REVIEW ARTICLE

Tissue Engineering and Regenerative Medicine 2015: A Year in Review

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This may be the most exciting time ever for the field of tissue engineering and regenerative medicine (TERM). After decades of progress, it has matured, integrated, and diversified into entirely new areas, and it is starting to make the pivotal shift toward translation. The most exciting science and applications continue to emerge at the boundaries of disciplines, through increasingly effective interactions between stem cell biologists, bioengineers, clinicians, and the commercial sector. In this “Year in Review,” we highlight some of the major advances reported over the last year (Summer 2014–Fall 2015). Using a methodology similar to that established in previous years, we identified four areas that generated major progress in the field: (i) pluripotent stem cells, (ii) microtissue platforms for drug testing and disease modeling, (iii) tissue models of cancer, and (iv) whole organ engineering. For each area, we used some of the most impactful articles to illustrate the important concepts and results that advanced the state of the art of TERM. We conclude with reflections on emerging areas and perspectives for future development in the field.

The Aim, Scope, and Methods of the Review

TISSUE ENGINEERING AND regenerative medicine (TERM) is an incredibly complex field that spans from molecular level studies in genetics and developmental biology to whole organ engineering. Historically, the objective of TERM has been to apply the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve the function of a tissue or whole organ.¹ While this objective remains intact, in recent years, we have been experiencing two important changes. The main focus of the field—to repair or replace tissues and organs lost to injury or disease, has been extended to an equally important application of engineered tissues for drug testing, disease modeling, and precision medicine, an effort enabled by the advent of human induced pluripotent cells (iPS cells).²

Clearly, both the regenerative medicine applications and *in vitro* tissue platforms have vast potential for understanding and treating human disease. The second change is in the approaches used to generate, use, and analyze engineered tissues, which are becoming increasingly diverse, sophisticated, and interdisciplinary. Our review attempts to cover exciting research contributing to both the regenerative medicine and *in vitro* research applications of engineered tissues along with the novel methodological approaches that are advancing the field.

This is the fourth *Year in Review* in *Tissue Engineering*. The previous three articles of the same kind^{3–5} helped establish methodology for selecting the areas of focus and the

articles to highlight. We adopted the basic methods used in previous years to identify the most exciting advances in the field. As in previous years, we started by searching the ISI Web of Knowledge database for articles in “tissue engineering” and “regenerative medicine” published during the period of June 2014 through November 2015. We chose a 3-month overlap with the previous review to not miss impactful articles, given that the focus of this review is different. This search showed that the areas most active in the previous year included human pluripotent stem cells (PSCs; basic studies, application in tissue engineering), microtissue platforms (organs on a chip, tissue chips, drug testing, disease modeling), human cancer (tissue engineered models), and whole organ engineering (use of native tissues and organs as biological scaffolds for tissue engineering).

We also introduced some modifications by including other criteria to determine the impact of recently published articles. The well-established metrics, such as the ISI citation index, are reliable for evaluating the impact of articles published years ago but not for very recent articles that were our focus. We therefore also took into account the continuity of research (analyzing recent articles from groups leading the field), quality of journals (analyzing TERM articles appearing in top journals, both general and specialized), and the immediate reaction of the field to recently published articles (such as commentaries and highlights in peer-reviewed scientific press). Our approach was somewhat holistic as we based our choices on an aggregate of all the above criteria, in an attempt to select the most highly meritorious and influential recent work. Clearly, there is no

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completely objective way to determine the impact of recent research. The selection we offer to the reader reflects our personal views and does not include all highly significant work published over the year.

Using these methods, we selected four topics for this review (Fig. 1): (i) PSCs, (ii) microtissue platforms for drug testing and disease modeling, (iii) cancer, and (iv) whole organ engineering. For each section, several articles are used to illustrate the important concepts, especially in the context of advancement beyond the state of the art in the field. We then provide perspectives on some future developments.

Area I: PSCs: Understanding and Exploiting Pluripotency

The introduction of iPS cell technology by Shinya Yamanaka in 2006 is probably best described as a “game changer” for the whole TERM field.² This transformative development has alleviated the need to compromise between easy access to adult, multipotent stem cells, and truly pluripotent, but ethically contentious, embryonic stem cells (ESCs).^{6,7} Since 2006, reprogramming methods have markedly improved, as have the differentiation protocols for creating mature cell types.^{8–10}

Today, iPS cells are vector-free and can be routinely derived from various tissues (skin, blood, and many others) of healthy individuals and patients with genetic mutations.^{11,12} Furthermore, owing to recent gene editing technologies, iPS cells can be modified to create isogenic lines with or without a given allele type or mutation.^{13–15} This ability has greatly amplified the utility of iPS cells in tissue engineering for regenerative medicine, and for drug testing and disease modeling, which will be described in greater depth in the following sections.

Mechanisms of pluripotency

It is important to keep in mind that iPS cells are not identical to their embryonic counterparts. While all our tissues develop from ESCs, iPS cells do not come into play during natural development. Moreover, even native ESCs do not stay in a prolonged undifferentiated state during the early stages of gestation, as is the case during expansion and maintenance of these cells *in vitro*. These observations suggest that the development of methods for maintaining pluripotency and the understanding of differences between ESCs and iPS cells will be critical to their use in research and clinical applications.

FIG. 1. Overview of the key areas of tissue engineering and regenerative medicine research. Highlighted regions reflect topics covered in this review article.

Relevant to the maintenance of pluripotency, a recent article by Theunissen *et al.*¹⁶ discriminates between the naïve, ground-state pluripotency of ESCs and iPS cells and the “primed” pluripotent state of epiblast stem cells. Because the Oct4 gene is driven by a distal enhancer in naïve cells versus a proximal enhancer in primed cells, the authors used gene editing in mouse cells to create an Oct4-2A-GFP element that has the proximal enhancer removed such that GFP+ cells are suggestive of a naïve pluripotent state. This reporter was used to screen small molecules for their effect on cell pluripotency, and the authors describe five different kinase inhibitors that, when combined, led to GFP+ colonies without the additional need to drive KLF2 or Nanog expression. This combination of inhibitors could thus be used to convert blastocyst cells to ESCs with epigenetic and transcriptional features reflective of ground-state pluripotency.

On a similar note, Hussein *et al.*¹⁷ explored the multi-stage process of reprogramming somatic cells at the genetic and epigenetic levels, detailing the regulatory roles of histone methylation and noncoding RNAs. The authors describe a new “F-class” of PSC (named for their “fuzzy” appearance). These cells exist in a more stable steady-state than traditional “ESC-like” cells and notably exhibit a much higher transgene expression. Finally, an intriguing article by Ohnishi *et al.*¹⁸ identified that keeping reprogramming factors under an inducible promoter *in vivo* can lead to cell dysplasia and tumor formation. Importantly, when the same tumor cells were reprogrammed into iPS cells and seeded *in vitro*, they formed non-neoplastic derivatives. These findings support the notion that failed reprogramming of somatic cells and the accompanying epigenetic changes can be responsible for the initial *in vivo* tumor formation (vs. genetic mutations). Epigenetic changes of this kind could thus play a fundamental role in studies of cancer pathophysiology.

It is clear from these articles and those published in recent years^{19–21} that reprogramming is a multistage process that can lead to varying outcomes. It may be misleading to think of PSCs as a single, distinct cell type, or to view ESCs and iPS cells as a black-and-white dichotomy. There seem to be many differences even among iPS cells, depending on the tissue source, and the method and stage of reprogramming. The field is still learning about the differences that are critically important for the safe and reproducible use of stem cells for tissue formation. Micro-tissue models, discussed as a separate topic, are becoming invaluable for assessing the functionality of iPS cells and their derivatives in response to genetic and epigenetic modifications.

Immunogenicity of iPSCs

Better understanding of the mechanisms involved in inducing cell pluripotency is not only relevant to minimizing batch-to-batch variability and improving differentiation efficiency, but also to assessing potential immunological responses. While it has been generally assumed that autologous iPS cells and their derivatives will not elicit an immune response, a provocative article in 2011 challenged this idea.²² The authors showed that iPS cell-derived teratomas exhibited rejection and T-cell infiltration in syngeneic mice, which was not seen with ESC-derived teratomas in the syngeneic setting. The authors attributed these iPS cell-related

responses to the expression of specific proteins (Zg16 and Hormad1). Since then, several groups have sought to determine the actual immune compatibility of iPS cells, focusing more on their differentiated progeny as these are more directly translatable into the clinic. Paradoxically, T-cell infiltration was seen following transplantation of autologous iPS cell-derived cardiomyocytes,²³ whereas no rejection was seen when autologous iPS cell-derived dopaminergic neurons²⁴ or cells differentiated to lines from each germ layer (i.e., endothelial, hepatocyte, and neuronal) were transplanted into syngeneic models.²⁵

In the most recent investigation into iPS cell immunogenicity, Zhao *et al.*²⁶ advance their previous studies by using integration-free human iPS cells in autologous humanized mouse models. Teratomas that formed in mice showed infiltration of CD4+ and CD8+ T-cells with tissue necrosis, although to a lesser degree than in similar experiments using allogeneic ESCs. Sequencing of the T-cell receptor repertoire for reacting T-cells showed they were oligoclonal, consistent with the notion of an antigen specific response. Most interestingly, the authors found that T-cell infiltration was greater around smooth muscle cells (SMCs) within the teratomas than in tissue that looked like retinal pigment epithelium (RPE). They subsequently differentiated iPS cells to SMCs and RPEs *in vitro* and found that, when these cells were transplanted into nonocular sites (since the eye is immune privileged), the RPE cells showed minimal T-cell infiltration in serial sections whereas SMCs had extensive infiltration (Fig. 2). Consistent with their 2011 findings,²² the authors attribute this to expression of Zg16 and Hormad1 in SMCs but not RPEs.

Based on these data, one could postulate that certain iPS cell differentiation protocols may permit expression of proteins that can elicit an immune response, potentially warranting modifications of currently used methods. As reviewed elsewhere, there may be several other contributors toward immunogenicity of autologous iPS cells.^{27,28} Since the responses are not mediated by major histocompatibility complexes, and are thus weaker, the relevance to clinical translation remains unclear. Most authors strongly advise detailed preclinical testing for any potential immune response. This would be especially necessary for lines in which gene editing is used to correct a genetic defect in the patient, as this would lead to the expression of a protein/epitope that the patient’s immune system has not been exposed to during development.

PSC banking

The potential immunogenicity of autologous iPS cells and their derivatives is not an absolute barrier to using these cells in the clinic. Indeed, the need for creating iPS cell banks, similar to cord blood banks,²⁹ is being strongly articulated by the scientific and clinical community. These banks could adopt several models for matching haplotypes of human leukocyte antigens (HLAs), which are described briefly below.^{30–33} One model would account for patient-specific iPS cells, which would be expected to have only the relatively weak antigenicity that was detailed in the previous section. While providing the best HLA match to the patient, this method suffers from the late onset for therapy development. As it is unfeasible in terms of resources, cost, and storage space to proactively create iPS cell lines for every individual, any autologous line would have to be made on-

FIG. 2. Immunocompatibility of patient-specific induced pluripotent (iPS) cell derivatives. iPS derived cells have been considered as nonimmunogenic due to their autologous nature. By differentiating iPS cells into smooth muscle cells (SMCs) and retinal pigment epithelium (RPE), Zhao *et al.*²⁶ demonstrate that depending on the iPS cell derivation protocol there is a greater or lesser immune cell infiltration when these cells are implanted into autologous humanized mouse models. These findings support the notion that there may be residual antigens on the cells following differentiation. Reproduced with permission from Zhao *et al.*²⁶

demand and be subjected to functional evaluation and quality control. As a result, there would be a substantial time (several months) and cost involved before the cells or tissue graft would be ready for use.

Nevertheless, since methods for rapidly creating GMP-compatible, integration-free iPS cell lines from a variety of accessible sources (e.g., urine, blood)^{34–36} have been rapidly improving, it may be possible to create banks of allogeneic PSC lines that span a diverse array of HLA-haplotypes. Several investigators have looked at the minimal number of lines to match the majority of a given national population. As expected, the greater the ethnic diversity, the more lines will be needed.^{37–40} Residual major and minor histocompatibility antigen mismatch may still lead to an immune response, but this could be treated using immunosuppressive regimens that are common practice elsewhere in transplantation. Furthermore, if cells or tissues are transplanted into immunologically privileged regions of the body (e.g., CNS, eye), allogenicity may not be an important issue.

From a resource and time stand point, allogeneic therapies would make it easier to have cells ready as needed, which is important particularly when acute pathologies are to be addressed. Ultimately, the strategy used (autologous or allogeneic) will depend on the acuteness of the pathology. To facilitate monitoring of the available iPS cell lines and their regulatory restrictions, endeavors such as the Human Pluripotency Stem Cell Registry have been initiated.⁴¹ While it remains to be seen whether access to iPS cell banks will become as streamlined as access to blood banks, cell banking could be a huge asset for the development of rapidly available clinical therapies.

Summary

At this point, the field needs to determine whether we have enough understanding and control of PSC for use in patients. Clinical trials using iPS cells are still in the “lag”

FIG. 3. Engineered brain micro-tissues. (a) The brain contains multiple cortical layers of gray matter and white matter tracts. (b) The different layers of gray matter are recreated using six concentric rings of silk scaffold, which are each seeded with cortical neurons. The center of the disk contains collagen and serves as an axon reservoir. The complete, assembled scaffold is shown: (c) in original color (d) with each concentric ring stained with a different food color, and finally (e–g) with live cells stained with either DiI (red) or DiO (green) to demonstrate that the scaffold permits alternating cellular layers. Scale bar: 1 mm. Reproduced with permission from Tang-Schomer *et al.*⁶⁶

phase of their growth curve,⁴² and some investigators have started replacing iPS cells by implementing direct reprogramming of somatic cells. Recent advances in reprogramming across germ layers using nonviral methods are particularly exciting.^{43–45} While it is impossible to know what the distant future will hold, PSCs remain a powerful cell source for meeting the TERM objectives. These cells will likely be central to the field for at least the next decade. The achievements highlighted in this section are all relevant to clinical translation, as insights into the mechanisms of pluripotency will enable iPS cells to be cultured in a more standardized and long-term fashion as is necessary for cell banking. Currently there are several integration-free methods for producing iPS cells,^{8–10} which should mitigate fears about introducing genetic mutations into the body through cell reprogramming. Since, by nature, all pluripotent cells can acquire mutations through extended culture, the development of high-throughput, cost-effective methods for monitoring the maintenance of iPS cell lines and the regulations that must accompany these technologies are perhaps the most immediate hurdles to clinical translation of iPS cells as regenerative therapies.

Area II: Microtissue Platforms for Drug Testing and Disease Modeling

Some of the most exciting progress in the last 5–10 years has come from the ability to engineer heterogeneously cellular 3D environments that lead to tissues that can authentically mimic specific organ functions. These tissues, often called organoids, have greatly expanded our ability to model *in vitro* both acute injury and chronic disease. Indeed, recent years have seen an accelerated commercialization of microtissue or “organ-on-a-chip” platforms in the area of drug screening. This direction, which is pursued in parallel to the “classical” regenerative medicine goals, appears to be an easier path toward the development of new treatment modalities. The small size of such tissues alleviates difficulties with maintaining cell viability and tissue function. Moreover, the commercialization of drug screening and disease modeling platforms does not require the application of long and expensive regulatory procedures. In this section, we highlight some recent progress in engineering tissues for *in vitro* applications, using bone and brain as two diverse examples of structurally and functionally different tissues.

Bone microplatforms

One of the earliest objectives for engineering bone tissues was to provide an alternative to autologous bone grafts in patients with large structural defects that would not require harvesting bone from the patient.^{46,47} This goal still holds today, and we have seen major progress in the ability to shape grafts to mirror patient anatomy^{48,49} and incorporate an osteochondral interface.^{50,51} Miniaturization of bone tissue engineering is now leading to *in vitro* models for studying primary and metastatic bone cancer,^{52–54} and for better recapitulating the physiological and pathophysiological states of bone marrow hematopoiesis.

As a compelling example of engineering an *ex vivo* bone marrow niche, Di Buduo *et al.*⁵⁵ recently designed a silk-based system for modeling platelet formation and related disorders. At the center of their platform were endothelial-

lined silk microtubes designed to mimic the bone microvasculature into which megakaryotes normally shed platelets. To put the microvasculature in the context of trabecular (spongy) bone, the tubes were encased within a porous silk scaffold, and the whole system was perfused with culture medium. The investigators found that megakaryocytes seeded into the surrounding spongy region of the bone migrated to the silk microvascular tubes and began releasing platelets into the lumens within only 24 h. These processes were enhanced when the silk tubes contained stromal cell-derived factor 1 (SDF-1), a cytokine known for strong chemotactic features. While the system was not intrinsically thrombogenic, recovered platelets were shown to be capable of activation and clotting functions. Intriguingly, the platelet producing function of megakaryocytes derived from patients with myelodysplastic disorders paralleled levels found in the patients' blood.

This article follows an earlier 2014 article⁵⁶ in which a bone marrow compartment with a more complete set of cell types was created by subcutaneously implanting a bone-forming graft into mice. The newly formed marrow was extracted 8 weeks later and could be incorporated into an *ex vivo* culture system. By harnessing the methods and results of these and other studies,^{57–59} it will be interesting to see whether more complete marrow compartments can be engineered using human cells *in vitro*. Given the critical role of hematopoiesis, one could imagine great utility of bone marrow platforms for investigating drug toxicity, radiation damage, blood disorders, and hematopoietic stem cell expansion and engraftment.

Brain microplatforms

The human brain is arguably the most intricate organ in terms of its composition and multiple, largely unknown functions. While recent endeavors such as the *Human Connectome Project* offer hope that we may be able to better understand its inner workings in health and disease,⁶⁰ neural tissue microplatforms can complement *in vivo* studies in animals and humans (mostly using imaging modalities) and serve as controllable models for drug testing and disease modeling.^{61,62} Compared to other tissues, 3D culture systems for *de novo* brain tissue engineering are relatively new. Notably, this past year saw a number of interesting developments.

For example, in their *Nature* article,⁶³ Choi *et al.* attempted to create a better *in vitro* model for human Alzheimer's pathology. Previous work with murine and human cells has been able to recapitulate the formation of amyloid plaques but has been less successful in demonstrating the other classic pathological features, such as tau-based neurofibrillary tangles.^{64,65} The authors focused on using lentiviruses to overexpress Familial Alzheimer's Disease mutations in the β -amyloid precursor protein and presenilin 1 genes of human neural stem cells. These stem cells were then differentiated into neuronal and glial cells in a 3D Matrigel system. The authors found that 3D culture promoted differentiation and resulted in higher levels of tau isoforms. Furthermore, they observed the formation of extracellular amyloid β -aggregates and increased phosphorylated tau levels, which could be ameliorated using β - and γ secretase inhibitors. Through various other characterization studies, this study demonstrated that their *in vitro* system

recapitulates several features of Alzheimer's disease, suggesting the utility of the platform for further studies of disease pathophysiology and treatment options.

In contrast to trying to mimic a specific neuropathology, Tang-Schomer *et al.*⁶⁶ focused on recapitulating key aspects of complex cortical brain architecture, including both gray matter with its multiple laminar layers and white matter. They designed a template for tissue formation by shaping porous silk scaffolds into different sized rings that were coated by polylysine and seeded with primary neurons. These "layers of gray matter" were assembled as shown in Figure 3. Collagen hydrogel filled the empty space within the scaffold and served as a "white matter-only" region at the center of the tissue construct. The combination of silk scaffold and collagen gel promoted neuron adhesion, network formation, and electrophysiological responses that could be attenuated by drug treatment. The authors propose that the scaffold stiffness was important for neuron adhesion, while the slow-degrading collagen gel permitted axon spreading and network formation. As a test of the utility of their model, they simulated a traumatic brain injury by dropping a weight on their engineered tissue. The injury induced a glutamate spike and neural hyperactivity that were similar to those observed in animal models of brain injury.

Lastly, bringing together elements of both of the above studies, Park *et al.*⁶⁷ attempted to create a biomimetic model of cortical tissue and to use this model to demonstrate elements of Alzheimer's disease pathology. In the 3D culture system, the innovation came from the use of a microfluidics platform that permitted fluid flow. Such flow is important as it mimics the interstitial flow of cerebrospinal fluid, which is known to affect cell metabolism and communication in the brain. In the experimental setup, rat neural progenitor cells were seeded into wells within the microplatform, and they spontaneously formed neurospheroids after 10 days of culture. Compared to static culture, neurospheroids exposed to the interstitial flow at rates comparable to those in the brain were larger, had greater neurite outgrowth and neural network formation, and showed greater differentiation into mature cortical neurons. Having shown the benefit of flow in creating cortical-like tissue, the investigators demonstrated the pathological effect of β -amyloid protein, which was exacerbated by flow, presumably due to greater penetrance of the β -amyloid protein into the neurospheroids. The investigators postulate that microfluidic studies could be used to further investigate the role of β -amyloid clearance in maintaining cortical neuron integrity.

Summary

Using examples of engineering micro-sized bone and brain tissues, we have described how microtissue platforms can be used to form tissues that are complex enough to be used to interrogate disease status or predict drug response. While the field of tissue engineering remains influenced by the original expectation that "the more complex the tissue the better," this intuition is not necessarily true, particularly for *in vitro* modeling work. Whether studying responses to a specific environmental factor (such as drug, hypoxia or inflammation) using a single or multi-tissue device,^{68–71} the purpose of a platform should be to make the simplest system

that has a desired functional readout. The complexity of an individual tissue type or the necessary combination of multiple tissue types will naturally depend on the factor being studied. In our first example, a marrow-like compartment was used to study a marrow-related damage or disorder. If the goal were to instead model arthritis, the design parameters for the microplatform would include the need for mechanical loading, inflammatory cells and cytokines. Similarly, in the brain examples, each study attempted to model something unique: (i) Alzheimer's disease using gene mutations, (ii) the layered architecture of the cortex, or (iii) the role of interstitial flow in neural tissue development and disease. Clearly, the detailed design and the level of complexity vary from one application to another, with models of disease generally requiring more sophistication than models of drug toxicity.

Area III: Tissue Models of Cancer

Studying human cancers in laboratory settings has been a difficult endeavor. Primary tumor cell lines are known to divide over extended periods of time without senescence, but in the absence of the right signals—growth factors, blood vessels, an array of immune cells, continually remodeled architecture, physical signals—most *in vitro* models fall short of recapitulating the actual *in vivo* tumor environment and its influence on cancer cell behavior. The current standard in the field is the use of animal models. However, animal models are not readily controllable and do not always correspond to human conditions in terms of the mutations involved, local environmental factors, and cell–cell interactions. Better tumor models would recapitulate *ex vivo* the actual tumor microenvironment. Fortunately, with the advent of sophisticated gene editing strategies and advanced 3D culture methods, such models have started to emerge.^{72–74}

Gene editing and cancer

The CRISPR-Cas9 and TALEN methods for targeted gene editing have made it easier to follow the natural history of tumor development in a more controlled setting. The details of these methods are reviewed elsewhere,^{75,76} to show that their utility comes from the ability to take healthy cells in culture and introduce sequential "hits." The significance of each mutation for tumor development and metastatic capacity can then be followed and compared in otherwise isogenic cell lines.^{77,78} This approach has also been used to study hematological malignancies, as was shown by an elegant study last year, in which up to five mutations were introduced in mouse hematopoietic stem cells to create models of acute myeloid leukemia.⁷⁹ In a more recent study, investigators used sequential mutations to interrogate the role of driver pathways in colorectal carcinoma development.⁸⁰ Their system was based on intestinal organoids, which spontaneously form by seeding human intestinal stem cells in Matrigel supplemented with factors normally present in the intestinal stem cell niche. Since these factors are less essential for the growth and maintenance of colorectal carcinoma, the investigators were able to use growth advantage in the absence of certain factors as a readout for the influence of specifically introduced mutations. These are but a few examples of semi-guided tumor development by gene editing.

3D cancer platforms

Another major achievement has been the design of 3D tissue culture environments, as they have enabled *in vitro* studies of cancer processes that have previously proven very elusive—tumor extravasation and metastasis. In an impressive feat of engineering design, Bray *et al.*⁸¹ constructed scaffolds comprised of four-arm starPEG-heparin hydrogels with tunable stiffness. RGD sequences were incorporated to promote cell binding, matrix-metalloproteinase (MMP) sensitive cross-linkers were used to permit natural tumor remodeling, and endothelial and stromal cells were added to promote angiogenesis. With this system, the investigators were able to screen multiple breast and prostate cancer lines and show differential responses of 2D versus 3D cultured cells to numerous common chemotherapeutic agents and angiogenesis inhibitors.

Using a completely different approach, Jeon *et al.*⁸² created a microfluidic model of breast cancer extravasation. As shown in Figure 4, the model consisted of two flow channels that surrounded a central cavity containing undifferentiated MSCs, those differentiating toward an osteogenic phenotype, and endothelial cells encased in fibrin. This tri-culture of cells was shown to form microvascular networks and was meant to mimic a bone microenvironment. To test whether factors secreted from this central cavity affect tumor extravasation, breast cancer cells were injected into one side of the microfluidic channel and their migration across vasculature was monitored in real time. Migration toward the bone microenvironment was shown to be superior to a comparable acellular or muscle-mimicking environment, and the investigators identified that an adenosine receptor mediated pathway may be related to the reduced metastatic capacity toward skeletal muscle.

Summary

Given the variability in tissue of origin, microenvironmental factors, gene mutations, and the intrinsic differences between animal models and humans, our ability to understand human cancers continues to face significant challenges. However, through combined innovations in gene editing and 3D culture systems, we are now able to have much greater control over the initial genetic “hits” and tissue architecture including angiogenesis and remodeling. As a direct consequence, it is now possible to study cancer initiation and mutational progression *in vitro*, with recapitulation of highly complex processes of tumor cell extravasation and metastasis.^{83–85} Just as not all diseases are caused by a single gene polymorphism, not all cancers have been defined by a specific initial mutation. Therefore, the 3D tumor platforms that emulate multiple aspects of native tumors could offer more complex insights into cancer biology, are translatable to a variety of tumor types.

Area III: Whole Organ Engineering

Engineering a whole functional organ such as a heart, lung, or liver is still way beyond our current capabilities. At least one major challenge is that the complex matrix architectures of these organs (anisotropy of the heart, multiple generations of the airway tree intertwined with the vascular network in the lung, and highly structured parenchymal tissue of the liver)

cannot be reproduced using our current scaffold synthesis technologies. However, tissue decellularization has long been used as a strategy to generate tissue/organ specific extracellular matrix (ECM) scaffolds for engineering tissues,^{86,87} and while ECM scaffolds are less tunable than their synthetic counterparts, they benefit from having a structural, mechanical, and biochemical composition that resembles native tissue and displays low immunogenicity following removal of cells.^{88–90} Furthermore, the retained molecular, biophysical, and structural cues, which are both tissue specific and regionally specific, have been shown to help direct stem cells to differentiate into the appropriate mature cell fates. For these reasons, the last decade has witnessed the emergence of whole organ decellularization, with studies involving complete cell removal from rodent to human sized organs.^{91–95} This research has created an alternative top-down approach to tissue engineering.

Vascular considerations

One of the major limitations of a holistic approach to decellularization is that it treats all cell types as equally expendable. While whole organ decellularization intrinsically overcomes the limitations in scaffold size, shape, composition, and biomechanical properties that pose as obstacles to scaffolds synthesized *de novo*, it generally compromises the organ vasculature. Since incomplete re-endothelialization invariably leads to fluid leakage, insufficient pressure, thrombosis, and proinflammatory cascades, there has been an increasing effort toward more effectively re-endothelializing the vascular tissue compartment. Two creative studies reported in the past year address the challenges of revascularizing the decellularized liver and lung.

The liver is an interesting organ from the vascular perspective, as it has two incoming blood supplies—the hepatic artery and the portal vein. These dual supplies provide oxygen to sustain cell metabolism and enable the liver to process drugs and factors originating from the intestine.⁹⁶ In their recent article, Ko *et al.*⁹⁷ focused on the process of re-endothelializing livers following whole organ perfusion decellularization using Triton X-100 and ammonium hydroxide. Rather than relying on endothelial cells to passively attach to native matrix, the investigators actively facilitated their attachment by treating the scaffolds with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/N-hydroxysuccinimide (EDC/NHS), and by perfusing anti-CD31 antibodies through the vasculature. With these antibodies in place, endothelial cells were infused using both static and perfusion culture methods. Not surprisingly, scaffolds that had been re-endothelialized using this method showed improved patency and resistance to platelet adhesion compared to acellular scaffolds. As a result, re-endothelialized livers implanted into a heterotopic porcine model were able to withstand physiological flow for 24 h. While a much longer time span will be necessary for therapeutic purposes, this article demonstrates how engineering strategies can be used to deliver new cells to specific regions within decellularized organs.

In addition to maximizing cell adhesion, another challenge to reseeding vasculature comes from the loss of pressure that accompanies decellularization. In native vasculature, there is a delicate balance between the osmotic and hydrostatic pressures, and this balance is regulated by an intact vascular bed. Using a decellularized rat lung model,

FIG. 4. Engineering a bone cancer niche. Peripheral media channels enable cancer cells (CCs) to extravasate into the central tissue-mimicking gel comprised of endothelial cells (ECs), Mesenchymal stem cells (MSCs), and osteoblast-differentiated cells (OB). The ECs form vasculature within the gel whereas the MSCs and OBs serve to mimic a bone microenvironment. Migration toward this bone microenvironment was found to be greater than a muscle-mimicking environment, and the investigators implicate the role of the adenosine receptor in inhibiting CC migration. Reproduced with permission from Jeon *et al.*⁸²

Ren *et al.*⁹⁸ showed that microbeads introduced through the pulmonary artery were able to pass through the capillary bed. This effect was attributed to the loss of hydrostatic pressure that is caused by the increased permeability of the basement membrane following decellularization, and the same effect was also shown for human umbilical vein endothelial cells (HUVECs). Thus, one strategy that was tested was to introduce HUVECs from both ends—via the pulmonary artery and the pulmonary vein. A second strategy was to co-introduce perivascular cells. This improved regimen enabled better endothelial coverage—up to 75% compared to native tissue, which improved barrier function, reduced edema, lowered vascular resistance, and decreased thrombogenicity when compared with acellular lungs.

Since rat lungs are clearly much smaller than human lungs, to demonstrate the feasibility of this approach for human transplantation studies, the authors used the same methods with human lungs that were seeded with several hundred millions of human iPS-ECs and iPS-PCs (perivascular cells). While this approach resulted in <10% coverage, the authors note that they only reseeded the vasculature with about 5% of the number of endothelial cells found in the normal human lung (3–6 billion). A takeaway from this study is that while some principles of whole organ engineering may be consistent across mammalian models (e.g., increased basement membrane permeability following decellularization), the ability to recellularize human-sized grafts will require efficient methods for mass production of cells on the scale of billions of cells per batch.

Multi-compartment recellularization

The previous section focused on the imperative role of re-endothelializing the vascular component of acellular scaffolds, but the investigators explored this role in isolation. Clearly, to reconstruct functional organs from scaffolds requires the ability to distribute a variety of cell types to their respective regions. One elegant example of achieving this type of organized, heterogeneous recellularization was published by Jank *et al.* early in 2015.⁹⁹ Using a rat forearm model, detergent was introduced through the brachial artery for 50 h leading to the elimination of >90% DNA. Imaging and mechanical studies demonstrated preservation of structural components of muscle fascicles (i.e., endomysial sheets), mechanical properties of bone, and integrity of vascular conduits. Acellular limbs were then moved to a custom bioreactor (Fig. 5), where a stepwise reseeded protocol prioritized re-introduction of HUVECs into the brachial artery, followed by the injection of HUVECs, C2C12 mouse myoblast cells, and mouse embryonic fibroblasts into the acellular muscle. Switching to low serum differentiation medium promoted

FIG. 5. Stepwise regeneration of decellularized rat forelimbs. (a) Schematic of perfusion bioreactor used for electrical stimulation of engineered muscle tissue. (A) Electrical field stimulation. (B) Medium perfusion. (b) Composite grafts are engineered in a stepwise fashion. Initially, acellular scaffolds are perfused with human umbilical vein endothelial cell (HUVECs) through the brachial artery. Subsequently myoblasts, HUVECs, and fibroblasts are introduced. Electrical stimulation is used to facilitate muscle maturation. Autologous skin is then grafted onto the construct to create a barrier against environmental stressors. (c) Photo of regenerated composite tissue (left) with a tissue cross-section (right). Scale bar: 5 mm. Reproduced with permission from Jank *et al.*⁹⁹

myotube fusion. In addition, electrical stimulation was used to facilitate fiber growth, alignment, and differentiation. Finally, autologous skin was grafted atop the tissue composite. Engineered muscle was able to achieve 80% of the force generated by neonatal rat muscle when interrogated via isometric force measurement. Orthotopically transplanted limbs demonstrated wrist flexion upon electrical stimulation of forearm muscles. While this model only focused on three cell types, the cells were shown to distribute and align properly within their respective compartments. By nature, innervation of composite grafts cannot be performed before transplantation and, in this sense, the design criteria for the graft are made simpler, although the lag-time toward a completely functional tissue is increased post-transplant.

Summary

The development of *ex vivo* perfusion methods has enabled tissue decellularization to be conducted at a new level—that of an entire organ.^{100–102} Resulting acellular scaffolds will always have the advantage of being anatomically, compositionally, and mechanically authentic at a physiologically relevant scale, but they are still far from being functional. An underlying assumption in using these scaffolds for whole organ engineering is that they can be revitalized in a way that achieves the integrity and diversity of tissues formed naturally during development. Significant challenges to be overcome include effective distribution of specific cells to specific regions, particularly the vascular compartment, and developing methods to produce cells on the order of billions. Lastly, while it is known that acellular scaffolds retain structural and biological cues to aid precursor cell differentiation, it has yet to be determined which optimal level of differentiation the cells must achieve to recognize matrix signals and to mature in the scaffold.

Concluding Remarks

It is rather difficult to write an objective review of such an active field as TERM, over a period of time as short as 1 year. The common metrics of the impact—such as the citation index—are not directly applicable and may give an unfair advantage to the articles with a longer “citation lag time.” Still, we embraced this challenge because we felt that a summary of most recent developments in this burgeoning field, even if less than perfect, will be of interest to the reader. To this end, we developed a methodology for objectively selecting the most meritorious work, which helped identify the areas and topics that received most attention by the research community. However, the final identification of the continuing and new areas with the highest potential, as well as the most interesting recent articles was largely based on combining the adopted methodology with our own judgment. We sincerely hope that our view of the last year in TERM is free of bias and instead provides an inspirational account of recent advances and potential of this most exciting field.

Interestingly, one of the leading themes covered here also appeared in the last year’s review—human iPS cells—demonstrating the confluence of the fields of stem cell research and tissue engineering, and the continuing major impact of iPS cell technology on the whole TERM field.

The other three areas we selected: microtissue platforms for drug testing and disease modeling, tissue models of cancer, and whole organ engineering, appear for the first time, and we believe they will be defining TERM in the years to come.

The availability of iPS cells from healthy individuals and patients carrying disease, and specialized methods for their directed differentiation, is bringing the use of engineered tissues for testing of drugs and modeling of disease to an entirely new level. The use of precise gene editing for derivation of isogenic cell lines that differ only in specific gene mutation(s) allows identification and study of the mechanisms of disease. We are excited about the prospects of this technology for implementation of precision medicine approaches, through the use of iPS-derived cells in microtissue platforms. The implementation of tissue specific microenvironments (with tissue-specific matrix, molecular and physical signals) to tissues engineered from such iPS-derived cells is now leading to the creation of *in vitro* models of unprecedented biological fidelity. One can imagine “virtual” clinical trials carried out *in vitro*, for studies of rare and pediatric diseases, combinations of diseases that present high risk for drug interactions, and for determining individualized therapeutic regimens.

Cancer is emerging as one of the most challenging and potentially most impactful areas of application for tissue engineering. For many decades, progress in finding new modalities to control and treat cancer remained limited by the lack of predictive models for testing promising drug candidates. To coax human cancer cells into behaving more realistically *in vitro*, advanced tissue engineering methods are now starting to be used for building tissue models that mimic the native milieu of human tumors. We reviewed some of the most interesting recent studies that established *in vitro* models for tumor extravasation and metastasis. It is encouraging to see that tissue engineering can overcome the limitations of cancer cell cultures and animal models, and markedly advance our ability to study human cancer *in vitro*, under patient-specific and controllable conditions. This is another area expected to accelerate in the coming years and to make a major impact in treating human disease.

Finally, we discussed whole organ tissue engineering, which is central to regenerative engineering of several metabolic organs including heart, lung, liver, and even whole limbs. The use of decellularized organs as scaffolds for regeneration via stem cells or their differentiated progeny has many advantages. The resulting cell-free matrix, generated by the methods that remove all cellular material while largely preserving the composition, architecture, and mechanical properties of the matrix, provides a cell-instructive scaffold for directed differentiation of regenerative cells. This way, one major component of the tissue engineering system is out of the way, and our focus can be directed to the developmental stage of the cells to be used and the necessary environmental signals. This approach appears to be most relevant to engineering of organs with multiple and complex functions. Advances made over the last year suggest that this approach to tissue engineering will continue to gain in importance.

In summary, the recent research has markedly advanced the field of tissue engineering, both in the existing and the entirely new areas of application. It will be most interesting

to follow the further progress and the expected and unexpected leaps forward that will be shaping the field in the coming years.

Acknowledgments

The authors gratefully acknowledge funding of their work by the NIH, New York State, and the Mikati Foundation.

Disclosure Statement

No competing financial interests exist.

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Received: November 29, 2015

Accepted: December 15, 2015

Online Publication Date: February 22, 2016