.e. complex medical disorders in physical models that surgeons can handle and study even before the first cut is made in surgery.

The 3D printing field is notorious for the lack of consistent nomenclature to describe similar printing technologies; however, the American Society for Testing and Materials (ASTM) currently categorises

3D printing technologies into seven categories: binder jetting, directed energy deposition, material extrusion, material jetting, powder bed fusion, sheet lamination, and vat photopolymerisation.[19] These technologies have all been developed for non-living materials, but in addition, 3D printing has recently evolved into 3D bioprinting in which cells and extracellular matrix can also be used as raw materials for printing.[20] 3D bioprinting relies on two of the seven categories of non-living 3D printing technologies listed above and one additional technology; thus 3D bioprinting can currently be divided into three technologies. The first and simplest is the inkjet method, which is inspired by the material jetting method of 3D printers and is in principle the same inkjet technology applied in desktop paper printers. In this technique, rapid electrical heating or piezoelectric/ultrasound generated pressure pulses are applied to propel cell containing droplets from a nozzle to the build surface. The second 3D bioprinting technique, microextrusion printing, is widely applied and fundamentally applies the same method as material extrusion 3D printers to pneumatically or mechanically dispense a continuous thread of cell containing material. The final and most complex technology is laser-assisted 3D bioprinting in which a laser is briefly and repeatedly focussed on an absorbing substrate coated in cells thereby generating a pressure that propels cell-containing materials onto a collector substrate that becomes coated by cells in a pattern defined by the laser path. Following the initial deposition of cells and substrates, the layered construct generated in all three bioprinting methods is cured and hardened to support its own structure either by designed polymers in the cell medium that respond to cooling, heating, chemical treatment, or more commonly by light-activated polymerisation in a fashion that is comparable to vat photopolymerisation in non-living material 3D printers.

The three 3D bioprinting technologies currently available have specific advantages and limitations. The simplest and most affordable technology is inkjet bioprinting, which also operates at a high printing speed and with a high cell viability because of a relatively gentle printing process. The spatial resolution of the technique is, however, relatively low and cell density in the construct is also low, which is important for the subsequent maturation of the construct. On the other hand, microextrusion based bioprinters provide high-cell densities but at the cost of viability (often <40%), primarily due to sheer

stress during deposition, and printing speed is lower than for inkjet printing. Laser-assisted bioprinters are fast and cell viability is very high (>95%) and so is the printing resolution; however, these systems are highly expensive and challenging to maintain.

Early experiments using 3D bioprinting have been reported for a number of tissues and organ structures ranging from skin, blood vessels, trachea, cartilage, kidney, and various cardiac tissue (e.g. myocardium and valves).[20] A central goal of this technology has however been in the construction of cardiac tissue which represents a relatively simple organ (compared to secretory organs often with manifold cell types) and a model system where the success of the construction can easily be tested in terms of function compared to baseline values. Thus, the attempts to construct cardiac tissue serves as a good model for the current status of 3D bioprinting technology. Several impressive attempts of printing different cardiac tissues have been made in recent years.[21] From a regenerative biology viewpoint the attempt by Gaetani et al.[22] in 2012 to apply microextrusion to 3D bioprint small alginate scaffolds with fetal human cardiomyocyte progenitor cells is interesting. These myocardial-like scaffolds both showed high-cell viability and importantly imbedded cardiomyocytes retained their commitment for the cardiac lineage. The implication of this is that the deposited cells in fact remain in their desired lineage and behave in a natural fashion. Another study by Gaebel et al.[23] in 2011 used the laser assisted bioprinting technique on human umbilical vein endothelial cells and human mesenchymal stem cells to construct various capillary like patterns of cells on a polyester urethane urea cardiac patch. Several of these vascular patterns were successful and resulted in the two cell types arranged into a capillary like network. These patches with patterned cells were thereafter cultured and matured and subsequently transplanted *in vivo* into infarcted rat hearts. Intriguingly, the study reported increased vessel formation and additionally a significant functional improvement of the infarcted hearts. The results of this study underline the importance of considering the construction of an appropriate vascular supply in regenerative therapies.

To date, no successful attempts to 3D bioprint complete organ constructs similar to the ones generated by decellularisation/repopulation procedures have been reported, but efforts in using extracellular matrix for building material have been made with success.[24] Assuming that the

3D bioprinting technology eventually matures to the state where the option of full organ printing becomes possible, it is not unlikely that constructs may suffer from the same deficiencies in terms of function and force production as described above for the repopulated heart constructs. To overcome these obstacles, it may be fruitful to consider some aspects of naturally occurring regenerative phenomena and implement these in artificial organ construction.

LEARNING FROM NATURAL REGENERATIVE MECHANISMS

Obviously, not all details of naturally occurring regenerative phenomena have been revealed. In that case artificial tissue construction would be redundant; however, several key aspects of some of these phenomena have been described and potentially hold some information on how to yield functional artificial tissue. Regeneration of extremities, such as the limb in the salamander, is an example of complex tissue regeneration that has been studied in great detail and serves well as an instance for some of these considerations.[3-5,25-28]

The regenerative process of the salamander limb falls into three non-discrete steps: wound healing, blastema formation, and regrowth.[3-5] Within the first couple of hours following amputation of a limb, the wound is sealed with a wound epidermis by migrating cells from the adjacent epidermis. The wound epidermis thickens and becomes several cell layers thick and then forms an apical epidermal cap with a protective outer surface and inner layers that anatomically and functionally resemble the apical ectodermal ridge formed during vertebrate limb development.[29] Signalling from the wound epidermis as well as neurotrophic signalling from severed nerves induces dedifferentiation of differentiated cells adjacent to the amputation site, leading to the formation of a structure termed a blastema within 1–2 weeks containing dedifferentiated cells with varying origin (e.g. connective tissue, muscular tissue, bone, and nerves). Finally, dedifferentiated blastema cells proliferate, redifferentiate, and regrow just part of the missing limb. The conclusion of this mechanism is that a process very similar to embryonic development can be initiated if the right factors are present. In the case of the limb, these are signalling from the wound epidermis, neurotrophic signalling from severed nerves, and the existence of dermal fibroblasts with a different positional identity. If any of these factors are removed, the regenerative process comes to a standstill. On the other hand, if these three factors are expressed artificially it is possible to induce limb regeneration at uncommon places and produce ectopic limbs.[25] The implications of this in an artificial tissue context is that it may not be crucial to construct an anatomical replica of the final organ of interest but rather focus on building a meshwork of cells that can be stimulated to undergo differentiation and growth in a process that recapitulates embryonic development of the organ. This approach has already been implemented in 4D bioprinting, in which printed objects can change functionality and shape after printing by the application of an external stimulus.[30]

Another important aspect of intrinsic limb regeneration is the origin of cells taking part in regeneration. It has long been speculated that blastema cells represent true pluripotent stem cells with the potential to differentiate into any cell type in the regenerating limb. However, in 2009 Kragl et al.[26] demonstrated, using GFP+ transgenic axolotls, that most cell types involved in limb regeneration are lineage restricted. The lesson from this is that artificial tissue endeavours are likely to have a higher chance of being successful if relying on multiple cell types that can differentiate to the exact type of cells needed rather than a single homogenous population of pluripotent stem cells.

CONCLUSION

In terms of tissue regeneration, it is stimulating to think that we have both examples of intrinsic tissue repair found in nature as well as a multitude of toolsets to construct artificial tissues possible with 3D bioprinting being the most sophisticated method to date. Combining the knowledge from natural phenomena with the ingenuity of biomedical engineers it is not unlikely that anatomically correct tissue constructs can be generated within a reasonable timeframe but also constructs that function just as well as their endogenous counterparts.

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