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Developments in tissue engineered and regenerative medicine products

A practical approach

Joydeep Basu and John W. Ludlow

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Joydeep Basu: I dedicate this book to the memory of my father, Dipak Basu (1939–2011). Without his love and encouragement, none of this would have been possible.

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His other responsibilities include bringing new product candidates into bioprocessing research and development and taking these candidates to demonstration in preclinical studies. Tengion currently has two product candidates, the Neo-Bladder Augment™ and the Neo-Urinary Conduit™, in clinical trials.

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In 2000 he was recruited to direct the cell therapy program at Incara Pharmaceuticals (Research Triangle Park, NC) where he led the research and development of a liver cell therapy product. This work culminated in the successful allowance by the Food and Drug Administration of an Investigational New Drug application and an issued patent on the use of primitive and proximal hepatic stem cells for treating liver disorders and producing bioartificial organs.

John continued this area of research and development as Senior Director of the cell therapy program at Vesta Therapeutics (Research Triangle Park, NC) until 2005, when he joined Tengion Inc. His responsibilities include bringing new product candidates into bioprocessing research and development, taking these candidates to demonstration in preclinical studies, good laboratory practice (GLP) and non-GLP production of neo-organs, and initiation of clinical trial sites. John is a member of the Tissue Engineering and Regenerative Medicine International Society (TERMIS).

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Preface

Tissue engineering and regenerative medicine are two terms often used synonymously. Both describe a field focused on discovering and developing new ways to help the body's innate ability to restore organ and tissue function using a combination of cells, biomaterials and engineering. A subtle difference between these terms may be that tissue engineering emphasizes the biomaterials and engineering components, while regenerative medicine accentuates the cellular contribution. Like many disciplines having a potential to affect public health, expectations surrounding the state of the technology and the benefit to human health are at times overstated. Although we may in the future be able to produce complete and functioning organs *ex vivo*, the current state of the technology relies on tissue culture to expand subsets of cells which are then combined with a suitable biomaterial to produce a construct destined for patient implantation. The construct then serves as a facilitator for the regeneration of functional tissue.

Developments in Tissue Engineered and Regenerative Medicine Products – A Practical Approach has been written in response to the growing numbers of industry professionals and entrepreneurial academics contemplating new start-ups who are seeking information on what is involved in manufacturing these types of products. As is, we hope, apparent, it takes much more than a good idea for a product concept to become a reality. The topics covered provide a level of detail regarding the infrastructure and document filings required for manufacturing tissue engineered and regenerative medicine products in accordance with national and international regulatory agency guidelines. We also include a detailed example of a product currently in clinical trials which we were intimately involved in developing. Also covered is the importance of securing intellectual property for the product to provide a measure of economic protection for the start-up company. We hope that you find this work helpful and informative; treat the contents as a road map enabling one to plot a route from product inception to manufacturing.

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Overview of tissue engineering/ regenerative medicine

1.1 Introduction

Tissue engineering/regenerative medicine (TE/RM) refers to a broad range of emerging technologies that employ cells, biomaterials or cell/biomaterial combinations (referred to as 'constructs') to reconstitute functional tissue or organ-like structures *ex vivo* (neo-organs) and/or to catalyze organogenesis *de novo* by utilizing the body's innate ability to regenerate itself. Emerging approaches to TE/RM encompass the full spectrum of cells, biomaterials and cell/biomaterial combinations. However, from a biotechnology perspective, perhaps the most significant recent developments in TE/RM product development include:

- Approval by the Food and Drug Administration (FDA) for initiation of a Phase I clinical trial for Geron's embryonic stem cell-derived oligodendrocyte progenitors (GRNOPC1) as a cell-based therapeutic for spinal cord injury (2010).
- Successful implantation of a tissue engineered trachea by surgeons in Spain (2008).
- Initiation of a Phase I clinical trial for Tengion's Neo-Urinary Conduit™, a smooth muscle cell/biomaterial-based bladder-like neo-organ facilitating urinary diversion (2010).

The present volume is not meant to serve as an exhaustive academic review of recent successes and failures in the TE/RM space. Rather, we have attempted to create a complete, 'how-to' manual for entrepreneurially minded scientists contemplating the creation of a new start-up focused on design, creation, evaluation and maturation of TE/RM products. Such

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individuals, we presume, will be well versed in the basic science underlying TE/RM and will no doubt have access to adequate existing resources in this area. However, not all academic TE/RM projects are appropriate for commercialization, and not all academics have experience or expertise in creating TE/RM start-up companies. Therefore, our objective here is to use our own personal experiences in TE/RM biotechnology companies such as Tengion Inc., Athersys Inc., Incara Pharmaceuticals and Vesta Therapeutics Inc., to provide guidance to the budding entrepreneur, as well as to highlight some general foundational principles to be considered during the start-up process.

To this end, the first part of the book will only briefly outline the foundational science underlying emerging TE/RM technologies, concentrating instead on illustrating application and product development based on detailed examination of the Neo-Bladder Replacement™ (NBR) and Neo-Urinary Conduit™ (NUC), the first TE/RM neo-organ products to have been commercially produced and successfully implanted in human subjects within the context of a clinical trial. We highlight features of these TE/RM products that have facilitated successful commercialization and examine how these central, foundational themes represent product development platforms that may be extended to development of other tubular neo-organ products, including the lung, gastrointestinal tract, blood vessels and reproductive tracts. Such foundational technology platforms are also under development for solid neo-organs, including the kidney, liver, pancreas and brain. We focus on identifying critical aspects of these emerging TE/RM products that will facilitate commercial feasibility and result in practical, marketable products in the near future (<10 years).

In Table 1.1, we have outlined a number of companies currently active in the TE/RM space. This list is by no mean exhaustive, but is intended simply to illustrate the point made in text Box 1.1: successful TE/RM products may be based on cells, biomaterials or a combination of the two. Table 1.1 illustrates another general theme: products based on simpler components with established safety profiles (e.g. porcine small intestinal sub-mucosa) will be further along the regulatory approval process and closer to market than novel product candidates utilizing emerging TE/RM technologies such as the NUC. This conclusion may seem self-evident; nevertheless, it may still serve as a guiding principle when considering what classes of biomaterial may be best suited to drive a TE/RM product candidate from laboratory to manufacturing. We examine this issue in more detail in Chapters 3–7. For the moment, a brief overview of the underlying principles of TE/RM will be of value.

Table 1.1 Companies currently (2011) operating in tissue engineering/regenerative medicine

Company	Product type	Product	Indication	Status
Osiris Therapeutics (USA); osiristx.com	Cell therapy (bone-marrow-derived MSC)	Prochymal™	(1) GvHD (2) Crohn's (3) Cardiac infarct	Phase III Phase II Phase II
Geron (USA); geron.com	Cell therapy (ES-cell-derived oligodendrocyte progenitor)	GRNOPC1	Spinal cord injury	Phase I
Tengion (USA); tengion.com	Cell/biomaterial composite (neo-organ)	Neo-Urinary Conduit™ (NUC)	Cystectomy secondary to bladder disease	Phase I
Organogenesis (USA)	Cell/biomaterial composite	Apligraf™	Foot ulcers	Market
Cook Biotech (USA); cookbiotech.com	Acellular biomaterial (porcine small intestinal sub-mucosa)	Surgisis™	Dural graft	Market

ES, embryonic stem; GvHD, graft-versus-host disease; MSC, mesenchymal stem cells.

Box 1.1 Components of TE/RM products

TE/RM products may be based on one or more of the following:

- Cells: stem and progenitor cells including embryonic stem cells, mesenchymal stem cells and induced pluripotent cells, committed cell types such as smooth muscle cells[1] or primary renal cells[2]
- Biomaterials: natural origin (e.g. gelatin, alginate) or synthetic polymers
- Cell/biomaterial combinations ('construct')
- Delivery system: needles, catheters, laparoscopic devices

1.2 Cells

The search for bioactive agents capable of modulating disease and catalyzing the body's inherent ability to repair itself has evolved from the identification, isolation and application of biologically derived or chemically synthesized small molecules and protein-based biologics to the conceptual recognition that the cell itself may be regarded as an active biological ingredient (ABI). Mechanistically operating through action-at-a-distance paracrine signaling pathways, the cell in its capacity as a therapeutic agent may serve to recruit and mobilize native (i.e. host-derived) stem and progenitor cell populations, or may release cytokines and growth factors that promote angiogenesis and neo-vascularization, trigger neurogenesis, modulate inflammatory, fibrotic and apoptotic cascades, and generally function to interfere with the onset of pathology while promoting self-repair and regeneration. Furthermore, the cell as medicinal agent may itself directly contribute towards regeneration of native tissues and organs by niche-specific directed differentiation towards defined developmental lineages as regulated by contextual signaling cues derived from the surrounding tissue or organ parenchyma. As advances in molecular embryology and developmental biology begin to unravel the mechanistic pathways underlying organogenesis, the directed recapitulation of these defined developmental pathways may provide additional avenues for novel TE/RM products.

Cell populations with application in TE/RM may be broadly divided into two categories:

- stem and progenitor cells
- committed cell types

The main classes of stem cells currently under active investigation for TE/RM include cell types classed broadly as mesenchymal stem cells or mesenchymal stromal cells, embryonic stem cells and induced pluripotent cells. These stem cell populations may be used directly as cell-based therapeutics utilizing the cell itself as the principal medicinal agent, or may be used to derive populations of fully differentiated cell types for TE/RM. Alternatively, committed cell types such as smooth muscle cells and endothelial cells may be isolated directly from *in vivo* sources and harnessed as a building block for the construction of artificial vasculature, bladder and other tubular neo-organs, or, as in the case of primary renal cells, may be directly applied as a cell-based therapeutic.[2] In Chapter 2, we examine these cell types in more detail, outline their putative mechanism(s) of action

and examine what features of these different cell classes make them amenable or recalcitrant towards successful commercial exploitation.

1.3 Biomaterials

In its simplest iteration, the role of biomaterials in TE/RM is to provide a passive, structural framework or scaffold for the adhesion, migration, expansion and differentiation of seeded cell populations with the concomitant formation of bioactive extracellular matrix (ECM). In addition, the biomaterial scaffold may serve to recapitulate aspects of the complex, three-dimensional micro-architecture of the targeted organ. Finally, the biomaterial may actively interact with the cell in a manner mimicking signaling interactions between cell and ECM during early embryonic development and organogenesis. Ultimately, the biomaterial scaffold will degrade completely and coordinately with the deposition of ECM and development of laminar or radial multicellular organization within the regenerating neo-organ. Biomaterials may be entirely synthetic compounds or may utilize the properties of naturally occurring polymers such as alginate or gelatin. We explore these biomaterial candidates in more detail in Chapter 3, with an emphasis on highlighting selection criteria for identifying biomaterials appropriate for successful TE/RM product development.

1.4 Therapeutic product delivery

Central to the creation of any TE/RM product is the question of its delivery. Methodologies for TE/RM product delivery are clearly dictated by product composition. The presence of a biomaterial will fundamentally impact the selected delivery strategy. For cell-based therapeutics, the product may be delivered in a general, systemic manner throughout the patient, or more precise targeting to a particular tissue or organ may be desirable. Cell/biomaterial composites or cell-based therapeutics may be dependent on specialized delivery technologies to be effective and, as such, the selected delivery strategy represents an integral component of the overall product package. The delivery device must be minimally invasive, straightforward to use and readily manufactured at low cost, and may be required to facilitate long-term storage of the cellular ABI. The most commonly applied cell delivery techniques combine some form of needle or catheter system

with ultrasound or magnetic resonance imaging-based guidance technology to achieve the desired outcome. Implantation of larger neo-organ constructs will usually be accomplished through specialized surgical techniques, although laparoscopic delivery of certain product candidates may also be possible.

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Cells

Abstract. The cell is the primary active biological ingredient in TE/RM. In this chapter, we examine the principal classes of stem and progenitor and committed cell populations currently under active commercial development for application in TE/RM. We highlight what features of these cell populations make them amenable to successful commercialization and discuss the possible mechanisms of action by which they present therapeutic potential. Additionally, we point out aspects of their phenotype and function that may impede their successful negotiation of the regulatory approval process. Throughout, we illustrate these concepts with specific examples of products currently under consideration by the US Food and Drug Administration.

Keywords: mesenchymal stem cells, embryonic stem cells, induced pluripotent cells, mechanism of action, gene therapy, genome modification

2.1 Introduction

Our current understanding of the role of the cell as active biological ingredient (ABI) in TE/RM products may be traced back to the maturation of preliminary hypotheses regarding the *in vivo* mechanism of action (MOA) by which stem cells were thought to ameliorate disease phenotypes. From this perspective, stem and progenitor populations have been categorized on the basis of their developmental plasticity, or ability to differentiate along one or more embryonic lineages, as summarized in Table 2.1.

Early literature on the isolation, characterization and expansion of stem cells typically focused on extensive analysis of the *in vitro* and *in vivo* developmental lineage plasticity of these cell populations.[1,2] Inherent within these studies was the assumption that the observed therapeutic effects of these stem cell populations were based on the principles of site-specific engraftment and directed differentiation. In this model, the cell-based therapeutic was envisioned to operate by homing towards the site of injury, integrating within the host's injured tissue or organ, multiplying to generate

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Table 2.1 Lineage plasticity of cell-based active biological ingredients

Category	Lineage potential	Example
Totipotent	All embryonic and extra-embryonic lineages	Mammalian zygote
Pluripotent	All embryonic lineages	Embryonic stem cells
Multipotent	Multiple, but restricted	Mesenchymal stem cells
Committed progenitor/unipotent	Single, defined lineage	Smooth muscle progenitor cells
Differentiated	N/A	Committed smooth muscle cells, primary tubular renal epithelial cells

a localized, clonal population of stem cells and then differentiating in a manner appropriate to the local organ micro-environment, thereby repopulating the injured tissue or organ with new, healthy cell populations instead of with fibrotic or scar tissue.

An example of this strategy is the application of mesenchymal stem cells (MSC) for treatment of cardiac infarcts. Targeted injection of MSC within the area of infarct has been suggested to ameliorate *in vivo* therapeutic outcomes.[3] However, compelling evidence demonstrating that delivered MSC specifically integrate and repopulate the infarcted tissue with healthy cardiomyocytes derived via the directed differentiation of delivered MSC has not been forthcoming. To this end, it appears that companies developing MSC-related cell types (Osiris, Inc., Athersys, Inc.) seem to have moved from claims of tissue and organ repopulation from delivered cell-based therapeutics towards claims of therapeutic efficacy leveraging paracrine and other trophic cell signaling mechanisms.[4]

These hypotheses notwithstanding, the recent widely publicized failure of Osiris' MSC-based cell therapeutic Prochymal™ in Phase II clinical trials targeting GvHD (graft-vs-host disease)[5] has called into question many fundamental assumptions regarding the potential of these cell types for treatment of disease, as the rigorous demonstration of therapeutic relevance *in vivo* within humans by any cell-based therapeutic in large-scale clinical trials remains to be achieved. In this regard, the outcome of Geron's recently initiated Phase I clinical trial of embryonic stem (ES) cell-derived oligodendrocyte progenitors will be of fundamental importance.[6] [Note added in proof: Geron has recently terminated this Phase I study; see http://ir.geron.com/phoenix.zhtml?c=67323&p=irol-newsArticle&ID=1635764&highlight= for additional details.] Any indication of therapeutic efficacy beyond the initial demonstration of clinical safety will represent a major step forward for the field.

2.2 Mechanism of action of cell-based therapeutics

Precisely how cell-based therapeutics function mechanistically to modulate disease pathologies and promote regenerative outcomes is fundamental to questions of process development and commercialization. Cell-based therapeutics that purport to utilize the 'classical' properties of stem cells (self-renewal, multi-lineage differentiation potential) must be isolated, expanded, maintained and stored under conditions that are optimized to promote the full expression of these stem-like characteristics. To this end, any associated quality assurance regimens must focus on evaluation of 'stemness' of the final product throughout the manufacturing process. Alternatively, if the qualities of self-renewal and multi-lineage differentiation potential are irrelevant for therapeutic functionality, focus may be directed towards other aspects of cellular bioactivity.

To illustrate this, we will examine briefly the currently understood MOA for Geron's ES cell-derived product GRNOPC1, which was until recently undergoing Phase I clinical trials. At a superficial level, these oligodendrocyte progenitors are claimed to mediate functional recovery of locomotor activity in rats with induced spinal cord injury by migration of the progenitor cells towards the site of injury followed by their subsequent differentiation into mature oligodendrocytes with the associated production of regenerative neurotrophic factors.[7] However, when examined more carefully within the context of a rat spinal cord injury model, these ES cell-derived neuronal progenitors failed to migrate and localize to the site of injury and did not differentiate into functional neurons; in fact, progenitor cell maturation was observed only at locations not directly associated with the induced site of injury.

Adult spinal cord may therefore not represent a location primed to facilitate the differentiation of mature neurons, being instead a principally gliogenic environment, as shown by the propensity of neuronal progenitors to differentiate towards a glial lineage *in vivo*, particularly when activated by injury.[8] Despite the fact that partial functional recovery was secured, this observation has significant consequences for cell-based therapies targeting the spinal cord that rely on differentiation *in vivo* of stem and progenitor cell populations. Such differentiation is entirely unregulated by the clinician and may be influenced by numerous factors which themselves cannot be controlled, including the specific location and nature of the injury, and the age of the injury and of the host. Furthermore, although directly demonstrated by this study only for the spinal cord, similar principles may potentially impact the *in vivo* differentiation potential of any stem cell population within any organ or tissue type.

This observation notwithstanding, if these ES cell-derived neuronal progenitors are not localizing to and maturing into neurons at the site of injury, how is therapeutic efficacy generated? Neuronal progenitors have been demonstrated to secrete numerous neurotrophic factors, including NT-3 (neurotrophic factor 3), NT-4 (neurotrophic factor 4), NGF (nerve growth factor) and VEGF (vascular endothelial growth factor). Furthermore, conditioned medium from cultures of neuronal progenitors has been shown to be capable of positively influencing neurite outgrowth in cortical neural cultures *in vitro*. *In vivo*, transplantation of neuronal progenitors led to modulation of key intracellular kinase signaling cascades potentially impacting apoptosis, inflammation and immune modulation.[8]

However, although suggestive, these data do not demonstrate causal relationships between trophic signaling on the part of the transplanted neuronal progenitors and the resultant rescue of locomotor functionality. Unambiguous demonstration of cause and effect would require silencing the secretion of defined cytokines or groups of cytokines by siRNA or other related transcriptional knockout technology, followed by comparative analysis. Such lack of clarity regarding MOA directly impacts the development of potential quality control assays to monitor regenerative cell bioactivity. Although *in vitro* multi-lineage differentiation assays demonstrating acquisition of markers associated with mature neurons is a standard protocol in the characterization of stem cells with neuronal differentiation potential within the literature, such assays may have little or no relevance towards the ultimate prediction of *in vivo* therapeutic efficacy.[9]

Another recently initiated clinical trial involving human ES cell-derived cell populations is the application of retinal pigment epithelial cells (RPE) procured by the directed differentiation of human ES cells for treatment of Stargardt's macular dystrophy.[10] Advanced Cell Technology (ACT), the company undertaking development of this platform, plans shortly to initiate evaluation of ES cell-derived RPE for treatment of age-related macular degeneration.[11] [Note added in proof: ACT has published preliminary clinical data on safety of ES-cell-derived REP transplanted into two patients with macular degeneration, see: http://download.thelancet.com/flatcontentassets/pdfs/S0140673612600282.pdf.] Importantly, the fate of ectopic RPE introduced within the eye may be directly evaluated in real time by the clinician, with the option of removal of the introduced RPE should abnormalities be detected. This ability to directly track cell-based therapeutics from the time of introduction into the body will be crucial in clearly defining MOA.

The MOA of MSC and related cell types (Prochymal™, Osiris Therapeutics) and MAPC (multipotent adult derived progenitor cells) or Multi-Stem™ (Athersys, Inc.) continues to be the subject of scientific debate. The reported ability of MAPC to reconstitute cells from all three embryonic developmental

lineages (see Table 2.1) both *in vitro* and *in vivo* led to suggestions that MAPC represented an adult-derived stem cell functionally equivalent to ES cells.[12] This in turn led to the acquisition by Athersys, Inc. of all intellectual property and product development rights associated with MAPC. Engraftment and localized, niche-specific differentiation of MAPC within developing mouse embryos further established the idea that MAPC represented a cellular therapeutic able to home directly to sites of injury and, through a process of clonal self-replication and localized directed differentiation, reconstitute the cell types native to that particular organ or tissue micro-environment.

However, key early publications describing the isolation and characterization of cells with MAPC potential have been retracted. (For a discussion of background, see reference 13.) Much like MSC, MAPC/Multi-stem™ now appear to be marketed principally as a cellular factory for the secretion of extracellular growth factors into the neighboring cellular microenvironment, thereby potentially creating a regenerative milieu within the surrounding tissue. There is little or no published evidence for site-specific engraftment and directed differentiation as a MOA, resulting in the trophic signaling hypothesis becoming a default explanatory pathway for the observed therapeutic bioactivity of MSC-like cell types.

The ability of MSC to undergo *in vitro* tri-lineage differentiation into chondrocytes, osteocytes and adipocytes led to similar expectations that MSC-based cellular therapeutics would utilize site-specific engraftment and directed differentiation to modulate disease pathologies.[1] Little, if any, direct experimental evidence for this hypothesis has been forthcoming, despite numerous reports of functional efficacy within small-scale studies *in vivo*. This has led to the current 'touch and go' model for MSC bioactivity, which emphasizes trophic activity and direct cell/cell contact. However, this interpretation may itself not be adequate to explain how intravenous injection of MSC appears to positively impact multiple organ systems simultaneously, given that essentially all injected MSC are trapped as lung embolisms (reviewed in ref. 14).

Additional mechanisms of cell/cell communication involving action-at-a-distance signaling interactions between MSC and non-stem cell populations are currently the subject of active investigation. In particular, considerable focus is being placed on the role of exosomes/microvesicles (MVs) in mediating aspects of cell/cell signaling and interaction. MVs are circular membrane fragments present in numerous biological fluids including plasma, breast milk and urine that may mediate the horizontal cell–cell transfer of surface membrane receptors, other functional proteins and/or genetic information in the form of micro-RNAs. These micro-RNAs are potentially capable of reprogramming injured cells towards dedifferentiation, proliferation and subsequent expression of trophic factors that facilitate the targeted recreation of a regenerative micro-

environment (reviewed in ref. 15). The transfer of information may also be bidirectional. In this regard, MVs derived from injured lung or kidney epithelial cells appear capable of mediating the partial reprogramming of MSC towards acquisition of lung- or kidney-specific phenotypes.[16,17]

Some combination of these mechanisms may possibly be responsible for the observed functional efficacy *in vivo* reported by multiple investigators for MSC in multiple experimental systems. Why, then, did Phase III clinical trials of Osiris's Prochymal™ MSC treatment for GvHD show no evidence of functional recovery over placebo?[5] Careful analysis of the results of Osiris's clinical trial has revealed that statistical significance of treatment over placebo is in fact achieved for a small sub-group of patients participating in the Phase III trial. Understanding exactly what factors within these subjects facilitated positive response to treatment will be of crucial importance to future efforts to successfully develop MSC and related cellular therapeutics.

These ambiguities notwithstanding, pharmaceutical companies, traditionally slow in identifying and investing within emerging technologies, have continued to demonstrate interest in MSC-based cell therapies. Osiris's Phase II efficacy data on Prochymal™ for GvHD were sufficient to trigger a $130 million investment by Genzyme for rights to both Prochymal™ and Chondrogen™ (the latter an MSC product for knee cartilage repair) in 2009.[18] In 2010, Cephalon, Inc. structured a deal worth up to $1.7 billion with the Australian firm Mesoblast to commercialize MSC therapies for a range of disorders including congestive heart failure.[18] We can expect to witness a continued, concerted effort to drive MSC-based cell therapeutics through clinical trials and into market in the next few years.

2.3 Other stem cell-based therapeutics currently under development

In addition to Geron's GRNOPC1, a number of biotechnology companies are attempting to target neurological indicators similar to spinal cord injury with neuronal stem cell populations. For example, StemCells, Inc. is initiating Phase I clinical trials of CD133+ human fetal-derived neural stem cells (HuCNS-SC) for chronic spinal cord injury.[19] Similarly, NeuralStem, Inc. is pushing forward their human spinal cord-derived stem cell line NSI-566RSC into Phase I clinical trials for amyotrophic lateral sclerosis.[20] Finally, UK-based Reneuron, Ltd is entering Phase I trials for ischemic stroke with ReN001, a genetically modified, conditionally immortal neural stem cell line derived from a 12-week-old human fetus.[21] Genetic engineering of cell-based therapeutics is associated with its own unique subset of

process development challenges. We will briefly outline recent developments in genome modification technologies pertinent to TE/RM in section 2.4.

Another interesting stem cell technology platform currently in vogue is iPS (induced pluripotent stem cells). The demonstration that lineage-committed, adult mammalian cells could be dedifferentiated into a pluripotent state recapitulating aspects of an ES cell phenotype has been the subject of considerable recent research activity. The forced expression of certain key transcription factors including (but not limited to) KLF4, SOX2, c-MYC and OCT4 was shown to lead to acquisition of ES cell-related developmental markers as well as partial lineage contribution towards all tissues of developing mouse embryos, a defining feature of ES cells. Injection of certain iPS lines into tetraploid mouse blastocysts has led to creation of 'all-iPS' mice, demonstrating that at least certain iPS cell lines may reconstitute most aspects of ES cell functionality. iPS lines have now been generated in multiple species and from multiple differentiated cell types (reviewed in ref. 22).

However, although of significant interest from an academic perspective, we believe that the application of iPS cells for cell-based therapeutics is unlikely to be practical in the near future. Factors negatively impacting commercial development include:

- requirement for delivery of reprogramming factors, either by gene transfer vectors or by direct microinjection of protein factors;
- extremely low efficiency of cellular reprogramming (0.001–1% depending on vector platform and cell type being reprogrammed);
- considerable length of time (up to 2 weeks) required to identify iPS clones; and
- finally, recent reports indicate that iPS cells may be less ES-cell-like than previously believed and more prone to tumorigenesis and reduced functionality.[23]

These factors notwithstanding, patient-specific iPS clones may be useful for small molecule or protein-based drug development screens, and start-up companies such as California-based Iperian, Inc.[24] are currently exploring this space.

2.4 Development of genome modification technologies: gene-based cell therapies

The synergy of gene therapy techniques with autologous cell-based therapies offers the potential for permanent correction of many severe, single and

multi-factor-based genetic disorders as well as cancers, neurological and cardiovascular diseases, and HIV/AIDS. Viral vectors including retroviruses, lentiviruses and adenoviruses continue to represent the gene delivery platform of choice, although non-viral vectors including plasmid DNA and human artificial chomosomes have been proposed (but are not being currently developed) as commercially viable gene transfer vectors (reviewed in ref. 25). Although the continued commercial development of gene therapies came to a crashing halt in 1999 following the death of a young volunteer named Jesse Gelsinger who had received an adenoviral vector containing a gene for the enzyme ornithine transcarbamylase,[26] the field appears to be currently enjoying a renaissance, with multiple biotechnology companies pursuing Phase I/II clinical trials targeting numerous clinical indications with a broad range of vector types and transgenes (reviewed in ref. 26).

In an effort to bypass the limited expansion potential associated with neuronal stem cells (and also with other classes of non-ES stem cells), Reneuron, Ltd has generated a human fetal-derived neuronal stem cell line conditionally immortalized by transformation with a retroviral vector carrying a single copy of the oncogene c-MYC, fused to the mouse estradiol receptor. Immortalization may be activated or suppressed at will by the addition of 4-hydroxytamoxifen to the cell culture media.[27] ReN001 is currently in Phase I clinical trials in patients presenting with ischemic stroke.

From a TE/RM product development perspective, the addition of genome modification technologies to the standard cell-based therapy platform triggers intense scrutiny by regulatory agencies, serving to substantially increase expenditure and time to market. Indeed, it has taken Reneuron, Ltd over 5 years to secure regulatory approval from the UK's Gene Therapy Advisory Committee. Some of the questions raised include:

- How is the transgene integration site controlled?
- How will transgene copy number be controlled?
- What is the impact of transgene integration on host cell biology? Insertion of an ectopic transgene represents an act of insertional mutagenesis. What is the effect on the stem cell potential of the host cell?
- What is the susceptibility of the integrated transgene to position effect silencing?
- What is the potential for incidental activation of oncogenes?
- How will cells transformed by the transgene be screened or selected away from those that remain untransformed?

The need to address these issues will substantially increase cost of goods, requiring the development of specific assays to monitor transgene integration

and copy number as well as impact on host cell phenotypes. Furthermore, few institutions currently have the experience to manufacture viral vectors at large scale under good manufacturing practice (GMP) conditions. Taken together, these difficulties lead us to conclude that genome modification technologies are not recommended for application in TE/RM product development, and will probably require 5–10 years of further optimization within the context of an academic research organization before beginning to acquire commercial relevance.

2.5 Committed cell types: ideal candidates for TE/RM product development

As we have seen, current interpretations of the MOA of stem cell-based cellular therapies principally emphasize action at a distance through secretion of bioactive growth factors and signaling mediated by paracrine factors or MVs. There is little, if any, direct evidence for site-specific integration and directed differentiation. As the secretion of regenerative growth factors is not a feature unique to stem and progenitor cell populations, it is reasonable to question if aspects of stem cell functionality may be recapitulated simply via populations of committed cell types. Such committed cell populations have significant advantages over stem and progenitor cells from the perspective of process development of TE/RM products. We illustrate these through a side by side comparison of MSC with committed smooth muscle cells (SMC) in Table 2.2. Both cell types are currently under development for application in organ regeneration platforms focused on regeneration of hollow organs such as bladder and intestine with MSC being used as a potential source of SMC (reviewed in ref. 28).

These characteristics underlie our decision to use committed smooth muscle cells over MSC for continued commercial development of Tengion's Autologous Organ Regeneration Platform™ (see Figure 2.1). This is in marked contrast to strategies focused around the manipulation of MSC to achieve the same objectives of hollow neo-organ tissue engineering.[29] Similar principles may be applied towards ES cells, iPS cells and other stem cell platforms.[30] Our overall conclusion based on this analysis is that the use of committed cells substantially lowers costs of goods and development timescales when compared with stem and progenitor cell populations.

In addition to tissue engineered organs, the advantages of committed cell types may also be utilized for cell-based therapeutics. To this end, a number of stem cell-based therapies have been proposed for the treatment of acute and chronic renal disease. These include bone-marrow-derived MSC, renal

Table 2.2 Comparison of mesenchymal stem cells (MSC) and committed smooth muscle cells (SMC) for application in commercially viable organ regeneration platforms (based on data from ref. 28)

Mesenchymal stem cells	Committed smooth muscle cells
Variation in proliferative and tri-lineage differentiation potential (donor effects, number of passages in culture, etc.).	Autologous sources may be sourced from multiple tissues using existing minimally invasive procedures. SMC phenotype does not exhibit donor variability.
Ex vivo expansion of MSC triggers loss of differentiation potential.	SMC phenotype is stable during *ex vivo* expansion (eight or fewer passages).
Evaluating tri-lineage differentiation potential during *ex vivo* expansion is lengthy and expensive.	No requirement to monitor multi-lineage potential.
Directed differentiation is an uncontrolled, inefficient process.	No requirement to evaluate directed differentiation.
Directed differentiation requires defined, inductive cytokine addition during *ex vivo* expansion, increasing cost of goods.	Defined, inductive cytokines not needed during *ex vivo* expansion.
Multiple molecular, proteomic and functional tests required to characterize differentiated cellular components. Test results may be misleading and unreliable.	SMC phenotype is consistent during *ex vivo* expansion, allowing standardization of cell characterization procedures.
Long-term effects of *ex vivo* exposure to inductive cytokines (e.g. transforming growth factor-β) are unknown and there is potential for generating tumor cells.	Long-term experience with media used for *ex vivo* SMC expansion exists as well as defined procedures for achieving approved dilution of residual levels of media components.
Differentiation protocol significantly increases process times for generating SMC (e.g. up to six weeks for differentiation in 1% fetal bovine serum-based media). Cells not expandable during differentiation.	No requirement for directed differentiation.
Limited *in vivo* preclinical and clinical testing with clinically relevant models to date.	SMC-based organ regeneration platforms have been tested *in vivo* across multiple organ systems and species using clinically relevant preclinical models and Phase I and II clinical trials.

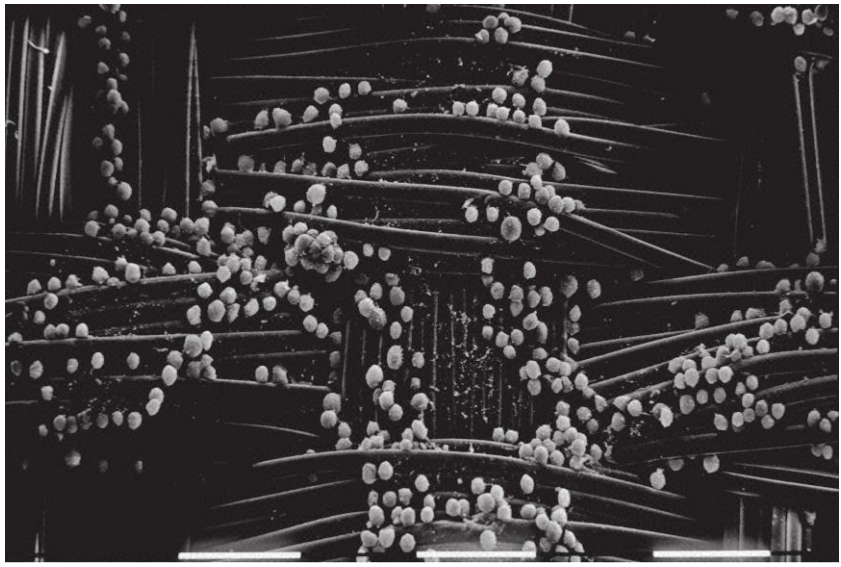

Figure 2.1 Scanning electron micrograph of committed rodent smooth muscle cells (SMC) on a woven PGA (polyglycolic acid) mesh. Such SMC may be used to regenerate native-like urinary neo-tissues and neo-organs such as the Neo-Bladder Augment™ (NBA) and Neo-Urinary Conduit™ (NUC).

stem cells, reprogrammed adult renal cells and ES cells. Although preliminary evidence of limited *in vivo* therapeutic efficacy has been generated for some of these cell types in acute animal models of renal disease, commercial development into cellular therapeutics is rate-limited by the factors we have outlined above.[31,32] However, primary renal cell populations composed principally of fully committed renal tubular cells have been shown to positively impact aspects of renal physiology associated with onset of chronic kidney disease in rodent models.[33] Such committed renal cell types may be isolated directly from renal tissue biopsies in an autologous manner and expanded to clinically relevant doses without any requirement for selection on the basis of surface marker profiles, maintenance of a pluripotent state or *in vitro* directed differentiation towards defined renal lineages prior to delivery.[34] These bioactive primary renal cells provide direct evidence that aspects of therapeutic efficacy traditionally associated with stem cells may in fact be recapitulated by committed cell types in a more technically straightforward and cost-effective fashion, thereby directly challenging long-held assumptions about which cell types may be effective for development of TE/RM products.

2.6 Summary: key features favoring commercial development of cellular ABIs

Although multiple distinct populations of stem and progenitor cells are currently under development for application as cellular therapeutics, it is our position that the rapid transitioning of any cellular ABI candidate through bioprocess research and development and into clinical trials and eventual production will be greatly facilitated through the use of committed cell types (Table 2.2) instead of stem cells. Such committed cells have the potential to reconstitute aspects of the currently held 'touch and go' model for action at a distance believed to underlie the MOA of most therapeutic stem cells, as well as to secrete ECM and additional growth factors and cytokines that may potentially recreate a regenerative micro-environment, a feature crucial for the development of neo-organs and other tissue engineered products. Ultimately, simplicity of isolation, characterization and expansion is the key to commercial success. Each requirement for maintenance of 'stemness', directed differentiation, specific media formulations with recombinant cytokines or genome modification serves only to increase development times and cost of goods.

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3

Biomaterials for TE/RM products

Abstract. Biomaterials may be required to support the cellular active biological ingredient within the context of a TE/RM product. In this chapter, we briefly review the principal classes of natural and synthetic biomaterials commonly used within the TE/RM industry. We illustrate the selection criteria for identification of appropriate biomaterial candidates for TE/RM product application using 'real world' examples from Tengion's ongoing development of Neo-Kidney Augment™ and Neo-Bladder Augment™ and Neo-Gastrointestinal tract (Neo-GI™) regeneration programs.

Keywords: biomaterials, extracellular matrix, decell/recell, hydrogel, chronic kidney disease, gelatin, poly-caprolactone, poly-glycolic acid, poly-lactic-*co*-glycolic acid, Neo-Kidney Augment, tissue engineering

3.1 Introduction

Any TE/RM product or product candidate incorporating a cellular active biological ingredient (ABI) may be defined as a bi-component 'construct' or combination product composed of:

- a cellular ABI (primary committed or stem cell population), and
- a biomaterial or scaffold. (In its broadest sense, the term biomaterial may even be used to refer to a basal diluent such as buffered saline. By this definition, even a 'cells-only' therapeutic may be regarded as a 'construct'.)

Alternatively, acellular biomaterials such as small intestine sub-mucosa (SIS) (see Table 1.1) have also been developed into successful TE/RM products. In the following sections, we briefly review biomaterial strategies pertinent to TE/RM methodologies, before proceeding to examine in detail

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how we have selected biomaterial candidates for incorporation into Tengion's internal product development pipeline. We hope through these illustrative examples to identify key properties of biomaterial candidates that facilitate commercialization and successful process development of TE/RM product candidates.

The role of biomaterials within TE/RM products has evolved from merely providing a passive structural framework within the body to facilitate repair or regeneration, as with the use of gold or porcelain to repair dental cavities. Instead, development of more bioactive biomaterials through incorporation with defined stem or committed cell populations may form regenerative scaffolds capable of facilitating deposition of extracellular matrix (ECM) by therapeutic cell populations as well as mediating the formation of a regenerative milieu to catalyze induction of neo-tissues or neo-organs. Ultimately, it is envisioned that biomaterials may replace cells altogether within regenerative constructs by incorporating elements of ECM, signaling factors and cytokines that are capable of manipulating the behavior of host-derived cells in the absence of any ectopic cellular component.

As currently understood, the role of biomaterials within today's TE/RM products may include:

- providing space for tissue repair and regeneration,
- providing a foundation for the expansion and delivery of therapeutic exogenous cell populations,
- serving as a framework for the deposition of ECM and paracrine signaling factors, and
- providing a foundation for the regeneration of neo-tissue and neo-organs in a manner appropriate to the local micro-environment.

In this manner, biomaterials act to recreate aspects of the endogenous ECM native to the host. With a few exceptions (steel or ceramic pegs for orthopedic applications, for example), biomaterials are designed to be fully biodegradable upon implantation within the body, with rates of degradation predetermined by the needs of the particular application under consideration.

The most straightforward classification of biomaterials is based on source, which may be of natural or synthetic origin. Examples of naturally occurring biomaterials include gelatin, fibrin, hyaluronic acid (HA), chitosan, silk, collagen and alginate. Such naturally derived biomaterials are typically well tolerated upon introduction within the body and tend to reflect properties of native ECM well. However, from a process development

and manufacturing perspective, naturally sourced biomaterials are potentially problematic, presenting difficulties in sourcing, quality control, reliability and reproducibility across lot to lot. In contrast, synthetic biomaterials such as poly-*co*-glycolic acid (PGA), poly-lactic-*co*-glycolic acid (PLGA) and poly-L-lactic acid offer better reliability and reproducibility, greatly facilitating process development and manufacturing. For these reasons, development programs at Tengion focused on the creation of neo-organs have typically been based on application of synthetic biomaterials. However, as our experiences with selection of biomaterials for tissue engineering of the kidney show, other factors may override the putative selective advantages of synthetic sourced biomaterials.

3.2 The ECM: comparator for biomaterials

The ultimate objective of any biomaterial used for TE/RM is to recreate the ECM associated with the native organ being regenerated. It is therefore reasonable to briefly consider some of the key functional attributes associated with native ECM before we examine how these may be mimicked by a biomaterial scaffold. At its simplest, the ECM is the structural framework within which cells are organized. Long believed to be merely a static gelatinous network for cellular bioactivity, the ECM is currently understood to be in continual, bi-directional dynamic interaction with the cells it is supporting. ECM serves to regulate cellular proliferation, migration and differentiation. Remodeling of ECM is constant and continuous, and is especially active during embryonic development and during repair and regeneration. The basic composition of ECM is generally well conserved across organ types and between species, although upon detailed examination, organ-specific compositional differences become manifest. Components of the ECM include:

(i) *Collagens*. Collagen is the single most abundant protein within ECM and in fact is the most abundant protein by weight within most animals. Over 20 iso-forms of collagen have been identified in mammals, with Type I collagen being the most abundant and therefore one of the principal components of any mammalian ECM. Collagen is a fibrillar, structural protein serving to provide foundational support for cellular proliferation and migration. The physico-mechanical properties of ECM may be modulated by the presence of alternative iso-forms of collagen, such as collagen I and III. The conservation of collagen sequence and structure across species has facilitated the application of bovine-derived collagen I for

multiple applications in TE/RM, including Apligraf™ (made by Organogenesis, Inc. as a cell-seeded collagen I-based skin substitute).[1]

(ii) *Elastins*. As suggested by the name, the role of elastins is to confer elasticity to tissues, allowing them to stretch as required yet recover their original shape. This property is critical in the vasculature, and considerable research time and effort has been devoted in attempts to recreate the elastic properties of blood vessels in the context of a tissue-engineered vessel (TEV), with variable success to date. In fact, elastin is the principal component of the arterial wall ECM, and may constitute up to 50% of its dry weight.[2] Although we will examine TEVs in more detail in a later chapter on tubular organ regeneration, we discuss them here to illustrate why an understanding of ECM composition and biology can be critical to successful development of a viable TE/RM product.

Elements of the vasculature are composed of three layers (Figure 3.1): the *tunica intima* is the endothelial cell monolayer lining the luminal surface of the vessel. Paracrine bioactivity by the endothelial cell layer is responsible for inhibition of platelet activation and associated thromogenic activity. The *tunica adventitia* is the external layer composed principally of collagen and fibroblastic cell populations. The central layer, the *tunica media*, consists of vascular smooth muscle cells organized in concentric orientation around the circumference of the vessel. Elastin is a secreted product of these

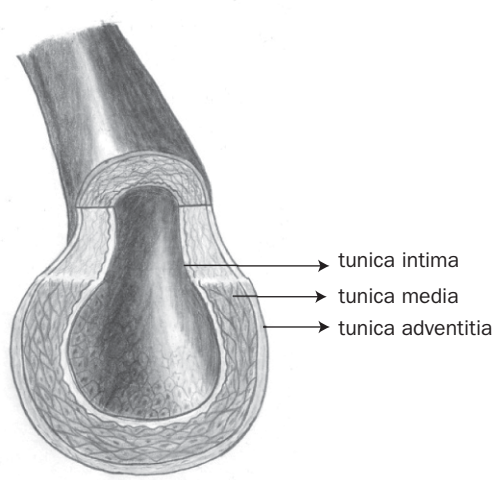

Figure 3.1 Cartoon image of a blood vessel illustrating tri-laminar organization: tunica intima (luminal layer), tunica media and tunica adventitia (external layer)

vascular smooth muscle cells. Post-translational modification of secreted elastin monomers results in concentrically organized rings of elastic polymers or lamellae in the tunica media. Together with collagen, elastin serves to modulate the mechanical properties of the vasculature in response to hemodynamic stresses associated with circulation, by providing elastic recoil to the vasculature after successive cycles of vessel inflation.

The inability to recreate a *tunica media* within TEVs with elastic properties reflecting native vasculature has significantly limited their commercial development. Compositional analysis of TEVs synthesized *in vitro* relative to native vasculature has demonstrated the general absence of elastic lamellae within TEVs compared with native vessels, despite similar overall collagen densities.[3] Furthermore, inadequate recreation of the elastic lamellae within the *tunica media* may be observed upon implantation of TEVs *in vivo*, probably leading to dilation of the TEV by six months post-implantation.[4]

(iii) *Fibronectins*. The fibronectins are another important component of the ECM and exist as multiple iso-forms generated through alternative splicing. Fibronectins serve as intermediates between the structural collagen fibrils and cells, binding cells through integrin receptors and acting as signaling ligands to regulate cellular proliferation and migration through the matrix. The minimal integrin binding domain has been identified as arginine–glycine–aspartate (RGD). The cell-adhesive properties of fibronectins and fibronectin-derived peptides are well established and have been extensively utilized in cell culture and tissue engineering applications.

(iv) *Laminins*. The laminins are important components of the basal laminae, where they form networked protein structures interacting with other ECM components including collagen. The cell-adhesive properties of laminin are also well known in cell culture and tissue engineering.

(v) *Glycosaminoglycans* (GAGs). GAGs are carbohydrate polymers characterized by a high overall negative charge, which serves to facilitate the entrapment of growth factors and cytokines as well as significant amounts of water. This property enables small amounts of GAGs to gel relatively large amounts of water. Key GAGs include heparin sulfate, chondroitin sulfate and keratan sulfate. Of these, heparin sulfate functions as an important regulator of growth factor-mediated cell signaling. HA is another important GAG that has been extensively used for TE/RM product development. Hyalgan™ is used for visco-supplementation of osteoarthritic knee. Injection of HA directly into the knee can augment joint lubrication and maneuverability.

(vi) *Growth factors and cytokines*. Analysis of bladder sub-mucosal matrix generated by bladder-derived smooth muscle cells has shown the presence of multiple key paracrine signaling factors, including vascular endothelial growth factor (VEGF), bone morphogenetic protein 4 (BMP4), platelet-derived growth factor (PDGF-ββ), keratinocyte growth factor (KGF), transforming growth factor (TGF-β1), insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and transforming growth factor (TGF-α), as resident components.[5]

All of these components are synthesized intracellularly and delivered into existing ECM by exocytosis. Degradation of ECM is mediated through proteolytic cleavage by enzymes such as matrix metalloproteases as well as serine and cysteine proteases. The balance between synthesis and degradation is central to remodeling during development, repair and regeneration, and interference with this homeostasis may lead to the onset of pathology. The detailed composition of ECM is organ specific, with a unique mixture of structural proteins and potentially regenerative growth factors.

The regenerative potential of ECM is illustrated by the observation that porcine-derived ECM may stimulate regeneration of amputated fingertips in adult humans.[6] This observation suggests that the ECM represents the ABI or at least a significant component of the ABI in any TE/RM product. This in turn raises the intriguing possibility that acellular biomaterials, composed of organ-specific ECM, may be sufficient to catalyze regenerative outcomes. Alternatively, it might be possible to define a set of synthetically derived ECM components and/or growth factors or growth factor mimetic peptides that could be introduced within the context of a biomaterial scaffold, and be potentially capable of recapitulating key aspects of organogenesis following implantation.[7] The potential absence of cells would considerably simplify process development and manufacturing. Unfortunately, it is clear that acellular biomaterials alone are not efficient in catalyzing a regenerative response upon *in vivo* implantation (reviewed in ref. 8). It is likely that the presence of living cells as part of a cell/biomaterial composite or construct is a prerequisite for the induction of a more sustained, physiologically relevant regenerative response.

3.3 Decell/recell: the ultimate biomaterial platform?

Given that the objective of any ectopic biomaterial is to recreate aspects of native ECM as precisely as possible, it is reasonable to extrapolate that the ideal biomaterial candidate for TE/RM applications is native ECM itself. After all, what better biomaterial to tissue-engineer the heart, for example,

than ECM derived from cardiac tissue? In this regard, a number of interesting results have been described in the popular media, most recently the reconstitution of partial pulmonary function from decellularized lung that has been reseeded with pulmonary epithelial and vascular endothelial cells.[9,10] In these rodent studies, the tissue-engineered lung, upon orthotopic transplantation within the body cavity of the animal, was partly functional for at least a few hours. Perhaps even more remarkable because it was performed in a human patient, a decellularized tracheal segment was reseeded with patient-derived epithelial cells and chondrocytes to form a tissue-engineered trachea that upon implantation led to successful regeneration of neo-trachea and recovery of pulmonary function.[11] Although these studies are no doubt impressive as proof-of-concept, can decellularization/recellularization of cadaveric organs really be viable as a commercial organ regeneration platform?

Strategies for decellularization/recellularization or 'decell/recell' of mammalian organs use a combination of chemical agents and mechanical agitation to achieve tissue decellularization with concomitant maintenance of key constituents of the ECM. Chemical agents may include acids/bases, hypotonic and hypertonic solutions, ionic, non-ionic or zwitterionic detergents, and solvents. Biological agents may include enzymes and chelators. Mechanical or physical agents can utilize the effect of temperature, force, pressure or electricity. Methodologies for application of chemical or biological decellularization can make use of whole organ perfusion, induction of pressure gradients across the target tissue, application of supercritical fluid and immersion/agitation (reviewed in ref. 12). Although theoretically ideal and doable in practice within the context of an academic research environment, a number of factors suggest that decell/recell may not be a viable commercial strategy for organ regeneration technologies:

- Decell/recell is dependent on a source of organs for scaffold formation, principally human cadavers. It is therefore limited by the same problem that the field is ultimately trying to solve, i.e. the shortage of human organs for transplantation.
- How will potential cadavers for organ donation be standardized? Will they be screened for pathogens? Will certain age groups be specifically targeted? These issues will raise cost-of-goods issues and introduce regulatory obstacles.
- Organs derived from different donors will result in considerable variability for the resultant neo-organ scaffold. The quality and composition of ECM will vary as will its likely regenerative potential upon implantation. How will these factors be controlled?

- Decellularization protocols will have to be established individually on an organ by organ basis. Each organ is unique in terms of volume, density, lipid composition, etc.
- Xenogeneic sources have been proposed to address these issues. In particular, the pig has been examined as a potential organ source owing to the reasonable size match between porcine and human organs. Transgenic pigs have been generated that have key immunogenic epitopes genetically deleted. However, such xeno-grafts still continue to induce an immune response in primate models and will inevitably be burdened by constant regulatory concerns over potentially pathogenic bio-burdens.[13]
- Methodologies for decell/recell typically involve a combination of mechanical agitation and salt/detergent action to gently lyse the cells open in a manner that removes most of the endogenous DNA and potentially immunogenic surface molecules while retaining key components of the native ECM. However, such methodologies vary widely between individual laboratories and from investigator to investigator. No standardized, universally accepted protocols have yet been developed.
- Despite best efforts, the removal of immunogenic epitopes can never be 100% complete. The potential always exists for induction of an adverse immune response from the cadaver-sourced scaffold. Quality control assays must therefore be developed to carefully evaluate the outcome of decellularization. Cost-of-goods and time to delivery are therefore significantly increased.
- Additional quality control assays are required to evaluate the bioactivity of the residual ECM. Are the levels of key matrix components within acceptable limits? What are these parameters, and how exactly will they be defined? A minimal set of criteria for effective decellularization has been proposed, based entirely on evaluation of residual DNA fragments: <50 ng dsDNA per mg ECM dry weight, <200 bp DNA fragment length, lack of nuclear material in tissue sections stained with DAPI or H&E.[12] Of these, only the first is an objective, quantitative measure appropriate for industrial standardization. It remains to be rigorously determined whether such criteria are adequate to predict the absence of a negative immunological response upon implantation.
- Although acceptable for preclinical and even Phase I academic studies, it is difficult to envision how cadaveric organs may be used for industrial production of neo-organs at the scales required to facilitate commercial viability.
- From a quality control perspective, it is important to define what functional assays will be applied to demonstrate that the tissue-engineered neo-organ is ready to go 'out the door' and into a patient. Evaluation of functionality for the tissue-engineered lung was provided

by implantation within living rodents, and doubtless numerous animals were killed prior to achievement of a successful outcome. For the tissue-engineered trachea made from cadaveric tissue, no functional evaluation or quality control protocols were applied – the neo-organ was simply implanted into the patient and the outcome left to good fortune. Such an approach, while acceptable for academic investigations, cannot be used for development of commercial TE/RM products under Federal or international regulatory guidelines (see Chapter 8). [Note added in proof: Synthetic biomaterial has now been used for tissue engineering of a tracheobronchial construct, see Jungebluth *et al*. (2011), "Tracheobronchial transplantation with a stem-cell seeded bioartificial nanocomposite: a proof-of-concept study", *Lancet* 378: 1997–2004.]

These inherent difficulties have led us at Tengion to avoid application of decell/recell as a foundational organ regeneration platform. Instead, by using the body's own inherent ability to regenerate, we avoid all issues related to *in vitro* engineering of neo-organs using decellularized scaffolds. For commercial TE/RM product development, we have focused instead on biomaterials with the following characteristics:

- Synthetic: straightforward to manufacture at large scale with reproducible, well-defined chemical and physical characteristics.
- Natural: applied in some cases; see below. Material should be readily obtained through commercial sources.
- Well tolerated by the targeted organ. Not all tissues, organs or localized micro-environments within the body will tolerate implantation of biomaterials equally well. Prescreening of biomaterials may be required, even for well-established biomaterials.
- Established history of usage as a biomaterial for implantation within human patients. For example, PGA has been applied for decades as a biodegradable surgical suture with no evidence of deleterious effects. Similarly, porcine-derived SIS is routinely used in multiple surgical applications. Such a history significantly streamlines the regulatory approval process, shortening time to market and costs of development as well as greatly alleviating risks associated with novel technologies.

In addition to the material composition, the form of the biomaterial is also of importance. Different organization of the same biomaterial may induce a different regenerative outcome and this may need to be systematically evaluated as part of the research and development process. A summary of the principal biomaterial configurations commonly dealt with is presented in Table 3.1.

Table 3.1 Material forms for biomaterials product candidates

Material form	Definition	Examples
Diluent	Fluid for suspending cells	PBS, HA solution, glycerin
Cell carrier	Small, injectable substrates to which cells attach	Microbeads/fragments
Gel, hydrogel	Mostly water, compressible but does not flow, cells imbedded, generally not porous, may set up *in situ* (injectable)	Alginate, HA, gelatin
Scaffolds (includes foams)	Solid, porous substrate for cell attachment. Stiffness can range from low (foams) to high (coral)	OPLA, PGA felt, electrospun collagen, lyophilized HA
Inert material	Does not promote cell integration or inflammatory response	Teflon sheets, stainless steel, titanium
Tissue expander	Balloon-like device with port. Fluid can be injected through port several times to slowly expand the tissue. Deflated and removed at time of implant	Silicon filled with saline, expander that expands in the presence of osmotic change

HA, hyaluronic acid; OPLA, open-cell polylactic acid; PBS, phosphate-buffered saline; PGA, poly-*co*-glycolic acid.

Rather than describe selection criteria for biomaterials during TE/RM product development in an abstract manner, we believe it will be more helpful for the reader for us to highlight the process through specific, 'real-world' examples from our own experiences at Tengion. In the following sections, we examine biomaterials decision points made during development of the Neo-Kidney Augment™ (NKA) and Neo-Bladder and Neo-GI tract.

3.4 Selection of biomaterials for tissue engineering: illustrative example – kidney

Chronic kidney disease (CKD) affects over 19 million people in the US and frequently develops as a consequence of chronic obesity, diabetes and/or hypertension. Patients in Stage 4–5 CKD receive dialysis and a complex drug regimen; the number of kidneys available for transplant is vastly insufficient to meet the need. Clearly, new treatments that delay or reduce dialysis

dependency are needed to fill this void. TE/RM products represent one approach towards reversal of the cellular pathologies associated with CKD.

Kidney tissue is composed of over 20 specialized cell types structurally organized into morphologically and functionally distinct compartments that act in concert to filter blood, produce urine, and regulate endocrine function as well as acid–base and electrolyte balance. Cell–cell interactions are critical to kidney function and are at least partially dependent on spatial and architectural relationships. Regeneration of complex solid organs such as kidney involves the defined reconstitution of multiple specialized cell types organized within highly complex three-dimensional micro-architectures. TE/RM approaches to CKD aim to re-establish homeostasis in part through restoration of cellular organization and intercellular communication.

The regenerative response of kidney to acute injury is generally understood to be mediated by dedifferentiation of resident tubular cell populations at the site of injury with concomitant acquisition of a stem/progenitor cell phenotype, followed by proliferation and re-acquisition of tubular characteristics.[14] One approach to identifying candidate treatments for regenerating organ function is to select native organ-specific cells that possess relevant bioactivity. Numerous studies on regeneration of renal architecture and function following acute kidney injury point to tubular epithelial cells as central in restoration of function.[15,16] Tubular cells can be separated from a primary kidney cell isolate prepared from the medulla, cortex and cortico-medullary junction compartments of kidneys based on differential buoyant density.[17–19] Selected bioactive primary kidney cell isolates, enriched for tubular epithelial cells, increased survival and enhanced renal functions when administered intrarenally to rodents with CKD secondary to 5/6 nephrectomy.[17,18] Such therapeutically bioactive cell populations may be valuable components of products developed to augment kidney function in patients with CKD.

Although these cell-based approaches to extending survival and enhancing renal function in rodents with progressive CKD are promising, additional efforts may be required to enable product delivery and extend application of these approaches to patients with severe fibrosis and end-stage renal disease. Combining bioactive cells with biomaterials could enhance the regenerative response by providing space and context to drive formation of new functional renal mass. The biomaterial component(s) of NKA constructs could also serve to enhance the shelf life of the product by preserving cell viability and to target delivery of bioactive component(s) to specific regions of the kidney to maximize effectiveness. Structural modifications that can be accomplished in a defined manner with biomaterials may provide an optimized environment for cell–cell interactions

in NKA constructs *in vitro* or complement and enhance the *in vivo* regenerative response elicited by the cells.

With these specific objectives in mind, how can biomaterial candidates be selected to support therapeutically bioactive primary renal cells, thereby creating a cell/biomaterial construct appropriate for renal tissue engineering? We envision the following sequence for such a development program:

Identification of prospective biomaterial candidates. Based on the literature and the criteria discussed above, the number of potential candidates suitable for implantation within the kidney is fairly small. Prospective biomaterials could include hydrogels such as gelatin, HA and alginate, as well as biodegradable synthetics such as PGA and poly-caprolactone (PCL). Note that at this stage, this is a theoretical list only and may be amended.

Evaluation of biomaterial candidates in vitro. The biomaterial candidates are evaluated through simple, *in vitro* studies to assess their biocompatibility with the primary renal cell population to be delivered, as well as their potential interaction with primary renal cell populations within the recipient kidney. Does the biomaterial trigger significant cell death, or does it alter the transcriptomic, proteomic or secretomic profile of the therapeutic cells?

Evaluation of biomaterial candidates in vivo in healthy rodents. Biomaterials are now evaluated for bio-response (inflammation, immune tolerance, infiltration by resident cells, biodegradation characteristics) by implantation of acellular biomaterials within the parenchyma of healthy rodent kidneys. Primary renal cell/biomaterial constructs may then be examined in a similar manner. Histological evidence of neo-kidney-like tissue formation from such constructs would be of particular interest, indicating the potential for true regenerative outcomes that may be catalyzed by such constructs. Finally, biomaterial constructs are evaluated in a hemi-nephrectomized rat (where one resident kidney is removed). The rationale here is that paired organs such as kidney mediate considerable redundancy of function, such that one organ can compensate for significant insult suffered by the other. To this end, to better identify potential systemic injury triggered by biomaterial implantation within one kidney, the contralateral kidney is removed.

Evaluation of biomaterial candidates in vivo in diseased rodents. By this point, biomaterial candidates have demonstrated absence of significant inflammatory or immune response, minimal fibrotic outcomes, acceptable biodegradation profiles (i.e. biomaterial degrades at rates similar to that of neo-tissue formation), and good infiltration of the biomaterial mass by host tissue and elements of the vasculature. Such candidates may therefore be

regarded as well tolerated by healthy kidney and are therefore appropriate vectors for delivery of bioactive therapeutic cell types. Constructs composed of such cell types complexed with candidate biomaterials may now be evaluated for therapeutic bio-efficacy in rodent models of CKD. Such models are created by 5/6-hemi-nephrectomy, i.e. removal of an entire kidney and five-sixths of the contralateral kidney, such that the animal retains only a sixth of the functional kidney remnant. These animals begin to manifest aspects of CKD over a period of several months post-surgery, typically dying no later than six months post-surgery. Correction of key aspects of the CKD phenotype may be mediated by direct implantation of therapeutically bioactive primary renal cells within the remnant kidney.[17,18] From a biomaterials perspective, questions to be addressed include: how well does the construct (cell/biomaterial) compare relative to cells alone (or biomaterial alone for that matter) in mediating functional correction of the rodent CKD phenotype? Is there any evidence that the biomaterial is exacerbating the disease condition?

Development or identification of potential delivery systems. The introduction of biomaterials within the body as opposed to cells alone represents a significant increase in technical complexity. Specialized delivery systems may need to be designed from scratch; alternatively, commercially available delivery systems may be suitable. Any such device has to be clinically relevant, which usually means it must have the smallest possible interventional footprint. For delivery of NKA to kidney, a number of catheter-like devices are currently under consideration, but no final decision has yet been made.

Evaluation of biomaterial prototypes in large animal models under good laboratory practice (GLP) conditions. Obviously, small animal models such as rodents can provide only limited, clinically relevant information. However, once biomaterial candidates have successfully passed preliminary screens for bio-safety and therapeutic efficacy as detailed above, the next step is to evaluate the cell/biomaterial construct for both bio-safety and therapeutic efficacy within a large animal CKD model under GLP conditions (see Chapter 9).

Phase I human clinical trials. Provided the biomaterial is well tolerated in large animal GLP studies and demonstrates evidence of therapeutic bioactivity in combination with defined therapeutic cell types, constructs may now be evaluated for both bio-safety and evidence of functional significance in human CKD patients. Any evidence that the TE/RM intervention is effective in delaying onset of the disease or in reducing the number of dialyses required will be of interest.

3.5 Biomaterials candidates for renal tissue engineering

For preliminary identification of biomaterials candidates potentially suited for applications in renal tissue engineering, we focused on material types already widely used for application in TE/RM. Such biomaterials offer the best chance for rapid and straightforward demonstration of key objectives: bio-safety and regenerative bioactivity. We have examined the following biomaterial prototypes:

- Naturally derived, gelatin-based hydrogels have been widely used for encapsulation of mammalian cells.[20] Gelatin hydrogel represents an obvious choice for a biomaterial potentially implantable within mammalian kidney.
- The naturally derived hydrogel HA has been shown to mediate significant regenerative bioactivity in the context of the renal micro-environment, including the directed branching morphogenesis of ureteric buds, induction of mesenchymal-to-epithelial lineage switching, up-regulation of aquaporin-2 (a key marker associated with tubular epithelial cells), and differentiation of both metanephric mesenchyme and ureteric buds *in vitro* in a molecular weight- and concentration-dependent manner.[21]
- PLGA is a synthetic biomaterial widely used in TE/RM applications. PLGA particles have been shown to provide a favorable substrate for the adhesion and proliferation of neural stem cells *in vitro*, as well as to facilitate the regeneration of neural-like tissue upon directed microinjection into defined lesions created within the brains of rats.[22] Such evidence of regenerative bioactivity led us to investigate bio-responses of kidney to implantation of particulate PLGA.
- PCL has been evaluated in multiple tissues, including bone, the vascular system, the neural system, cartilage and controlled release methodologies. PCL is similar in structure to PLGA, but has a much longer degradation time. We chose to investigate PCL in parallel with PLGA.

Tengion's screening of these material types represents the first systematic investigation of the responses of mammalian renal parenchyma to implantation of synthetic and natural biomaterials, both acellular and as bioactive renal cell/biomaterial composites (i.e. NKA constructs). We applied a combination of *in vitro* functional assays and *in vivo* regenerative outcomes to functionally screen candidate biomaterials for potential incorporation into a NKA construct prototype.[23] We observed the following:

- Implantation of acellular hydrogel-based biomaterials into renal parenchyma was typically associated with minimal fibrosis or chronic inflammation with no evidence of necrosis by four weeks post-implantation. Moderate cellular/tissue in-growth and neo-vascularization was observed, with minimal remnant biomaterial (Figure 3.2, top).
- In contrast, implantation of acellular PLGA particles or PCL beads into the renal parenchyma triggered fibrotic encapsulation of the biomaterial, with minimal concomitant neo-vascularization, cell/tissue infiltration and neo-kidney tissue regeneration (Figure 3.2, bottom).

These results demonstrate that despite widespread application of PLGA, PCL and related polymers in TE/RM, no assumptions can be made regarding the potential suitability of any biomaterial for implantation within a given organ.

Based on these *in vivo* data, hydrogel-based biomaterials were selected to produce NKA constructs with which to evaluate *in vitro* bio-functionality and *in vivo* regenerative potential. *In vitro* confirmation of material biocompatibility was provided through live/dead analysis of NKA constructs. Gelatin-containing

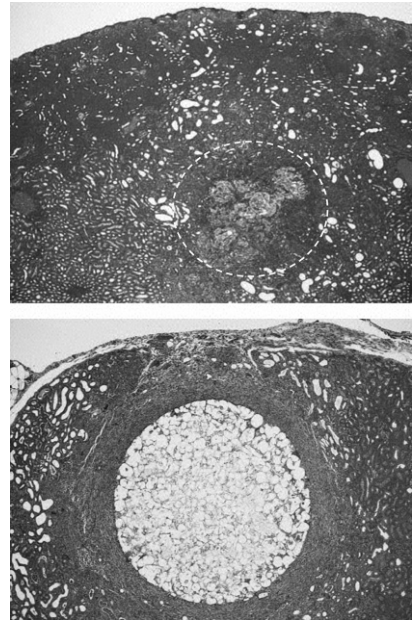

Figure 3.2 Implantation of gelatin hydrogel (top) and PCL beads (bottom) within renal parenchyma of healthy adult rodent kidneys. Note fibrotic encapsulation of PCL beads. 10× section of rodent kidney.[23]

hydrogels were associated with robust adherence of primary renal cell populations. Conversely, HA-based hydrogels did not provide a substrate for renal cell attachment, with spheroid formation being triggered instead. Neither cell attachment nor spheroid formation was observed upon PCL- or PLGA-based biomaterial, consistent with *in vivo* data.

Phenotypic and functional analysis of NKA constructs produced from bioactive primary renal cell populations and hydrogel biomaterials was consistent with continued maintenance of the tubular epithelial cell phenotype characteristic of the bioactive primary renal cells being supported by the biomaterial candidate. Transcriptomic, secretomic, proteomic and confocal microscopy analyses of NKA construct confirmed no significant differences relative to primary renal cells seeded in two-dimensional culture. Finally, implantation of hydrogel-based NKA construct into the renal parenchyma of healthy adult rodents was associated with minimal inflammatory and fibrotic response and with regeneration of neo-kidney-like tissue by eight weeks post-implantation.[23]

The strategy applied here involves screening acellular biomaterials to identify well-tolerated formulations, then assessing the viability, phenotype and function of bioactive cell/biomaterial composites *in vitro*. Screening the response of healthy kidney to NKA construct prototypes in parallel with *in vitro* characterization of potential mechanistic pathways represents a systematic and combinatorial approach to identifying and evaluating candidate regenerative treatments for CKD, and additionally may be applied to other diseases, organs and tissues.

3.6 Selection of biomaterials for tubular organs: bladder, esophagus and small intestine

The bladder is the foundational platform for TE/RM methodologies related to all tubular organs.[8] Thus, biomaterials selection criteria applicable to bladder are directly relevant to other tubular components of the genito-urinary tract, including urethra, ureter and vagina. Simply put, such biomaterials should be biocompatible, facilitate cell proliferation, migration and deposition of ECM throughout the volume of the biomaterial, and possess physical and mechanical properties similar to the native scaffolding associated with the corresponding tubular organ.

Regeneration of neo-bladder has been demonstrated using cell/biomaterial constructs based on bladder-derived sub-mucosa, small intestinal sub-mucosa and synthetic polymers including PGA, poly-lactic acid and PLGA. Clearly, bladder-derived sub-mucosa will best recapitulate the ECM

composition most closely associated with bladder, and preliminary optimization of decellularization protocols for bladder has been described.[24] Augmentation cystoplasty with bladder-derived acellular matrix grafts has been reported to mediate rescue of urologic functionality with concomitant regeneration of tri-laminar bladder wall architecture in rodent partial cystectomy models.[25] Similar studies evaluating application of SIS for bladder augmentation have yielded generally mixed results.[26,27]

These data notwithstanding, native organ-derived biomaterial is not suitable for TE/RM product development, for reasons we have already discussed. Therefore, to facilitate manufacturing and quality control concerns, we have sought to identify synthetic, biodegradable polymers capable of mimicking the physical and mechanical properties of bladder-derived submucosa in a reliable and reproducible manner, while simultaneously being easily obtainable at reasonable prices. With these criteria in mind, we selected PGA and PLGA for construction of scaffolds associated with Tengion's Neo-Bladder Augment™ (NBA), Neo-Bladder Replacement™ (NBR) and Neo-Urinary Conduit™ (NUC) urinary product portfolio. A detailed examination of studies demonstrating the suitability of these biomaterials for urinary organ regeneration will be presented in Chapters 4 and 5; for the moment, suffice it to say that native-like urinary neo-organ regeneration has been demonstrated in large animal clinical models using cell-seeded constructs composed of these polymers, and Phase I clinical trials of NUC are currently under way.

With these data in mind, and with successful bioprocess, quality control and manufacturing capabilities developed for production of neo-bladder and bladder-derivatives, our approach towards selection of biomaterials candidates for non-bladder tubular organs has been to regard these as extensions of the foundational bladder platform. Therefore, wherever possible, development of tubular neo-organs will utilize biomaterials, cells with regenerative bioactivity, construct seeding and maturation regimens and quality control assays that have already been applied to the neo-bladder. The esophagus in particular is directly comparable to the bladder in that both are laminarly organized hollow organs composed of a luminal epithelial layer surrounded by additional layers of directionally organized musculature.

We were able to take advantage of the observation that rodent esophageal tissue explants, upon culture *in vitro*, were associated with rapid proliferation and migration of smooth muscle cells out of the explant and onto the tissue culture surface. In fact, we observed that biomaterials could be implanted within esophagi of freshly killed rodents as patch implants; such constructs could be excised from the animal and cultured *in vitro* as *ex vivo* esophagi. Implanted biomaterials were rapidly colonized by smooth muscle cells migrating out of the native tissue and onto the construct (see Figure 3.3).

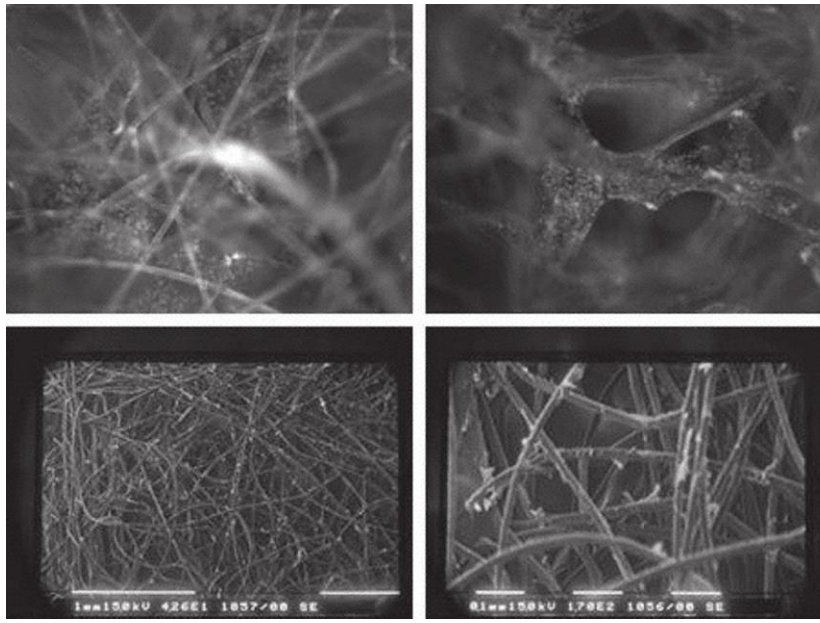

Figure 3.3 *In vitro* regeneration. Thin PLGA-coated PGA felt sutured to rodent esophagus and cultured for two weeks, then stained with DAPI to resolve migrated esophageal smooth muscle cells. Note formation of sheets of cells suspended from PGA felt fibers (top row). Scanning electron micrograph of similar construct (bottom row)

This allowed us to develop a straightforward *in vitro* assay to quickly screen potential biomaterials candidates for the ability to support esophageal smooth muscle cell migration and proliferation, a prerequisite for the application of such biomaterials in esophageal TE/RM (Figure 3.4).

Visible in Figure 3.3 are smooth muscle cells derived from rodent adipose[28] seeded onto a construct composed of PGA felt, organized as a spaghetti-like jumble of fibers. Note that this construct is composed mostly of open space, facilitating the continuous, circulating diffusion of nutrients into the interior of the construct as well as the efflux of waste products of cellular metabolism from the construct.

Using these methodologies, we identified PLGA-coated PGA felt as a suitable biomaterials candidate to explore for esophageal tissue engineering. This biomaterial has the added advantage of being in current use for production of NBR and NUC.

Preclinical rodent studies were initiated to evaluate the ability of constructs composed of smooth muscle cell-seeded PLGA-coated PGA

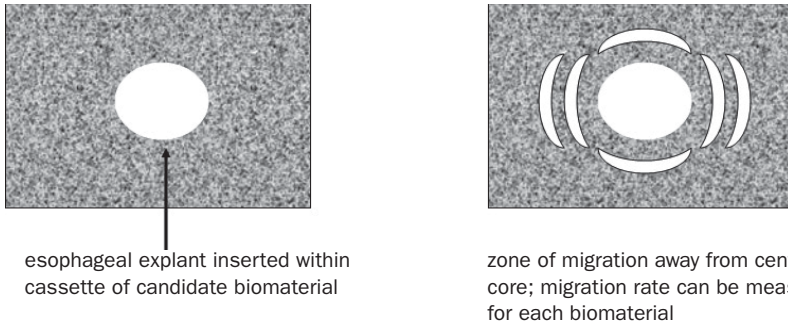

Figure 3.4 *In vitro* biomaterials screening: esophagus

patches to support regeneration of esophagus. In these studies, small (3–5 mm^2) defects were introduced within the esophagus of rodents, such that the entire esophageal wall was removed within the defect. Injuries were subsequently repaired with cell-seeded patch constructs and allowed to regenerate *in vivo* for up to 10 weeks post-implantation. Complete regeneration of longitudinal and circularly oriented musculatures and luminal epithelia were observed at 10 weeks post-implantation of the construct. These small animal data establish the utility of PGA felt-based biomaterials for tissue engineering and regeneration of the esophagus.

The next steps in development of an esophageal TE/RM product based on PGA felt constructs would involve similar esophageal regeneration studies carried out in a large animal such as swine or dog. Such studies would potentially also examine the regenerative potential of tubular constructs implanted subsequent to esophageal resection. We have found implantation of such constructs within rodent esophagi to be impractical. For additional details on regenerative outcomes associated with esophageal regeneration, we direct the reader to data presented recently by us.[29]

We attempted to use a similar approach towards screening of biomaterials candidates suitable for implantation within the small intestine (SI). As intestinal epithelium is highly proliferative, it might reasonably be expected that the SI will be highly amenable towards TE/RM methodologies. In this regard, we review strategies for SI tissue engineering in detail in a later chapter; in the context of biomaterials, however, it might be considered self-evident that the best biomaterial for regeneration of SI would be SIS. SIS would have the ideal mechanical and physical properties to recapitulate the behavior of native SI, would presumably contain elements of the ECM appropriate towards regeneration of SI epithelium and musculature, and has already been approved by the Food and Drug Administration for application in TE/RM products.

These properties notwithstanding, we nevertheless maintained our approach towards use of synthetic, biodegradable polymers as such biomaterials represent an extension of current bioprocess and manufacturing strategies applied to the bladder. When evaluated at a distance, efficiencies gained by regarding process development of non-bladder tubular organs as an extension of systems already in place for bladder are preferable to having disparate cell or biomaterial platforms for each tubular neo-organ.

Organ-specific differences in the migratory and proliferative potential of smooth muscle cells derived from disparate tubular organs may potentially impact strategies for the screening and identification of appropriate biomaterials candidates. In the case of the SI, we were unsuccessful in culturing smooth muscle or epithelial cells from SI tissue explants, a marked difference from the esophagus, which supported robust cellular outgrowth. The inability to culture primary SI cells implies that we are unable to effectively screen biomaterials *in vitro* for ability to support proliferation and migration of SI-derived smooth muscle and epithelial cells, as was the case for esophagus. Therefore, we had to proceed directly to small animal models, and evaluated biomaterials candidates in the context of both patch and tubular constructs for ability to support regenerative outcomes. Success with the esophagus using the same combination of biomaterials applied to bladder gave confidence that similar outcomes would result within SI from application of related biomaterial types.

As seen with esophagus, patch-based constructs composed of adipose-derived smooth muscle cell-seeded PGA felts were observed to support full regenerative outcomes upon implantation within rodent models of SI injury. Regeneration of intestinal epithelia with micro-villi as well as regenerated bundles of smooth muscle cells were observed within three months post-implantation. Unlike esophagus, implantation of tubular SI constructs upon excision of a tubular segment of native SI tissue and anastomosis of similarly sized tubular constructs was found to be practical. Therefore, we began studies to evaluate cell-seeded biomaterials constructs within the context of a tube. However, in addition to supporting the proliferation and migration of SI-derived cells, tubular constructs must have appropriate physical and mechanical properties that complement those of native tissue. In particular, within the gastrointestinal tract, tubular constructs must be capable of supporting peristaltic movement of food or fecal matter. This issue did not arise with the rodent esophagus because we did not evaluate biomaterials candidates for the esophagus in the framework of a tubular construct. However, for the SI, commercially sourced PGA-braided tubes were observed to have physical properties reminiscent of SI,

and were therefore applied in the context of tubular cell-seeded constructs for evaluation of regenerative outcomes within rodent SI.

As with the patch, complete regeneration of intestinal epithelia and partial regeneration of bundles of smooth muscle cells was observed. Implanted animals were capable of eating normally, gained weight and passed fecal material in a manner comparable with healthy controls. For additional information, we refer the reader to data we have recently presented.[30,31] These small animal data confirm that biomaterials used for regeneration of bladder may also be successfully applied to the SI. Again, the ability to apply the same combination of cells and biomaterials towards regeneration of multiple tubular neo-organs greatly facilitates development of a manufacturing platform with commonalities throughout bio-processing. These data notwithstanding, it is clear that the incomplete nature of regeneration of SI musculature from simple constructs derived from biomaterials used in bladder regeneration indicates that there are elements unique to SI currently missing from the candidate constructs.

Therefore, we are currently examining how biomaterials may be best used to facilitate complete regeneration of the SI. For example, perhaps the laminar organization of the SI may be mimicked within the biomaterial scaffold to encourage migration and proliferation of regenerative smooth muscle cells along longitudinal or circular pathways along or around the regenerating SI. Such 'encouragement' may come in the form of specific orientation of channels or pores within the scaffold or possibly by the use of specific growth factors or cell adhesion ligands tethered to defined layers within the biomaterial scaffold. Studies are currently ongoing to refine the organization of the biomaterial scaffold to better catalyze complete regenerative outcomes in rodents. Successful completion of these preliminary studies in small animals will lay the groundwork for evaluation of regenerative outcomes from tubular SI constructs in large animals such as swine or dog. Creation of appropriate disease models in large animals mimicking loss of adsorptive potential characteristic of small bowel syndrome (SBS) in humans will be essential to the further development of this product. Such models may be created by removal of progressively larger segments of native SI until evidence of malnutrition is manifest in the animal model. Once ascertained, similar sized SI tubular constructs can be implanted within the large animal model to evaluate functional recovery of symptomatology associated with SBS within the model. Additional questions to be resolved about the biomaterial candidate include:

- For each candidate, what is the maximum length of tubular SI construct that can successfully support complete SI regeneration?

- What are the temporal dynamics of degradation for each biomaterial candidate within the context of an SI construct? How does this compare with the dynamics of regeneration?
- Can biomaterial candidates cope with the uniquely hostile environment of the SI: low pH, constant passage of fecal matter, presence of bacteria, effect of digestive enzymes?
- How will constant peristaltic activity affect the physical integrity of the biomaterial during the regenerative process?
- How susceptible are individual biomaterials candidates towards induction of peritoneal adhesions? Such adhesions may be associated with extensive complications of the bowel, including blockage of passage of fecal matter leading to loss of construct integrity and leakage of fecal material into the peritoneal cavity.

Once these questions have been resolved, and appropriate biomaterials candidates that support regeneration of SI in large animal clinical models of SBS have been identified, preparations can be made for Phase I evaluation of safety and efficacy within human patients presenting with SBS.

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4

Neo-Bladder: a foundational technology platform for tubular organ regeneration

Abstract. This chapter examines the development of the Neo-Bladder Augment™ and Neo-Bladder Replacement™ as illustrative examples of TE/RM product development. We describe the patient population and current need for TE/RM products for regeneration of functional, native-like urinary tissue. We briefly examine preliminary proof-of-concept studies demonstrating regeneration of functional neo-bladder, including early clinical studies in pediatric patients. We then examine in detail how these experimental successes were translated to the development of practical neo-organ products currently undergoing Phase I clinical trials. Issues arising during the development of urinary neo-organs such as cell source, cell identity and bioprocess may be pertinent to additional, non-bladder hollow neo-organs.

Keywords: cystectomy, urinary diversion, bladder augmentation, bladder replacement, regeneration, alternate cell sourcing, mesenchymal stem cell, urothelial cell, organogenesis, adipose, smooth muscle cell, directed differentiation, peripheral blood, scaffold, neo-organ, organ regeneration, scaffold, biomaterials, histogenesis, stromal vascular fraction, peritoneal cavity

4.1 The need for urinary neo-organs

At its most fundamental level, the bladder is responsible for mediating storage and subsequent efflux of urine within a dynamically expandable and contractable container. Although relatively simple in terms of overall histology and structural organization (Figure 4.1), diseases impacting the bladder have the potential to significantly affect individual quality of life, resulting in continual incontinence or inability to effectively void urine as needed. Several congenital anomalies may result in abnormal bladder

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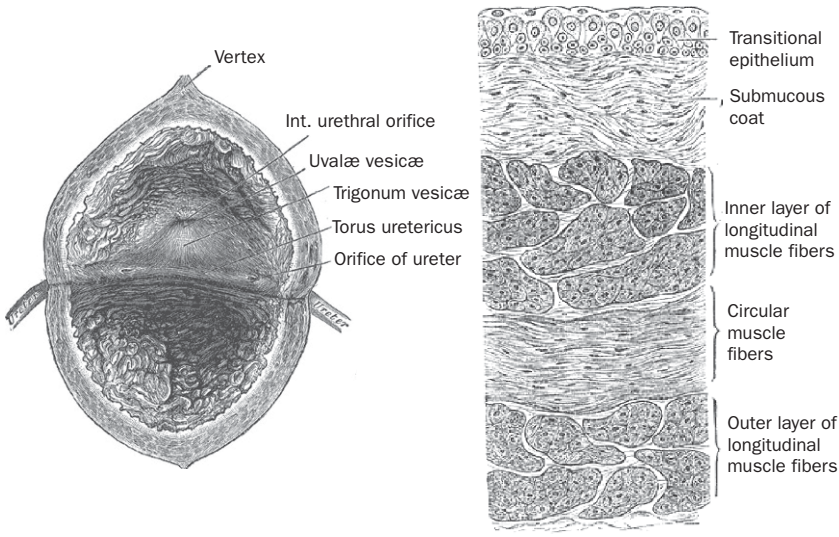

Figure 4.1 Macroscopic (left) and histological (right) organization of the bladder, illustrating tri-laminar cellular architecture of this hollow, muscular organ. Reproduced from Wikipedia Commons (Open Access) under terms of Open Access agreement.

development requiring surgical intervention, including posterior urethral valves, bilateral ectopic ureters, bladder exstrophy, cloacal exstrophy and spina bifida (myelomeningocele). The resultant clinical outcomes include incontinence and increased risk of renal failure from high pressures in the genito-urinary system.

The current standard of therapy for pediatric patients is bladder augmentation through enterocystoplasty, a procedure which involves the surgical removal of a section of large bowel that is then connected to the existing bladder to increase compliance, decrease pressure and improve overall urine capacity. These surgeries are relatively complex and costly. Even in patients with good technical outcomes, the procedure is associated with numerous immediate risks and potential chronic complications. A similar surgical procedure is performed in adults requiring bladder replacement, typically secondary to the onset of bladder-related malignancies. In this regard, cancer of the bladder may be manifested as a broad spectrum of disease presenting across distinct bladder compartments.

The most common types of bladder cancers originate from the internal epithelial lining of the bladder, the urothelium. Less common cancers of the bladder may include squamous cell carcinoma, adenocarcinoma, sarcoma

and small cell carcinoma. Estimates predict that almost 71,000 new cases of bladder cancer will be diagnosed annually in the USA, with some 14,000 patients succumbing to the disease.[1] Although many patients will have superficial urothelial tumors involving little or no metastasis within the smooth muscle layer, approximately 25% of bladder cancers will invade the detrusor musculature, with the majority of these presenting initially as invasive cancers with metastatic potential.

Invasive cancers often require multi-modality therapy which may involve extirpative surgery, i.e. radical cystectomy and construction of a urinary diversion. Despite the risk of adverse effects, there are approximately 10,000 of these procedures performed per year in the United States, including in 10% of children with congenital bladder abnormalities and 90% of adults with acquired disorders such as bladder cancer. In some cases of severe bladder cancer or other pelvic or abdominal cancers, removal of the entire bladder is indicated. In these circumstances, current standard of care also involves reconstruction of a bladder-like replacement using bowel tissue. Application of bowel tissue for reconstruction of urinary neo-organs is clearly problematic for a number of fundamental reasons, including the fact that bowel is a principally absorptive organ, whereas bladder is designed to store and excrete urine. Exposure of bowel tissue to urine or bladder tissue to bowel-derived micro-organisms has the potential to trigger multiple secondary complications. These may include any of the following:

- *Bowel complications*. Early complications are usually related to the bowel surgery required to harvest tissue for reconstructive use and typically consist of leaks, fistulas and obstructions. Because vitamin B12 is absorbed in the bowel tissue, loss of this tissue can result in anemia and neurologic abnormalities. Additionally, mal-absorption of salts and lipids can lead to diarrhea. Patients with neurogenic bladder are prone to bowel movement problems even before surgery, and the removal of bowel tissue may either exacerbate existing conditions or create new motility problems. These difficulties further contribute to substantial physical and psychological morbidity within these patients.
- *Absorption issues*. Use of bowel tissue often leads to electrolyte and metabolic imbalances, which can cause bone loss. Furthermore, certain drugs taken by the patient may be reabsorbed by the implanted bowel tissue, potentially leading to unintended toxic levels of these substances within the bloodstream. Exposure of the intestinal surface to urine also results in the inappropriate absorption of ammonium, chloride and hydrogen ions as well as potassium loss, leading to chronic metabolic imbalances or abnormalities.

- *Infection*. Persistent and recurrent infections are typical in patients with bowel tissue reconstruction. Such infections may reach the kidney and become life-threatening. Additionally, bacteria normally found in bowel tissue can serve as a source of infection and septic complications when repositioned into the urinary tract.
- *Stone formation*. One of the consequences of persistent infection is the development of stones, typically composed of calcium oxalate, or in some cases uric acid.[2] Stones are hard masses which can cause pain, bleeding, obstruction of urine or infections.
- *Mucus*. Bowel tissue, when repositioned in the urinary tract, continues to secrete mucus. Mucus increases the risk of stone formation and the viscosity of urine, and in the case of bladder augments, may require bladder irrigation and more frequent catheterization.
- *Cancer*. Malignancy, although rare, is a well-recognized complication following bladder augmentation using bowel tissue (enterocystoplasty), as well as from other reconstructive surgeries that incorporate bowel segments into the genito-urinary tract.[3]

In addition to cancers and developmental abnormalities, patients may present with neurogenic bladder or dysfunctional bladder due to some form of neurologic disease or condition. Treatment may often require an augmentation of the bladder in order to relieve high pressures and incontinence. Current therapies for neurogenic bladder include medical management through a combination of medication and clean intermittent catheterization and, in advanced cases, surgery. Surgical procedures, such as bladder augmentation, are often considered when other medical and less-invasive treatments fail to adequately lower bladder pressure or reduce the frequency of incontinent episodes.

Ultimately, it is self-evident that the ideal unit of anastomosis for urinary-like tissue is other urinary-like tissue. However, the lack of such material has generally precluded the widespread use of this option. There is clearly a compelling medical need for an improved approach that would eliminate or at least substantially reduce the complications potentially associated with the current standard of care. To this end, identification of bladder-like materials that may be applied towards bladder reconstruction in place of bowel tissue has been attempted. Pilot experiments in 1917 to augment bladder in dogs used fascia;[4] since then, numerous other biomaterials candidates have been evaluated, including skin, bladder sub-mucosa, small intestinal sub-mucosa, omentum, dura and peritoneum.

Synthetic materials candidates have included polyvinyl sponge, Teflon, gelatin, collagen, vicryl and silicone. Failure to achieve successful outcomes with such biomaterials types may be attributed to physical or mechanical

failure, lack of biocompatibility, and the induction of fibrosis and scarring leading to contraction of the implant and reduction in effective volume over time. Unfortunately, of these candidates the best biomaterial mimicking the physical and mechanical properties of bladder tissue has been bowel. Thus, a clear and present need exists for additional, novel technology platforms.

4.2 TE/RM methodologies for bladder replacement and augmentation

TE/RM approaches offer an alternative, potentially superior methodology to the use of bowel tissue for urinary diversion or replacement. In this methodology, the patient's own cells would be sourced from a bladder biopsy (or other, alternate cell source, see below) and applied to an appropriate, degradable biomaterial scaffold to create a neo-organ or organ-like construct. Construct implantation within the patient and anastomosis to native components of the urinary system would lead to regeneration of functional, urinary-like neo-tissue capable of storing urine and mediating voiding of urine as needed in response to appropriate neuronal signaling. Such a cell/biomaterial construct would catalyze the regeneration of urinary-like neo-tissue matching the native, laminarly organized bladder wall histo-architecture composed of a luminal urothelial layer and multiple smooth muscle layers, appropriately vascularized and innervated (Figure 4.1). Regeneration of urinary-like neo-tissue is accompanied by progressive degradation of the biomaterial, such that a seamless transition is achieved between the degrading biomaterial and the regenerating urinary-like neo-tissue.

In preliminary experiments to demonstrate the formation of tissue-engineered urothelial-like structures in the rabbit, bladder-derived urothelial cells were used to seed meshes of non-woven poly-glycolic acid (PGA), which were subsequently implanted within the peritoneal cavity of athymic mice. Upon recovery, structures composed of degrading biopolymer lined with urothelial cells were observed.[5] In follow-up studies, combinations of bladder-derived smooth muscle cells (SMC) and urothelial cells were used to seed tubular-like structures composed of non-woven PGA mesh. Implantation of these constructs subcutaneously within athymic rabbits led to regeneration of urinary-like tubular organoids composed of urothelial cells lining a central lumen and surrounded by layers of SMC, as observed within native bladder tissue. Evidence of neo-vascularization was also noted.[6] These studies provided preliminary proof-of-concept to support the potential for *in vivo* regeneration of urinary-like neo-organs through implantation of cell-seeded, synthetic bio-polymeric scaffolds.

Although current strategies for the creation of bladder-like neo-organs principally use non-bladder cell sources, initial work on neo-bladder tissue engineering was dependent on patient-derived bladder biopsies as a source of urothelial and SMC. For this approach to be commercially viable, the expansion dynamics of cellular growth for both biopsy-derived urothelial and SMC populations must be established. Although SMC could be reliably expanded from small bladder biopsies without difficulty, the demonstration that a single biopsy-derived source of bladder urothelial cells could also be expanded to the numbers required for effective seeding of urinary neo-organs was critical for establishing the preliminary bioprocess potential of this methodology.[7] The alternative would involve multiple biopsy sampling to generate sufficient cell numbers for urinary neo-organ seeding, greatly decreasing the attractiveness of this technology for practical application in the clinic.

From a biomaterials perspective, the application of synthetic biopolymers such as PGA for the seeding of urothelial and bladder-derived SMC permitted development of modified polymers with continuously tunable physical and mechanical characteristics. In this regard, the temporal sequence of polymer hydrolysis may be manipulated by altering the nature and sequence of individual monomer units. In addition, coating by other polymers such as poly-lactic-*co*-glycolic acid (PLGA) may be applied to further fine-tune the physical properties of the biomaterial scaffold. Finally, the open, fibrous networked structure of the biomaterial (Figure 4.2)

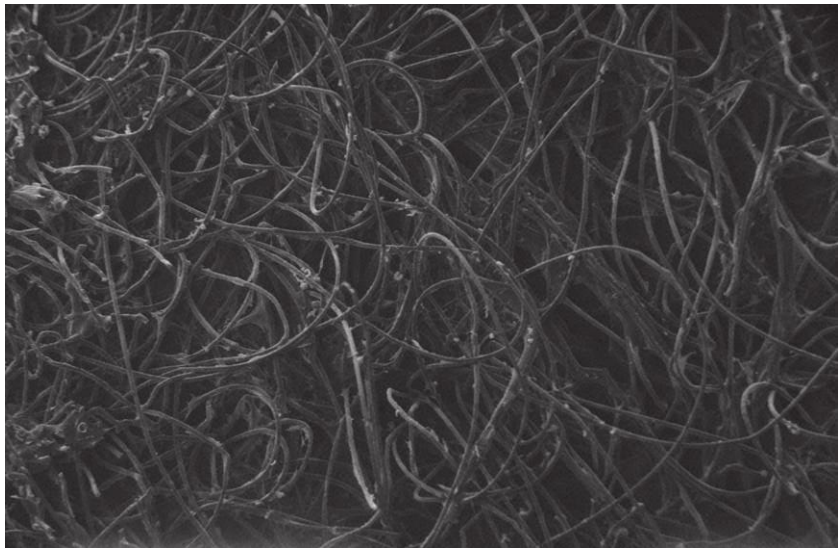

Figure 4.2 Low-magnification scanning electron micrograph of a SMC-seeded, PLGA-coated PGA mesh scaffold used for regeneration of urinary neo-organs.

facilitates angiogenesis and neo-vascularization of the developing neo-organ. Taken together, this binary cell/synthetic biopolymer construct was the foundational technology platform needed for initiating large animal clinical trials and proof-of-concept trials in humans.

4.3 Demonstration of Neo-Bladder formation in large animals

Subsequent to initial proof-of-concept studies showing *de novo* regeneration of urinary-like structures in a small animal model (rabbit), the next step in successful process development is evaluation of the technology in a large animal clinical model such as swine or dog. For the Neo-Bladder Replacement™, a key proof-of-concept study was provided using a canine total cystectomy (bladder removal) model. Fourteen beagles were sub-divided into three groups: group A ($n = 2$) underwent cystectomy without further intervention; group B ($n = 6$) underwent cystectomy followed by implantation with acellular, bladder-shaped biopolymer scaffold; and group C ($n = 6$) was subjected to cystectomy and implantation with cell-seeded biopolymeric scaffold. To create the constructs, PGA/PLGA biomaterial scaffolds (Figure 4.2) were shaped by hand into bladder-shaped structures. Urothelial cells and SMC were sourced from bladder biopsies and expanded separately to numbers sufficiently meaningful to warrant seeding onto the scaffolds (10^6 cells per cm^2 of polymer surface). Urothelial cells were used to seed the interior, luminal surface of the construct, and SMC were applied to the exterior, non-luminal surface.

Upon maturation, these constructs were implanted within group C animals. Implanted neo-bladder constructs were wrapped extensively with omentum during surgery, as omentum is a well-established source of pro-angiogenic growth factors.[8] All animals were monitored over a period of up to 11 months post-implantation and extensive urodynamic and radiographic measurements were taken to document urologic functionality. At the completion of the study period, animals were killed and bladder-like structures were examined histologically for evidence of tri-laminar bladder wall architecture from regenerated neo-organs. Structural and functional differences in regenerative outcomes were observed between all three groups of canines. Group A dogs regained only minimal urinary reservoir volumes without approaching pre-cystectomy parameters.

Group B dogs presented principally fibrotic neo-bladders with a regenerated luminal urothelial cell layer but minimal regeneration of bladder wall musculature. Urologic dynamics were also significantly affected. This ability of acellular constructs to regenerate a normal

luminal urothelial cell layer is noteworthy, providing a preliminary indication that prior seeding of constructs with urothelial cells may not in fact be a requirement to achieve full regeneration of native-like urinary neo-tissue from the implanted neo-organ construct. We will re-visit this observation and its implications later in this chapter. However, canines implanted with cell-seeded, bio-polymeric neo-bladder constructs were able to regenerate histologically and functionally appropriate neo-bladders with native-like tri-laminar wall histo-architecture and native-like urodynamics.[9]

It may also be useful at this point to clearly define functional outcomes that are commonly referred to as 'regenerative' versus 'reparative' in the context of TE/RM. Organ regeneration involves the replacement and restoration of cellular components and fully developed tissue mass as well as the reconstruction of the organizational, architectural and functional characteristics associated with the particular organ. In this regard, outcomes observed with group C canines above are principally regenerative. In contrast, a reparative outcome is associated with incomplete tissue replacement and may be distinguished by the extensive deposition of collagen and concomitant fibrosis. Outcomes observed with group B canines represent principally reparative pathways. This study and many others demonstrate clearly that regenerative outcomes are mediated by cell-seeded constructs only. Acellular constructs are unable to catalyze neo-organ regeneration. This is an important foundational principle for continued development of neo-organ regeneration technology.

Analysis of the dynamics of neo-bladder regeneration in subtotal cystectomized canines serves to further illustrate the dichotomy in outcomes between implantation of acellular and cellularized scaffolds. In another such study, bladder-shaped scaffolds composed of woven PGA felt or PLGA seeded with autologously sourced bladder-derived urothelial cells and SMC facilitated a regenerative response within one month post-implantation, as characterized by induction of an extensively vascularized, smooth muscle-like parenchyma. In contrast, acellular PGA/PLGA scaffolds triggered a principally fibrotic, reparative outcome featuring disorganized collagen fibers with minimal vascularization. Baseline urodynamics were recovered within four months post-implantation with cell-seeded scaffold, whereas urodynamic profiles of animals implanted with acellular scaffolds remained abnormal throughout the nine-month study.[10]

In a related cystectomized canine study, native-like tri-laminar bladder wall tissue architecture was observed at three months post-implantation with a bladder-shaped non-woven PGA felt scaffold seeded with 1.5×10^8 each of

autologously sourced bladder-derived urothelial cells and SMC, and normal compliance characteristics of a urinary bladder had developed by 12 months. Regenerated bladders in animals receiving these cell-seeded scaffolds have shown functional and structural stability for up to two years post-implantation. Importantly, although the volume of the cell-seeded scaffold was held constant, implantation of the construct within dogs of different sizes that had gained varying amounts of weight over the course of the study yielded organs that, as measured by the ratio of bladder capacity to body weight, adapted to the individual recipient animal's size, demonstrating that the regenerated neo-organ was capable of responding to homeostatic mechanisms regulating organ volume.[11]

Key results from these pre-clinical canine studies may be summarized as follows:

(1) Cell/scaffold combination constructs are a foundational technology platform for regenerating hollow organs

- Regeneration of a complex hollow organ with a cell-seeded biomaterial scaffold occurs in an ordered sequence: degradation of the scaffold occurs with associated angiogenesis, histogenesis and recovery of organ-specific structural organization prior to final, functional maturation.
- The regenerated neo-organ will adapt to growth of the host animal in a native-like manner.
- A neo-organ construct can regenerate a functional bladder within growing canines.

(2) Delivery of committed cell types within a biomaterial scaffold triggers regeneration of native-like functional urinary tissue

- Urothelial cells and SMC derived from normal bladder biopsies can regenerate native-like urinary tissue upon implantation within a large animal complexed with biodegradable polymers.
- Upon implantation within cystectomized canines, the cell/biomaterial construct permitted development of the detrusor muscle and stroma such that the regenerated bladder presented native-like visco-elastic mechanical properties and compliance.

(3) Bladder regeneration is a paradigm for directed organogenesis

- Tri-laminar histo-architecture was observed from regenerated neo-bladder within three months post-implantation in cystectomized canines. Functional capacity was observed within six months

post-implantation and normal compliance characteristics of native bladder by 12 months post-implantation.
- Regenerated neo-bladders were stable within canines for 24 months post-implantation.
- Despite the volume of the neo-bladder construct being constant at the time of implantation, upon introduction within different sized canines, the regenerated neo-bladder's volume adapted to that of the host animal, implying that the regenerated neo-organ is regulatable by normal homeostatic feedback mechanisms controlling organ size.

4.4 Can neo-bladder constructs be made from cells sourced from diseased patients?

Prior to initiation of clinical trials in human patients, it is critical to demonstrate that cells sourced from diseased patients (representative of the patient pool being targeted by the potential neo-organ product) may be used to seed the biomaterial component of the neo-organ construct, and that the resultant composite may mediate regeneration of a functional neo-organ as effectively as cells sourced from a healthy individual. In a study specifically designed to investigate this issue, SMC were sourced from normal, exstrophic and neurogenic bladders and evaluated *in vitro* (cell culture) and *in vivo* (as cell-seeded polymers implanted subcutaneously within athymic mice). Expression of smooth muscle-associated markers was found to be normal from diseased sourced bladders in both circumstances. Tissue-engineered musculature generated by constructs containing cells derived from diseased bladders showed the expected contraction–relaxation responses to electrical stimulation. Taken together, these data suggested that cells sourced from likely clinical candidates for neo-bladder replacement could be used successfully for induction of urinary-like neo-organ formation.[12]

4.5 Neo-bladder replacement in human pediatric patients – first clinical trials of a neo-organ

Studies in canines as outlined above established proof-of-concept for the application of cell-seeded biodegradable polymeric scaffolds for regeneration of functional, native-like neo-bladders in a large animal cystectomy model.[9] Additional data suggested that SMC sourced from diseased bladder could potentially be applied successfully to regenerate neo-urinary tissue.[12] These data

laid the groundwork for initiation of a proof-of-concept clinical trial in humans. In this seminal study, seven pediatric patients presenting with myelomeningocele (a form of spina bifida) were recruited to receive the first ever human neo-organ implants. As previously described in canines, both urothelial cells and SMC were isolated and expanded from autologously sourced bladder biopsies.

Up to five cell passages over 7–8 weeks were required to generate enough cells to seed the neo-bladder scaffold. Using a sterile pipette, the scaffold exterior was seeded with bladder-derived SMC at a seeding density of 5×10^7 cells/cm^3. After a 48-h recovery period, the luminal surface of the scaffold was seeded with urothelial cells at a density of 5×10^7 cells/cm^3. The construct was matured in a tissue culture incubator at 37°C for 3–4 days, prior to implantation. Subsequent to implantation, the engineered neo-bladder was cycled (i.e. subjected to serial volume expansion and contraction) as part of regular post-operative care for up to three weeks; the mechanical forces induced across the neo-bladder during cycling were found to augment regenerative outcomes. Engineered neo-bladders were found to functionally rescue urologic dynamics and were associated with tri-laminar bladder wall architectures upon histological examination of bladder biopsies recovered at 31 months post-implantation. A number of different scaffold iterations were evaluated, with changes being made over the course of the study to accommodate new data being made available from this and other related studies. Ultimately, an omentum-wrapped, collagen/PGA scaffold was found to present best overall regenerative outcomes (Figure 4.3).[13]

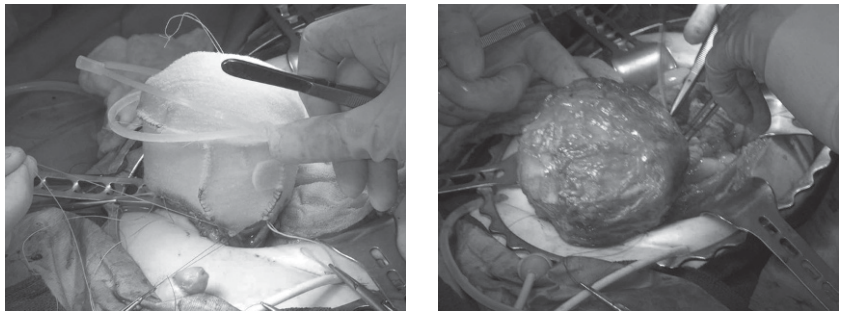

Figure 4.3 Left, implantation of Tengion's Neo-Bladder Augment™ at dome of native bladder during augmentation cystoplasty in a patient presenting with neurogenic bladder secondary to spina bifida. Right, wrapping of omentum around Neo-Bladder Augment™ to facilitate vascularization of regenerative construct.

4.6 Making the product: cell sourcing

The studies outlined above served as proof-of-concept examples from small animals to humans to demonstrate that native-like urinary neo-tissue can be regenerated from implantation of cell-seeded, biodegradable polymeric constructs. To create a viable *product* candidate, however, a number of modifications must be made to the foundational methodology that serve to streamline the overall process, thereby decreasing time to delivery and cost of goods associated with the final product. To this end, we have considered the following points:

(1) One cell type or two? Both autologously sourced bladder urothelial cells and SMC were applied for construct seeding in all preliminary proof-of-concept studies. At first glance, this appears reasonable. After all, if the construct to be implanted already mimics native-like urinary tissue as closely as possible, surely the regenerative process will be more efficient? However, implantation of acellular biopolymeric constructs within cystectomized canines is associated with complete regeneration of the urothelial cell layer,[10,11] suggesting that prior seeding of the luminal surface of the construct with urothelial cells may not in fact be a requirement for successful neo-organ regeneration. Switching from a binary cell system to a single cell platform has the potential to considerably simplify bio-processing, time to market and cost of goods. Based on data generated through evaluation of the Neo-Urinary Conduit™ (see Chapter 5), we were able to demonstrate that SMC alone are sufficient to catalyze the regeneration of native-like urinary tissue. Migration of urothelial cells from the ureters is presumed to be adequate to mediate regeneration of the urothelial layer.

(2) Bladder-derived or alternate cell source? SMC may be derived from the patient's own tissue, including the bladder, urethra, ureter and other urogenital tissue. However, there are challenges associated with dependence upon the development and maintenance of cell culture systems from the primary organ site as the basic unit for developing new and healthy engineered tissues, as for example during treatment of cancerous bladder tissue. Clearly, such cancerous cells are inappropriate for populating an implantable neo-bladder scaffold or matrix. Although the majority of bladder cancers are cancers of the urothelium, methodologies for isolation, harvesting and expansion of SMC derived from bladder biopsies are still subject to the risk of contamination by cancerous urothelial cells.

One approach to dealing with this issue is to develop assays capable of detecting cancerous cells at limits of at least one cancerous urothelial cell within 10^6 non-cancerous cells. In this regard, we have developed a novel methodology based on magnetic enrichment of sampled cell populations

for CD326, a marker known to be up-regulated in cancerous urothelial cells. Prior enrichment of sampled cell populations by magnetic activated cell sorting with antibodies specific to CD326 followed by RT-PCR analysis of RNA derived from the cell population with primers specific to CK-5, another marker specifically associated with cancerous urothelial cell lines, allowed us to detect cancerous urothelial cells at the required detection limit of $1/10^6$.[14]

These data notwithstanding, we have attempted to identify and characterize alternative sources of SMC capable of reconstituting urologic characteristics *de novo* upon implantation on appropriate synthetic, biopolymer scaffold constructs, and to demonstrate functionality *in vivo*. Potential sources should present the following characteristics to facilitate bioprocess development:

1. Sources should be easily accessible through minimally invasive clinical procedures.
2. SMC should be isolatable reliably and reproducibly, regardless of donor age, sex or secondary health conditions.
3. The source should be isolatable at sufficient mass to permit adequate numbers of SMC to be generated with minimal expansion in cell culture.
4. Finally, the source should contain fully committed or at least partially committed smooth muscle-like cells capable of being expanded without application of recombinant growth factors or other agents for directed differentiation of smooth muscle-associated phenotypic characteristics. This significantly reduces costs-of-goods associated with cell sourcing.

With these criteria in mind, we selected peripheral blood and adipose tissue as two potential sources of SMC suitable for bioprocess development. Peripheral blood is ideal – cells with smooth muscle-like characteristics have been reported to be isolatable from human peripheral blood.[15,16] Isolation of peripheral blood is a straightforward and minimally invasive clinical procedure that is eminently suitable for incorporation into patient-specific TE/RM products. However, as we shall see when we discuss the Neo-Urinary Conduit™ (NUC), we observed that SMC could only be isolated sporadically from human peripheral blood. Interestingly, isolation of SMC from porcine or canine peripheral blood was observed to be reliable and reproducible across multiple independent samples, providing a lesson in the dangers of extrapolating animal data (even from large animal clinical models such as swine or dog) to the human condition.

Although the isolation of adipose tissue is somewhat more invasive than isolation of peripheral blood, it is nevertheless still acceptable for widespread clinical application. We observed that unlike peripheral blood, human-sourced adipose tissue could be used reliably and reproducibly for isolation of SMC, independently of donor age, sex or disease status. However, from a product development and intellectual property perspective, use of adipose tissue is complicated by the fact that the stromal vascular fraction (SVF) of adipose tissue used to source SMC is also an established source of stem and progenitor cells with mesenchymal stem cell (MSC)-like bioactivity. Therefore, clear definition of the cell source was required to establish unambiguous distinctions between adipose-derived SMC and MSC.[17]

4.7 Definition of the cell source used for seeding neo-bladders: adipose

Adipose tissue contains a heterogeneous mix of cell types, including adipocytes, endothelial cells, pericytes, SMC and MSC. Adherent cells that were derived from adipose tissue using different conditions have been referred to as MSC without applying a systematic approach to defining the isolated cell composition; however, distinctly different cell types can share phenotypic characteristics. As more cell-based therapies move from preclinical to clinical evaluation, establishing a cell composition profile that provides a degree of distinction from other cell types will probably become increasingly important for protecting intellectual property rights, gaining regulatory approval and scaling up for manufacturing. The relevance of cell type characteristics defined from analysis of native tissue or initial cell isolates to expanded cell populations is unclear because gene and protein expression patterns can be altered by isolation and *in vitro* expansion. Such differences have been documented for MSC and we have observed the same to be true for adipose-sourced smooth muscle-like cells (Ad-SMC).

The more important questions from a product development perspective are what characteristics are associated with the cell population that will be administered and how indicative are those characteristics with regard to cell identity and product potency. Our comparison of Ad-SMC and MSC used established definitions of SMC and MSC and focused on the analysis of smooth muscle phenotype, growth kinetics, *in vitro* tri-lineage differentiation bioactivity and functional responses to small molecules that affect SMC-specific signaling pathways. Unlike methodologies for MSC, isolation and expansion of Ad-SMC are specifically promoted by growth of adipose

SVF-derived cells at low cell densities in the *absence* of positive/negative selection through magnetic bead-based separation, inductive cytokines or growth factors, high serum (>10%) concentrations or preselected lots of serum. The specific influences of media formulations on isolation and expansion of cells with MSC-like bioactivity have been extensively documented.[17] Expansion of Ad-SMC is therefore the *default* outcome from culture of SVF-derived cell populations in principally basal media formulations at high cell densities not supplemented by inductive cytokines, preselected lots of serum or high concentrations of fetal bovine serum. This property greatly facilitates process development and manufacturing.

From where do Ad-SMC originate and what is their relationship to MSC? Adipose is a heavily vascularized tissue and a number of studies have implicated the peri-vascular niche as a potential source of MSC as well as smooth muscle and endothelial cells. Pericytes with MSC differentiation potential have been isolated directly from blood vessels as well as from multiple organ systems throughout the body. However, although ACTA2/SMAA+ cells have been localized to all capillaries, arterioles and venules of the adipose-derived vascular bed, expression of STRO-1, a key MSC-specific marker, is closely associated with endothelium and additionally found only within a subset of blood vessels. Furthermore, expression of the stem cell-specific markers Oct4 and telomerase was observed only rarely, suggesting that truly pluripotent progenitors are uncommon within adipose.

In their entirety, these observations point to MSC, endothelium and smooth muscle occupying distinct spaces within the broader peri-vascular niche. Nevertheless, there remains the potential for considerable ebb and flow across developmental lineages. For example, endothelial cells appear to be capable of lineage switching towards an SMC phenotype in response to TGF-β or the depletion of pro-angiogenic factors and loss of endothelial cell–cell contact. In addition, adherent cell types with endothelial and smooth muscle phenotypes as well as limited mesenchymal differentiation potential have been identified to circulate in adult peripheral blood. Such circulating SMC may contribute to the population of adipose-derived smooth muscle cells, although we have been unable to purify them directly from human adult peripheral blood in meaningful numbers (our unpublished observations).

Given that MSC in long-term culture also follow an SMC differentiation pathway, we believe that, taken together, the published data as well as our observations are consistent with the peri-vascular niche of adipose SVF as a source for a broad continuum of SMC, smooth muscle progenitors, MSC and partially lineage-committed MSC-like cell types with variable and overlapping degrees of proliferative and differentiation potential.

Modulation of SVF-derived cell phenotypes and functionality away from MSC and towards SMC is possible through selection of media formulations during cell isolation and expansion. Our studies provide methodological validation for the utility of non-bladder sources of SMC for applications in urologic regenerative medicine, thereby bypassing the potential for isolation and expansion of transformed SMC associated with bladder biopsies derived from patients presenting with bladder-related malignancies.

4.8 Other approaches to tissue engineering neo-bladders

The sequential process of development for the SMC-seeded/biodegradable synthetic biopolymer scaffold neo-bladder regenerative platform, as outlined above, represents by far the most clinically advanced neo-organ product candidate developed to date. No other methodology for bladder regeneration has even come close to clinical trials in humans or to commercial manufacture. However, it is worthwhile critically examining some alternative approaches that have been presented in the literature and discussing why these have proven to be ultimately unsuitable for commercialization.

From a biomaterials perspective, we have seen in Chapter 3 why the application of synthetic, biodegradable polymers is preferable for industry. Briefly, such biomaterials may be sourced or manufactured with defined chemical composition and offer reliable and reproducible physical and mechanical properties that are continuously tunable by modification of basic polymer chemistries. In contrast, naturally sourced biomaterials such as bladder-derived sub-mucosa present variable chemical compositions that are donor specific, and may not be readily sourced, as is the case with human bladder-derived sub-mucosa.

This has led to the use of xenogeneically sourced biomaterials as substitute. Notwithstanding the fact that porcine-derived intestinal sub-mucosa is an established product for multiple surgical applications, scaffolds made of decellularized bladder sub-mucosa were actually evaluated in the first human neo-bladder clinical trials against scaffolds composed of principally synthetic polymers.[13] However, the most favorable regenerative outcomes were generated from implanted neo-bladders composed principally of PGA, whereas the least favorable regenerative outcomes were observed within patients implanted with neo-bladder constructs composed of decellularized bladder sub-mucosa. The course of the clinical trial was appropriately modified to incorporate these findings. Taken together, these observations reinforce the application of synthetic polymers over naturally sourced biomaterials for commercial viability.

From a cell sourcing perspective, a number of investigators are actively exploring the utility of SMC derived by the directed differentiation of MSC isolated from bone marrow or adipose. A key characteristic of such stem and progenitor cell populations is a requirement for exposure to combinations of exogenous growth factors, extracellular matrix components or other defined factors to drive differentiation along defined developmental lineages. For example, in one recent report, adipose-derived MSC were differentiated into SMC using inductive media containing 100 U/ml heparin for up to six weeks prior to seeding polymeric bladder dome-like scaffold structures that demonstrated some evidence of functionality in a rat cystectomy model.[18]

In related studies, TGF-β or small molecule agonists targeting the TGF-β signaling pathway (e.g. sphingosylphosphorylcholine, bradykinin and angiotensin II) have also been used to induce a smooth muscle-like phenotype from adipose or bone marrow-derived MSC.[19–22] In a less targeted approach, epigenomic reprogramming with the DNA demethylating agent 5-azaC has been used to direct bone marrow-derived MSC towards a cardiomyocyte-like phenotype.[23] Dedifferentiated adipocytes may also be driven along a smooth muscle lineage using TGF-β and have been reported to contribute towards bladder tissue regeneration in a mouse bladder injury model.[24] Finally, methods for TGF-β-induced differentiation of SMC from bone marrow-derived cells have been described.[25,26] These studies invariably involve demonstrations of *in vitro* directed differentiation of SMC-like cells derived from MSC, using various combinations of growth factors and cytokines over an extended time period. Any *in vivo* data are typically from small animals, are often subcutaneous and therefore are of little relevance to product development and manufacturing.

Finally, the use of the peritoneal cavity as a living bioreactor for maturation of tubular organs has been proposed. It is well established that implantation of foreign material into the peritoneal cavity may frequently trigger a fibrotic response, with encapsulation of the foreign matter by multiple layers of fibroblasts. We have observed similar, non-regenerative outcomes associated with implantation of certain synthetic biopolymers such as poly-caprolactone within the renal parenchyma.[27] However, this normally unwelcome outcome may be used for induction of tubular neo-tissue formation.

In the first such demonstration of this approach, silastic tubing was implanted into the peritoneal cavity of rats/rabbits. At two weeks post-implantation, the tube was observed to be coated with several layers of myofibroblasts, collagen and a layer of mesothelium. Removal of the silastic tube followed by eversion of the cellular construct resulted in formation of a vessel-like structure, with laminar wall architecture mimicking that of native vasculature. Anastomosis of this neo-vessel with

the vasculature of the original host animal was associated with evidence of function and patency for up to four months post-implantation.[28]

Similarly, neo-organs resembling bladder, uterus and vas deferens could be engineered by grafting biomaterials templates of appropriate shape within the peritoneal cavity of rabbits. After 2–3 weeks of implantation within the environment of this 'living bio-reactor', neo-organ-like frameworks composed of myofibroblastic layers could be harvested and grafted on to the appropriate organs of the original host to demonstrate functionality and eventual regeneration of urinary-like tissue.[29] Although interesting, it is difficult to envision how such a strategy can ever be feasible in practice. Clearly, undergoing major surgery and all associated post-operative care and monitoring simply to create the neo-organ implant will not be an attractive option, given the alternatives currently under development. Therefore, we do not anticipate that tissue engineering of bladder using the peritoneal cavity as a living bioreactor will become a commercially viable strategy.

4.9 Key results from development of the Neo-Bladder: factors facilitating commercial viability of an organ regeneration platform

The Neo-Bladder experience at Tengion highlights certain key factors that may impact the commercial success of an organ regeneration platform. These same principles are pertinent to discussion of non-bladder tubular organs as well as to regenerative technologies targeting solid organs such as kidney, liver and pancreas, which we will examine in a later chapter. We outline these principles below:

1. A biodegradable, synthetic scaffold based on well-characterized biomaterials (e.g. PGA, PLGA) that can be manufactured reproducibly with defined characteristics is desirable.
2. A population of committed cell types (e.g. SMC) that is easily isolatable and expandable is required for scaffold seeding to trigger regeneration *in vivo*.
3. A purified population of defined stem cells is neither needed nor desirable. Cost of goods increases significantly with requirements to monitor stem cell potential and to control directed differentiation.
4. Engineering of an entire organ *in vitro* or within the peritoneal cavity is neither needed nor desirable. Instead, an *in vitro* cell-seeded scaffold is adequate to trigger the innate regenerative response in the mammalian body, resulting in *de novo* organogenesis *in vivo*, including in humans.

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5

Neo-Urinary Conduit™

Abstract. The Neo-Urinary Conduit™ is a tubular neo-organ composed of native-like urinary tissue mediating efflux of urine from the body in patients requiring urinary diversion. Its development from concept to clinical trials is presented here as an illustrative example highlighting issues common to bioprocess development of novel TE/RM products based on cell/biomaterial composite constructs.

Keywords: scaffold, smooth muscle cell, regeneration, tissue engineering, urinary conduit, urinary diversion, biomaterial, neo-organ

5.1 Introduction

During efforts to transition the Neo-Bladder Replacement™ (Chapter 4) through the clinical trials process, alternative strategies for mediating efflux of urine from the body were considered. For example, current surgical treatment options for many urinary disorders caused by congenital conditions, injury or cancer involve the application of gastrointestinal (GI) tissue to create an orthotopic neo-bladder for urinary diversion. From a surgical perspective, there are three principal categories of urinary diversion:[1]

- *Ileal conduit urinary diversion*. Here, a portion of the ileum is surgically ligated to the ureters at one end, and opened through the abdominal wall via a stoma at the other end. The stoma may then be covered with a colostomy bag to receive the urine.
- *Indiana pouch reservoir.* In this approach, a portion of the large intestine is molded into a reservoir and the ureters are positioned to drain directly into this reservoir. Urine may then drain through a small stoma in the abdominal wall via a segment of small intestine.

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- *Neo-bladder to urethra diversion.* With this methodology, a portion of the small intestine is modified to form a pouch into which the ureters can drain. Urine may now pass from the kidneys through the small intestine-based neo-bladder and efflux the body via the urethra as normal, although occasional catherization may be required.

This established standard of care requires the resection and manipulation of GI tissue, consequently exposing the principally absorptive GI tissue to urine. This has the potential to considerably increase chances of infection and additional acute and chronic complications. Ideally, urinary diversion would be possible without the use of autologously sourced GI tissue – a TE/RM approach might provide one possible solution.

The possibility of creating a TE/RM product facilitating urinary diversion without using native GI tissue was the rationale underlying the development of the Neo-Urinary Conduit™ (NUC). In its simplest configuration, the NUC is a cell/scaffold construct that, upon implantation within the body and attachment to native ureters, mediates efflux of urine from the kidneys directly to the external surface of the body. As with the Neo-Bladder Replacement™ and related TE/RM products, the NUC construct serves as a template to catalyze the regeneration of native-like urinary tissue concomitant to degradation of the biomaterial scaffold following implantation.

This raises an interesting question. The NUC is the first of an entirely novel category of neo-organs: one designed entirely by scientists to fulfill specific functional roles largely not required in healthy individuals, in this case bypass of the bladder and direct efflux of urine from the body. Yet the expectation remains that the body will not only accept the novel neo-organ but also catalyze the regenerative process in accordance with the desired functional role, such that native-like urinary tissue is modeled within the degrading NUC scaffold, instead of, for example, retinal tissue. Studies in large animal clinical models provide evidence that this expectation is not unreasonable, opening the door to the design and engineering of entirely novel neo-organs for specific patient applications that have no counterpart in healthy individuals.

5.2 Assembly of the NUC

Design and assembly of the NUC reflects many of the principles we have discussed in earlier chapters. The NUC is assembled from two principal components:

- *Biomaterials*. The NUC scaffold is composed of poly-glycolic acid (PGA) polymer mesh fashioned into the required tubular shape and coated with a 50/50 blend of poly-lactic-*co*-glycolic acid (PLGA) co-polymer. The NUC is configured into a tapered, tubular structure using an automated engineering workstation (Figure 5.1). Specific structural parameters may be modified as needed. The choice of well-established, synthetic, degradable biopolymers reflects the same requirements for reliability and reproducibility inherent in the choice of these polymers for applications in other bladder-related neo-organs.
- *Cells*. Autologous smooth muscle cells (SMC) sourced from adipose tissue, peripheral blood or bladder tissue may potentially be applied for construction of an NUC. As with the Neo-Bladder Replacement™, adipose is the tissue of choice for isolation of SMC, owing to the reliable and straightforward manner in which SMC may be isolated and expanded from a clinically secured adipose sample.[2]

A more detailed examination of the NUC production process provides an excellent illustration of elements salient to manufacture, quality control, transport and delivery of a TE/RM product. To this end, the clinical manufacturing process for the autologous NUC construct contains the following key elements:

1. Adipose tissue collection and handling
2. Isolation and expansion of SMC
3. Preparation of the tube-shaped scaffold
4. Cell seeding of the scaffold
5. Maturation of the construct
6. Stability of the NUC construct

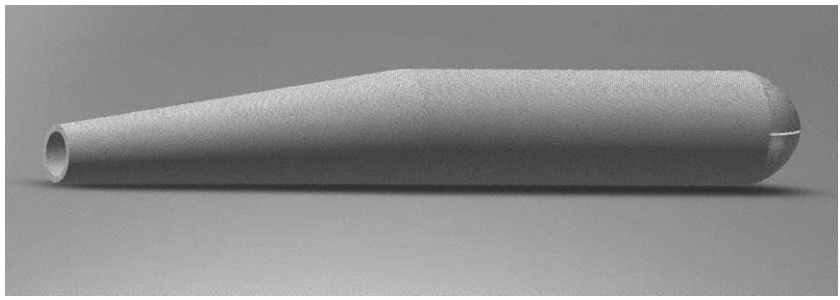

Figure 5.1 Tengion's Neo-Urinary Conduit™ scaffold, formed from non-woven PGA felt coated with PLGA. Total length 14.5 cm.

7. Testing and release of the final construct
8. Packaging and transport of the construct.

We will examine each of these steps briefly.

5.2.1 Adipose tissue collection and handling

A Tissue Shipper box is sent out to the collection site, i.e. the clinic where the patient receiving the NUC is being prepared for surgery. Adipose tissue is collected from the patient either by subcutaneous biopsy or through liposuction, and transported via the Tissue Shipper box at ambient temperature to Tengion's central processing unit. Upon receipt of the tissue, patient-specific ID and collection site ID are assigned. A sample of the tissue transport medium is collected to track the incoming bio-burden load, as well as to assess the presence of mycoplasma, endotoxins and overall sterility. The tissue is weighed to calculate potential SMC yields.

5.2.2 Isolation and expansion of SMC

Protocols for isolation of SMC from adipose have been presented in detail in the literature and are therefore only briefly outlined here.[2] The adipose sample is digested extensively with collagenase and centrifuged to isolate the stromal vascular fraction (SVF). Plating of the SVF under defined conditions of low cell density and absence of inductive cytokines or growth factors permits the expansion of a specific population of committed cell types best defined as smooth muscle-like cells.[2]

For the purposes of bioprocessing, however, the key production controls for cell culture media and associated ancillary materials, such as trypsin, fetal bovine serum (FBS) and DMSO (dimethyl sulfoxide), include use of qualified sources (see Chapter 10) from certified vendors and dilution of any animal-derived components (principally FBS) from the final product by extensive washing. Simple calculations may then be applied to demonstrate that the residual amounts of any such animal-sourced components are vanishingly small.

Given that the final adipose sample from any given patient will be unique, it is important to standardize the cell isolation and expansion protocol as far as possible to understand the key parameters affecting the final yield and quality of SMC. We have established how variation in initial

sample size affects cell yields as well as time in culture required to reach the minimum cell numbers needed to effectively seed the NUC scaffold. As far as possible, this SMC expansion step is performed within a closed system. SMC are expanded in T-flasks and Cell Factories by serial passaging. Four to six such passages are typically required to generate the $4–7 \times 10^7$ SMC needed for scaffold seeding. Testing for sterility and endotoxins is performed at passage one (P1) and three (P3).

Once SMC have been expanded to appropriate numbers, they may be functionally evaluated for suitability for incorporation within an NUC construct by analysis of the following parameters:

- *SMC morphology*. Healthy SMC in culture present a well-established flattened, spindle-like, fibroblastic morphology, with characteristic 'hill-and-valley' organization (Figure 5.2).
- *SMC phenotype*. Expression of the key SMC-associated marker smooth muscle alpha-actin (SMAA) is used as a quality control marker to confirm SMC phenotype.
- *MCP-1*. The cytokine MCP-1 (monocyte chemoattractant protein-1) is associated with regeneration and is known to signal native stem and progenitor cell populations.[3] Expression of MCP-1 from SMC is thus used as a functional assay to confirm the regenerative potential of SMC prior to scaffold seeding.
- *Cell viability*. Measurements of cell viability with passage are applied as a key production control.

5.2.3 Preparation of the scaffold

Biomaterial components of the NUC scaffold may include: PGA fibers, PGA mesh, PLGA, and vicryl sutures. For these materials, certificates of

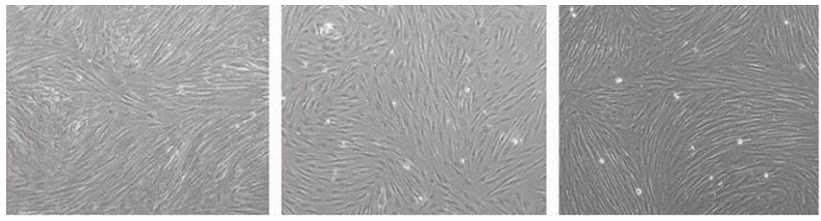

Figure 5.2 Left to right: bladder SMC, adipose-derived SMC and peripheral blood-derived SMC. 10x magnification.

analysis (COA) are typically provided by the manufacturer. Such COA are required to be filed to provide evidence that the mechanical and physical properties of the biomaterials are within established parameters. PGA non-woven mesh is secured in sheets. Sheets may be die-cut and formed into the appropriate tubular shape by suturing, using PGA/PLGA-based sutures. Visual inspection of the physical integrity of the structure, including sutures, knots, loops and seams, is applied as a production control (Figure 5.1). The shaped, tubular scaffolds are then coated with a 50/50 PGA/PLGA solution and air dried, then subsequently stored within a vacuum dessicator. Coating alters the surface area of biomaterial associated with individual polymer fibers, increasing surface area for SMC attachment and proliferation (Figure 5.3). Finally, coated scaffolds are sterilized by exposure to ethanol or ethylene oxide. Production controls include defined weight of the scaffold and visual inspection of the physical integrity of the scaffold structure.

Evaluation of scaffold structural integrity is also performed by scanning electron microscopy of the biomaterial micro-structure. Provided the scaffold integrity is acceptable, expiration data for sterilized scaffolds may be derived based on mechanical testing of experimental scaffolds stored within a vacuum dessicator for up to 12 months. Finally, after extensive washing, measurement of residual levels of chemicals used in the scaffold

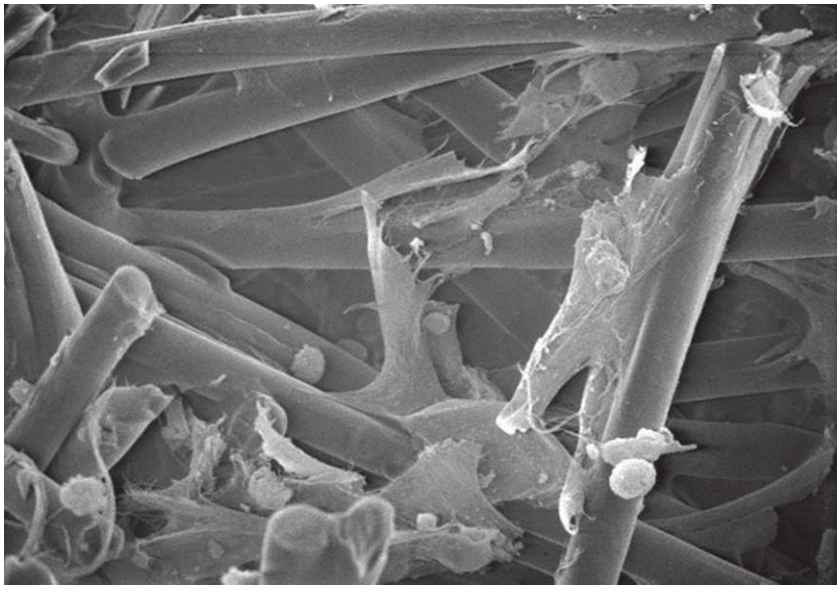

Figure 5.3 High-magnification scanning electron micrograph of SMC-seeded PGA scaffold showing bridging of scaffold fibers by SMC. 500x magnification.

preparation process, such as methylene chloride, is performed. Such residual levels are typically well within guidelines acceptable to the Food and Drug Administration (FDA).

5.2.4 Cell seeding of the scaffold

Seeding of the biomaterial scaffold with adipose-derived SMC is performed within a closed, custom-fabricated bioreactor (Figure 5.4). In preparation for seeding, the sterilized bioreactor containing the scaffold is removed from its vapor barrier bag. The scaffold is then pre-conditioned with ethanol to pre-wet the biomaterial. Residual ethanol is then washed out with phosphate-buffered saline (PBS), and the scaffold is soaked within the bioreactor with SMC growth medium for at least 15 min.

A key production control at this stage is testing of the preconditioning medium for sterility and the presence of endotoxins. Once sterility of the biomaterial has been confirmed, seeding can take place. The objective at this stage is to maintain a closed process as far as possible. To this end,

Figure 5.4 Bioreactor (no media) with NUC scaffold

SMC are pumped into a closed media bag, and the cell suspension is delivered into the bioreactor using gravity. Seeding of the scaffold is accomplished by rotating the bioreactor to facilitate maximum cell attachment. Key production controls at this stage include confirmation of SMC identity through evaluation of cellular morphology, phenotype and functionality, assessment of cell numbers and viability, and analysis of media sterility and mycoplasma and endotoxin levels.

5.2.5 Maturation of scaffold

The SMC-seeded scaffold is incubated within the bioreactor for 37 °C in a 5% CO_2 environment for 5 days to facilitate expansion of the SMC population. NUC growth media may be exchanged as needed. Key production controls include monitoring of salient metabolites such as glucose and lactate as well as assessment of the secreted protein MCP-1, a marker of the regenerative potential of the SMC population. The SMC-seeded biomaterial scaffold is referred to as a 'construct'. Taken together, the central theme emerging here is the application of a multi-faceted approach to characterization of the final construct. This involves:

- *Characterization of the SMC population during seeding of the construct*. Cells are evaluated prior to seeding by sampling the cell culture growth media for assessment of sterility, and mycoplasma and endotoxin levels. Cell viability is required to be at least 70%. Morphology of the SMC population is confirmed by comparison of images with an in-house library of images of established SMC populations. Phenotype of the SMC population is confirmed by demonstrating expression of SMAA. Finally, cell numbers used to produce the construct are verified to be within the defined range.
- *Evaluation of the physical integrity of the construct at time of maturity.* At the end of the maturation period, the construct is evaluated by assessing changes in physical integrity, performed by visual comparison of the construct against images of the scaffold taken prior to cell seeding. Criteria for evaluation can include scaffold shape and general appearance.
- *Monitoring of cellular metabolic activity at time of construct maturation.* During maturation, the metabolic and functional activity of SMC is assessed by analysis of glucose uptake, production of lactate and expression of the secreted regenerative marker MCP-1.

- *Verification of the physical integrity and cellular composition of constructs at maturity through destructive testing*. Visualization of viable cells inside the construct is performed by live/dead staining. Quantitation of the viable SMC population is executed by assaying levels of the metabolic enzyme lactate dehydrogenase.

5.2.6 Stability of the NUC construct

To evaluate how long the final NUC construct may be stored yet still retain functionality, NUC constructs are kept on the bench-top for an extended hold period at room temperature. Cell stability is then monitored by evaluation of the recovery of metabolic and functional indices, including glucose uptake and production of lactate and MCP-1 to levels similar to that prior to initiation of the storage period. Scaffold stability is assessed through measurement of the tensile strength and suture pull-out strength associated with the scaffold after the extended hold time.

5.2.7 Testing and release of the NUC construct

The final decision to release an NUC construct for implantation is based upon a combination of test results established by guidance documents published by the FDA for cell/biomaterial combination products. These include the in-process testing during SMC growth and NUC construct maturation discussed above as well as testing for release of the final product. A summary of the in-process testing is presented in Table 5.1.

Final product release will be based upon the product meeting acceptance criteria for all in-process testing results and the following final construct release elements:

- *Release testing on cellular components*. Cell culture media associated with the matured construct are sampled for evaluation of sterility, and the presence of mycoplasma and endotoxins. The SMC-like phenotype and functionality of cells is confirmed through previously outlined assays. Cell numbers and viability are indexed to previously established release criteria.
- *Assessment of physical characteristics of the final construct*. The general appearance of the final construct is visually compared relative to images of scaffold from an established image library.

Table 5.1 Acceptance criteria for release of the Neo-Urinary Conduit™

Test	Manufacturing step(s)/ sample	Method	Acceptance criteria
Bioburden	Adipose tissue Transport medium	Membrane filtration	Record
Endotoxin	Adipose tissue Transport medium Passages 1 and 3 Scaffold preconditioning	*Limulus* amebocyte lysate	Record < 5 EU/mL < 5 EU/mL
In-process sterility	Adipose tissue Transport medium Passages 1 and 3 SMC (spent medium) Scaffold preconditioning	Bac/T Alert	Record Record No growth in 5 days
Mycoplasma	Adipose tissue Transport medium	PCR method	Negative for mycoplasma
Cell growth/ passaging	Each passage/SMC (cells)	Visual examination under the microscope	≥ 70% confluence or no more than 12 days in culture
Cell count and viability	Each passage/SMC (cells)	Dye exclusion (Trypan Blue)	Minimum number of viable cells needed for each passage seeding density Viability (P1 – Final) ≥ 70%
Morphology	Each passage/SMC (cells)	Visual examination under the microscope	SMC cell shape and size as defined in image library
Phenotype	Final cell harvest/SMC (cells)	Flow cytometry or immunocyto-chemistry	SMC phenotypic expression of alpha actin

- *Assessment of 'fitness for use' or 'potency' of the final construct.* On the day of release of the NUC construct from manufacturing, two principal criteria must be met to establish 'potency' or 'fitness of use':

1. The final construct must have the potential to function as a conduit for passage of liquid. As the actual length of the final construct cannot be directly measured, scaffold shape will be evaluated against a reference library of established scaffold images.
2. Seeded SMC must be viable as demonstrated by metabolic activity. Viable cell populations that are metabolically active consume nutrients (e.g. glucose) from and excrete waste products (e.g. lactate) into the surrounding cell culture medium. Therefore, glucose uptake and lactate excretion parameters are measured from the culture medium surrounding the construct prior to release.

These indices of continued cellular bioactivity, together with evaluation of SMC phenotype of cells at time of seeding, provide information that may be regarded as sufficient regarding the potency or 'fitness for use' of the final construct. These criteria are summarized in Table 5.2.

5.2.8 Packaging and transport of the NUC construct

Upon release of the final NUC construct, the construct ID must be verified against the tissue collection site ID, and a final shipping label is prepared. IDs are verified by the Quality Assurance group prior to packaging. The NUC container is then packaged within a product transport box (Figure 5.5) and shipped to the clinical implant site by courier. Key production controls include verification of patient and clinical site ID, as well as courier shipment of the NUC construct.

For all incoming tissues, transport temperature and time of transport represent key production indices to be monitored. For outgoing NUC constructs, the final construct is shipped inside a bioreactor, within a leak-proof bag and placed within a qualified construct shipper. The latter represents an insulated container capable of maintaining a 15–30 °C temperature range during transport. The construct shipper will be qualified to verify that it remains sterile, retains the physical characteristics measured as part of final product release and continues to meet the criteria established for 'fitness of use'.

5.3 Preclinical evaluation of the NUC

Prior to initiation of clinical trials in human patients, formation of native-like urinary tissue from NUC constructs was evaluated in a porcine

Table 5.2 Criteria for evaluation of potency of the Neo-Urinary Conduit™

Manufacturing step(s)/sample	Test	Method(s)	Criteria
Cell expansion/ just before harvest	Identity– Morphology	Visual examination under the microscope	Expected SMC cell shape and size compared with image library
Cell harvesting/ spent medium at harvest	Sterility	Visual observation of turbidity	No observable evidence of contamination
		Bac/T Alert USP (United States Pharmacopeial) <71>	No growth in 5 days No growth after 14 days
	Purity (Endotoxin)	LAL	<5 EU/mL
Scaffold/before cell seeding	Physical integrity	Visual inspection	Shape: meets the scaffold shape as defined in image library Seams (if present): intact seams
Cell seeding/cell suspension just prior to seeding of the scaffold	Mycoplasma (cells + spent culture media)	PCR method, and culture method	Negative for mycoplasma (pre-shipping) Negative for mycoplasma (post-shipping)
	Identity– Phenotype	Flow cytometry or immunocytochemistry	Expression of smooth muscle α-actin Expression of calponin
	Viability	Trypan Blue Dye exclusion	≥70%
	Cell seeding number	Trypan Blue Dye exclusion	3.9×10^7–6.5×10^7
NUC construct maturation/ spent medium from bioreactor before last SMC culture medium exchange	Sterility	Visual observation of turbidity Stat Gram stain	No observable evidence of contamination
		Bac/T Alert	No growth; 3-day early read
	Mycoplasma	PCR method	Negative for mycoplasma

Table 5.2 *continued*

Manufacturing step(s)/sample	Test	Method(s)	Criteria
Final product/ NUC construct/ spent medium from bioreactor before exchanging to NUC construct transport medium	Sterility	USP <71>	No growth; 14-day culture (post-shipping)
	Mycoplasma	Culture method	Negative for mycoplasma (post-shipping)
	Purity (Endotoxin)	LAL	<5 EU/mL
	Physical appearance	Visual inspection	Shape: meets the scaffold shape as defined in image library Seams (if present): intact seams
	Potency ('fitness for use')	Chemistry analyzer	Detection of glucose uptake or lactate production
		ELISA	MCP-1 production

Figure 5.5 Product transport box for delivery of NUC construct to patient location

cystectomy model.[4] We will briefly outline the design and functional outcomes associated with this study as an illustration of requirements for product development in the TE/RM space. [Note added in proof. The following sections are exerpted from a study which was recently accepted for publication and is currently in press: Basu J, *et al*. Regeneration of native-like neo-urinary tissue from non-bladder cell sources. *Tissue Engineering Part A*, in press, 2012.]

SMC were isolated from bladder and adipose biopsies as well as peripheral blood draws for use in generation of an autologous NUC construct. A 1-cm^2 bladder biopsy specimen, 2-cm^2 adipose biopsy specimen and 50 mL of peripheral blood was obtained from each of 24 Gottingen swine approximately eight weeks prior to the planned implantation of the final NUC. For isolation of bladder-derived SMC, the urothelial cell layer was dissected away from the bladder biopsy and the remaining smooth muscle layer was cut into 1-mm^2 pieces and arranged onto the surface of a tissue culture plate. Biopsy pieces were dried in a biosafety cabinet for 10–30 min. SMC growth medium was added to the biopsy samples and the plates were incubated in a humidified 37 °C incubator at 5% CO_2. Adipose tissue (7–25 g) was washed three times with PBS, minced with a scalpel and scissors, transferred to a 50-mL conical tube and incubated at 37 °C for 60 min in a solution of 0.3% collagenase and 1% bovine serum albumin in basal medium. The tubes were either continually rocked or periodically shaken to facilitate digestion. The stromal-vascular fraction was pelleted by centrifugation at 600*g* for 10 min and resuspended in SMC growth media. The stromal-vascular fraction was then used to seed passage zero. Twenty-five milliliters of porcine peripheral blood was diluted 1:1 in PBS and layered with 25 mL Histopaque-1077 in a 50-mL conical tube. Following centrifugation (800*g*, 30 min), the mononuclear fraction was collected, washed once with PBS and resuspended in SMC growth media to seed passage zero.

5.4 Assembly of an NUC cell/scaffold composite

Bladder, adipose and peripheral blood-derived SMC were expanded separately for up to seven weeks to generate the 10^7 cells required for seeding an NUC scaffold. Bladder and adipose-derived SMC were expanded for two passages before harvesting of cells for seeding of scaffolds to produce the final construct. Peripheral blood-derived SMC cultures were expanded to passage 3–4 before harvesting for scaffold seeding. To make the NUC scaffold, PGA felt was cut to size, sutured into the shape of an NUC and coated with PLGA. This construct was then sterilized using ethylene oxide.

On the day prior to cell seeding, the NUC scaffold was serially pre-wetted by saturation with an ethanol/growth medium solution at room temperature overnight. The NUC scaffold was then seeded with either bladder, adipose or peripheral blood-derived SMC and the seeded construct was matured in a humidified 37 °C incubator at 5% CO_2 until being packaged for shipping prior to implantation in an autologous host pig by day 7.

5.5 GLP preclinical analysis of *de novo* NUC formation in a porcine cystectomy model

Thirty-two Gottingen swine with total cystectomy and incontinent ureterostomy (eight animals per data point, comprising four males and four females each) were used in a good laboratory practice (GLP) preclinical analysis to determine the safety and functionality of tissue-engineered NUC constructs seeded with autologous SMC derived from the bladder, blood or adipose tissue. Of the 32 animals, the first group (four males, four females) was implanted with NUC seeded with bladder-derived SMC. A second group was implanted with NUC scaffold seeded with adipose-derived SMC, a third group was implanted with an NUC scaffold seeded with blood-derived SMC, and the fourth group was implanted with unseeded NUC scaffold only. Device effect and performance was monitored through ultrasound imaging, pyelogram, as well as urine and blood analysis at different time-points of the study. At the completion of the recovery period (Day 84, ±5), all animals were killed and a necropsy was performed for harvesting the kidneys, conduits, and associated organs and tissues for histological preparation and pathological examination.

5.6 Alternate cell sourcing of SMC for seeding of the NUC

Tissue engineering principles have been successfully applied to provide implantable cell-seeded matrices for use in the reconstruction, repair, augmentation or replacement of laminarly organized luminal organs and tissue structures, such as a bladder or a bladder segment or component, typically composed of urothelial and smooth muscle layers. SMC may be derived from the patient's own tissue, including the bladder, urethra, ureter and other urogenital tissue. However, there are challenges associated with dependence on the development and maintenance of cell culture systems from the primary organ site as the basic unit for developing new and healthy engineered tissues, as for example during treatment of cancerous bladder

tissue. Clearly, such cancerous cells are inappropriate for populating an implantable neo-bladder scaffold or matrix. We have therefore attempted to identify and characterize alternative sources of SMC capable of reconstituting urologic characteristics *de novo* upon implantation on appropriate synthetic, biopolymer scaffold constructs, and to demonstrate functionality *in vivo*.

Numerous studies have indicated that adherent, fibroblast-like cells with typical SMC characteristics may be recovered from the mononuclear fraction of peripheral blood, cord blood or bone marrow. These smooth muscle-like cells may be pericytes, which are recruited to developing arterioles, capillaries and venuoles. Additionally, it is well established that adipose tissue is a readily available source of adherent cell types and may therefore also represent an alternative source of SMC useful for urologic application. Indeed, adipose tissue isolated during abdominoplasty procedures is rich with capillaries, providing a potential source of isolatable smooth muscle-like pericytes. Although SMC have also been isolated from other tissue sources such as skeletal muscle and omentum, we chose to focus on recovery of SMC from peripheral blood and adipose, as these represent the source tissue with maximum potential clinical utility. A porcine cystectomy model was selected to evaluate the performance of peripheral blood and adipose-derived SMC relative to bladder-derived SMC upon application in a cell/scaffold composite.

Direct plating of the peripheral blood-derived mononuclear fraction from swine resulted in outgrowth of colonies with typical SMC morphology (see Figure 5.2). All animals screened ($n = 24$) generated SMC colonies, with 2.44×10^3–2.37×10^6 SMC recovered at passage zero from 50 mL of peripheral blood. Recovery of SMC was unaffected by changes in media formulation, cell density or surface coatings. A similar approach was used to investigate the potential application of subcutaneous or lipoaspirate-derived adipose as a source of SMC. The SVF of adipose tissue represents a heterogeneous population of cells including endothelial cells, SMC as well as progenitor cells with limited mesenchymal potential. We were able to generate colonies (expandable into monolayers) of SMC from porcine adipose tissue with 100% efficiency ($n = 24$), with a cell recovery rate of 1.37×10^5–4.36×10^5 cells/g adipose tissue. In comparison, SMC could be isolated from bladder tissue with a recovery rate of 1.29×10^6–9.3×10^6 cells/g bladder tissue. Expansion of SMC colonies from peripheral blood or adipose resulted in the formation of a cell monolayer with a typical whirled, 'hill-and-valley' organization characteristic of cultured bladder-derived SMC (Figure 5.2). Enrichment of SMC was facilitated by the use of high cell densities and media rich in glucose, which has been shown to specifically select against the growth and expansion of mesenchymal stem cells.[2]

Analysis of the functional properties of peripheral blood or adipose-derived SMC *in vitro* demonstrates that they are indistinguishable from bladder-derived SMC. Increased expression of proteins associated with smooth muscle contractility is a characteristic feature of SMC differentiation and maturation. Myocardin is a key transcription factor required for SMC differentiation and acts to mediate the expression of smooth muscle markers essential for contractility, including SM22 (transgelin), smooth muscle α actin (SMAA), smooth muscle myosin heavy chain (SMMHC) and calponin (CNN). Expression of the smooth muscle markers SMMHC and CNN is generally regarded as diagnostic of mature SMC.

Application of adipose and peripheral blood-derived SMC for urologic regenerative medicine is contingent on being able to secure adequate cell numbers within an acceptable time frame. Towards this end, we have observed that SMC colonies (from a 50-mL sample of porcine peripheral blood or 7–25 g porcine adipose) are identifiable within seven days post-seeding and may be passaged within 14 days. In fact, tens of millions of SMC may be recovered from bladder, peripheral blood or adipose within 2–4 weeks ($n = 24$). Bladder and adipose-derived SMC were expanded for two passages prior to harvesting of cells for seeding a synthetic, neo-urinary conduit scaffold. Peripheral blood-derived SMC were expanded for 3–4 passages to generate equivalent cell numbers. On average, $30–40 \times 10^6$ SMC were used to seed an NUC scaffold.

We have previously shown that bladder-derived SMC may be used to seed a synthetic, biopolymer scaffold which upon implantation into an *in vivo* clinical model of bladder cystectomy resulted in the regeneration of a fully functional *de novo* bladder augment.[5,6] However, because use of bladder-derived SMC may not be clinically ideal, we proceeded to evaluate the *in vivo* clinical efficacy of peripheral blood and adipose-derived SMC in a three-month porcine clinical model of urinary incontinence. Bladder, adipose and peripheral blood-derived SMC were used to seed PGA/PLGA-based scaffolds to create a regenerative NUC permitting efflux of urine from the ureters directly to the external body surface.

We observed that constructs composed of SMC obtained from blood or bladder sources regenerated a patent conduit composed of an urothelial cell lining and smooth muscle layer that did not result in alterations to the upper urinary tract. No evidence was found for elevated creatinine, metabolic abnormalities or altered hematological parameters. Histological characteristics of the regenerated urological tissue forming the NUC were generally similar regardless of the origin of the SMC population. In contrast, scaffold-only implanted animals developed patent urothelial-lined conduits composed primarily of fibrous connective tissue and limited

smooth muscle development. This group also had a high frequency of hydro-ureter and hydro-nephrosis. In both groups, early post-operative management of the conduit lumen and stoma was required to maintain patency for the study duration.[4]

This study demonstrated that a synthetic, biopolymeric scaffold composite seeded with autologous SMC derived from multiple potential cell sources (blood, fat or bladder) is capable of being used to re-create a patent NUC. The regenerated conduit was not associated with sequela commonly found with urinary diversions generated from intestinal tissue or with non-cell-seeded synthetic scaffolds. This ability to create urologic structures *de novo* from synthetic scaffolds seeded by peripheral blood or adipose-derived SMC will greatly facilitate the translation of urologic tissue engineering technologies into clinical practice.

5.7 Clinical trials of the NUC

Based on successful outcomes in GLP porcine cystectomy models as outlined above, Tengion has initiated Phase I clinical trials of NUC constructs in human patients requiring urinary bypass. This Phase I study, 'Incontinent Urinary Diversion Using an Autologous Neo-Urinary Conduit',[7] is currently recruiting patients (as of time of writing), with the goal of implanting up to 10 patients by the end of 2012. The objective of the study is to evaluate if NUC constructs, made in the laboratory by a combination of the patient's own adipose-derived SMC in combination with defined, degradable biomaterial scaffolds, may be used to form a functional conduit to safely facilitate passage of urine from kidneys to outside the body subsequent to radical cystectomy in patients presenting with bladder cancer.

Primary outcome indices over a 12-month post-implantation time frame include structural integrity and conduit patency. Computed tomography (CT) scans will be used to demonstrate that urine may flow safely through the NUC construct. Additional measures of primary outcomes up to 12 months post-implantation include an evaluation of any product- or procedure-related adverse events. Similarly, secondary outcome indices will include analysis of NUC structural integrity and patency over a 12- to 60-month post-implantation time frame. CT and renal ultrasound will be applied to demonstrate that urine is able to flow safely through the NUC construct up to 60 months post-implantation. Procedural and product-related adverse events will also be monitored up to 60 months post-implantation. Finally, the overall safety of the NUC construct will be assessed through evaluation of non-product/procedural adverse events and

patient vital signs. Eligibility for this clinical trial has been adjusted to be as inclusive as possible while ruling out any patients who present with secondary conditions likely to negatively impact regenerative outcomes:

Eligibility

Ages eligible for study:	18–80 years
Genders eligible for study:	Both
Accepts healthy volunteers:	No

Criteria

Inclusion criteria:

- Male and female subjects 18–80 years of age
- Patients undergoing radical cystectomy for treatment of bladder cancer clinically staged as no greater than T2, N0
- Indicated and agreed between physician investigator and patient to have an incontinent conduit as the diversion mechanism of choice post-cystectomy.

Exclusion criteria:

- History of other cancer within the past five years (except non-metastatic prostate or non-melanoma skin cancer)
- Evidence of cancer metastasis
- History of any pelvic radiation or non-pelvic radiation within the past five years
- Debilitating cardiac or pulmonary disease
- Expected need for chemotherapy within three months post-cystectomy
- Life expectancy less than two years.

At the time of writing, three patients have been implanted with NUC constructs and are currently undergoing post-implantation evaluation.

5.8 Regeneration of muco-cutaneous region at the skin/conduit junction

Successful implantation of the NUC requires the regeneration of a native-like muco-cutaneous region at the skin/conduit junction following implantation. A muco-cutaneous junction is a region where the luminal mucosa transitions to skin. Such muco-cutaneous junctions occur throughout the body at orifices including the mouth, nostrils, anus and components of the genito-urinary

system. Typically, such junctions involve transitioning of epithelium to epidermis, lamina propia to dermis and smooth muscle to skeletal muscle. The regeneration of a muco-cutaneous region at the skin/conduit junction of a synthetic neo-organ such as the NUC, which has never before existed in nature, indicates that the body somehow understands what is required and regenerates the cell types relevant to the neo-organ.

In the urinary system, urine exits the body via the urethral meatus, a distinct structure incorporating features that defend the opening against local and/or ascending infections, emptying into the vaginal vestibule in females and the fossa navicularis in males. Specifically, the muco-cutaneous junction is a non-keratinized stratified squamous epithelium composed of glycogen-rich cells that provide substrate for a protective endogenous lactobacterial community. Also, as the epithelium nears the skin, it is associated with acid phosphatase activity and lysozyme-like immunoreactivity indicative of the presence of macrophages that secrete bacterial compounds. In a pre-clinical study in cystectomized pigs, an implanted NUC was observed to catalyze the formation of a native-like transition between the urinary mucosa and skin epithelium with structural features resembling that of muco-cutaneous junctions observed in native urethras.[8]

5.9 Speculations for the future

As previously discussed, it is noteworthy that implantation of a regenerative construct composed of adipose-derived SMC seeded onto a biodegradable, synthetic polymeric scaffold, and surgically attached to the ureters, is sufficient to catalyze the formation of a neo-organ composed of native-like urinary tissue. What signals indicate to the body that this construct is a template for urinary-like tissue regeneration, as opposed to GI or vascular tissue? Is it attachment to the ureters, specific location in three dimensions within the peritoneal cavity or a combination of these and other factors? What is the role of the cell type used to seed the construct? Very similar constructs may be applied towards the regeneration of other laminarly organized hollow organs; presumably, anastomosis of such a construct to the esophagus, for example, would result in regeneration of esophageal-like tissue and not urinary-like tissue. Migration of cell populations from the anastomosed native tissue (esophagus or ureters) may well mediate the initial sequence of events that establishes neo-organ identity. Controlled dissection of these specific factors mediating neo-organ regeneration will facilitate the development of better controlled, second-generation neo-organs potentially incorporating novel functionalities never before seen in nature.

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6

Tissue engineering of non-bladder tubular organs

Abstract. Methodologies developed for the tissue engineering of bladder and bladder-related neo-organs may be regarded as a foundational technology platform for the regeneration of alternative, laminarly organized hollow organs. These may include, but are not limited to, lung, elements of the vasculature, components of the gastrointestinal tract and male/female reproductive organs. In this chapter, we examine how key principles applied for the regeneration of bladder may be used more broadly. In particular, we focus on identifying elements of this platform that have facilitated the transition of tubular organ regeneration from academic proof-of-concept to clinical viability.

Keywords: tissue engineered vessel, chronic obstructive pulmonary disease, gastrointestinal tract, hollow organ, scaffold

6.1 Introduction

Two significant recent studies have brought the concept of *de novo* regeneration of tubular organs within humans towards proof-of-practice in the clinic. In the first, seeding of tubular, biodegradable scaffolds with autologous urothelial and smooth muscle cells was shown to catalyze the regeneration of a complete bladder with laminarly organized histology and associated urologic functionality upon implantation within seven pediatric patients.[1] In the second example, a tissue engineered, functional human trachea was created using a decellularized, cadaveric tracheal segment as scaffold and seeded with autologous respiratory epithelial cells and chondrocytes generated by the directed differentiation of the patient's own bone marrow cells.[2,3] In both studies, a scaffold seeded with autologous cells was used to trigger a regenerative response within the patient,

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ultimately leading to complementation of organ functionality with concomitant histogenesis of laminarly organized tissue structures. These approaches have focused on the regeneration of tubular organs and, together with additional recent developments in the regeneration of other hollow organs, provide perspective into overlapping technology platforms and insight into key methodological differences that may impact the commercial feasibility of tubular organ engineering.

6.2 Vasculature

No other tubular organ has advanced further into clinical trials and commercial development than tissue engineered vessels (TEV). Demand for TEV reflects the approximately 600,000+ coronary and peripheral vascular bypass graft procedures performed in the USA annually.[4] The saphenous vein or internal mammary artery may typically be utilized, but such tissues may not always be available or may be unsuitable due to concurrent vascular disease. In this regard, TEV represent a major commercial opportunity for application of a tubular organ platform technology in patients receiving bypass surgery. However, successful construction of TEV is contingent upon recapitulation of the unique physical and mechanical characteristics associated with the tri-laminar wall organization of native vessel (see Figure 3.1). For vascular grafts with internal diameter exceeding 6 mm, synthetic biomaterials such as Gortex and Dacron have been successfully applied. For example, in a recently published clinical trial, vascular grafts composed of autologous bone marrow aspirate were seeded onto a poly-glycolic acid (PGA)/poly-caprolactone (PCL) scaffold of internal diameter 12–24 mm and implanted into a cohort of 25 patients presenting with single ventricle physiology. All patients were asymptomatic 30 days post-implantation and 24/25 patients were alive at one year post-implantation.[5] However, for vascular grafts with internal diameter less than 6 mm, these biomaterials have proven susceptible to thrombus formation and compliance mismatch between the TEV and the native vasculature. As such, research on biomaterials development for vascular grafts has focused on bio-mimicry of aspects of native vascular grafts including tensile strength and visco-elasticity, as well as resistance to induction of thrombosis provided by the luminal endothelial layer (reviewed in ref. 6). Recent strategies for engineering TEV have therefore focused on techniques that eliminate the requirement for biomaterials altogether at the time of implantation.

In one recent approach, allogeneic smooth muscle cells (SMC) derived from cadaveric donors were seeded onto tubular PGA scaffolds. During

culture, seeded SMC secrete extracellular matrix (ECM) components (predominantly collagen) as the PGA degrades, to form vascular-like engineered tissue. Once the culture period is complete, SMC are removed by detergent action, leaving tube-like structures composed principally of collagen matrix. A large-diameter TEV engineered by this approach was tested as an acellular vascular graft of arterio-venous bypass within a baboon model and shown to retain patency for up to six months post-implantation. Smaller diameter vascular grafts, upon seeding with autologous endothelial cells, were implanted within canine models of peripheral/coronary bypass and shown to retain patency for at least one month post-implantation.[7] Furthermore, these TEV may be stored within PBS for periods of up to one year, while retaining patency. This ability to provide TEV as an 'off-the-shelf' product available immediately to patients requiring large-diameter vascular grafts or within a relatively short period of time for patients requiring smaller diameter grafts (pending isolation, culturing and seeding of autologous endothelial cells) represents a significant commercial opportunity.

In a related strategy, tissue engineered sheets of autologous skin fibroblasts are wrapped around mandrils and cultured to form TEV. Such TEV may then be further endothelialized prior to implantation. This approach, although showing some promise in clinical trials,[8] is unlikely to be commercially viable owing to significant (up to nine months) lead times and associated costs required to tailor such individualized grafts to the specific requirements of each patient. Attempts are being made to modify this methodology to facilitate application of allogenic cells for construction of tubular sheets, which may then be frozen to permit 'off-the-shelf' application.[9]

Efforts to seed PGA tubular neo-vessel scaffolds with SMC derived from the directed differentiation of bone marrow- or adipose-derived mesenchymal stem cells with TGF-β have also been described.[10,11] The requirement to induce directed differentiation with TGF-β or related agents as well as the prolonged maturation period under pulsatile conditions needed to achieve a mature smooth muscle phenotype will probably make this approach impractical for commercial application. A reliable source of committed SMC (e.g. the vascular fraction of adipose or omentum) may represent a more commercially feasible platform.[12]

6.3 Lung

Efforts to regenerate tissues of the lung have been focused on patient populations presenting with chronic obstructive pulmonary disease (COPD). COPD is a broad, blanket term encompassing a range of chronic conditions

including chronic bronchitis, chronic asthma and emphysema. In general terms, COPD covers a variety of symptoms, with considerable overlap between patients, but may be associated with loss of lung tissue and remodeling of airways, including bronchiolitis of small (<2 mm) airways and emphysema (dilation/destruction of lung tissue beyond terminal bronchioles, Figure 6.1). Additional characteristics include loss of elasticity, mucoid impaction and apoptosis of epithelial cells. More specifically, these disease manifestations are associated with the following features:

- *Emphysema*. Permanent enlargement of alveoli due to destruction of walls between alveoli, loss of elasticity, collapse of bronchioles and obstruction of airflow.
- *Bronchitis*. Chronic bronchitis involves inflammation and swelling of the lining of airways, leading to narrowing and obstruction of airways. In addition, inflammation stimulates production of sputum.
- *Chronic asthma*. Obstruction of airflow due to inflammation of airways and spasm of muscle surrounding airways. Inflammation leads to thickening of airway walls.
- *Pulmonary hypertension (PAH)*. Most relevant here is PAH associated with COPD and interstitial lung disease (WHO Group III PAH). Micro-vasculature of the lung is destroyed, blocking pulmonary blood

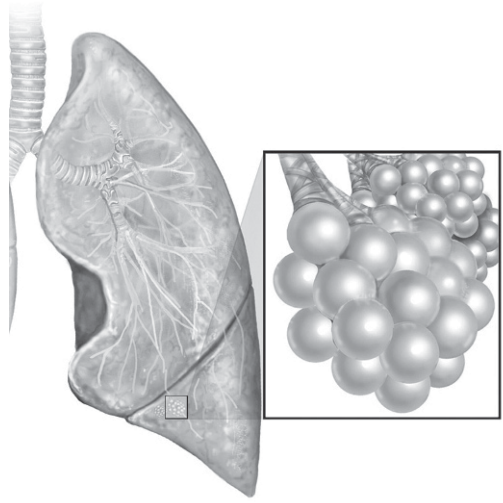

Figure 6.1 Anatomy of lung, showing close-up of terminal bronchioles and alveolar sacs (inset)

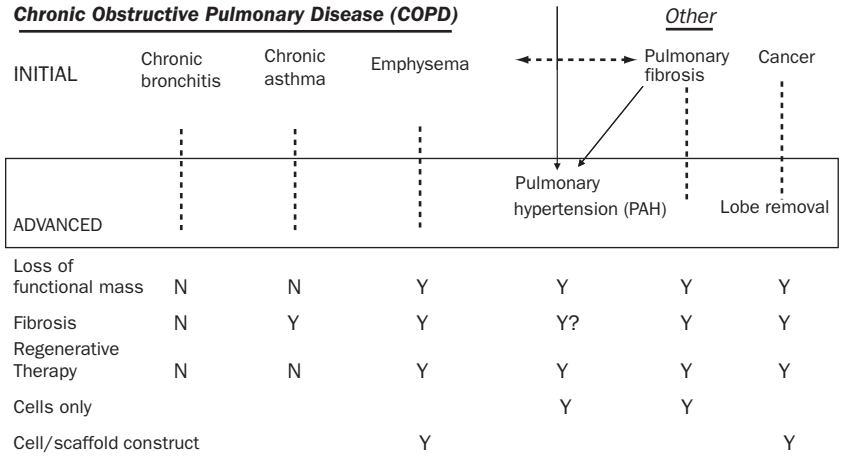

Figure 6.2 Relationships between different classes of respiratory diseases and potential (Y, yes; N, no) for TE/RM options

flow and therefore increasing pulmonary blood pressure. PAH may also be associated with genetics (WHO Group I), heart disease (WHO Group II) and thrombotic or embolic disease (WHO Group III).

- *Pulmonary fibrosis*. Pulmonary fibrosis is associated with excessive connective tissue and scarring or confluent fibrosis of the lungs. Many factors, including chronic inflammation, lupus, rheumatoid arthritis and exposure to asbestos or ionizing radiation, may lead to pulmonary fibrosis. Development of PAH may be a secondary outcome of pulmonary fibrosis.

The relationships between these respiratory diseases are outlined in Figure 6.2.

To summarize, although the causes of these differently classified respiratory disorders may differ, they typically share final outcomes characterized by:

- chronic inflammation
- fibrosis and scarring
- loss of functional lung tissue.

Such outcomes are candidates for treatment with TE/RM approaches.

The lung may be thought of as a highly specialized tubular organ amenable to regeneration with the bladder-based regenerative platform

outlined above. This would imply that regeneration of the lung would be made possible by implantation of a construct composed of committed primary cells and a degradable biomaterial. Evidence to this effect was provided by a study examining the regenerative potential of PGA felt sheets seeded with adipose-derived stromal cells in triggering pulmonary regeneration within a rat lung lobectomy model.[13] The cell-seeded PGA sheet was sealed onto the remaining lung lobe. Alveolar and vascular regeneration was observed within one week of implantation, with concomitant recovery of pulmonary functionality. Paracrine action by secreted factors including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) was suggested as a possible mechanism of action for triggering the regenerative effect.

In another study, fetal rat lung cells were seeded onto gel-foam sponge-based scaffolds and implanted into adult rat lung. Alveolar-like structures with apparent vascular networks were observed to regenerate within the degrading scaffold by four months post-implantation.[14] Importantly, the formation of these alveolar-like networks was observed to be strongly dependent on prior seeding of the scaffold with lung cells. As with the previous study, the authors suggest a paracrine action of secreted factors from the seeded lung cells acting to facilitate regenerative in-growth of native lung cells from the surrounding host lung tissue as the likely mechanism of action.

Growth of lung cells in three dimensions is essential to induce expression of epithelial genes related to lung morphogenesis, including FGFR2.[15] Appropriate combinations of exogenous fibroblast growth factors chosen to target specific receptor isoforms may facilitate appropriate lung epithelial and mesenchymal cell behavior conducive to tissue regeneration.[16] More recently, a number of groups have reported the creation of tissue engineered lungs made from recellularized scaffolds derived from decellularized lung.[17–19]

In these widely publicized studies, adult rodent lungs were decellularized with salts and detergents as previously outlined (Chapter 3). Removal of cellular material was confirmed by fluorometric assay for DNA content as well as by immunoblotting to establish loss of the major histocompatibility complex MHC-I and MHC-II proteins (important triggers of immune response). Key components of the ECM including collagen, elastin and laminin were shown to be retained. The decellularized lung was maintained within a specialized bioreactor, with negative pressure applied in a pulsatile manner to simulate rhythmic breathing. Culture medium was perfused into the lung scaffold through the pulmonary artery.

To repopulate the scaffold, neonatal rodent lung epithelial cells were introduced into the airway niche and micro-vascular lung endothelial cells were injected via the pulmonary artery. Both cell types presented good cellular adherence to the matrix. These tissue engineered lungs were associated with expression of certain key functional markers of lung cell bioactivity, including pro-surfactant proteins B and C, important in reducing alveolar surface tension and facilitating lung inflation at physiologically relevant pressures. Engineered lungs were shown to have compliance parameters resembling native lung, implying that no significant stiffening of the engineered lung tissue had occurred. Finally, engineered lung was orthotopically transplanted within adult rodents and shown to recapitulate aspects of functionality for up to two hours post-implantation.[17–19]

However, although interesting, it is difficult to see how these data will translate into a clinically relevant, commercially viable product. From where will the scaffold to be decellularized be sourced? How will it be evaluated? Can the seeding regimen be controlled in a reliable and reproducible manner? Importantly, the cell source used to seed the airways was neonatal in origin. What cell source will be used for human patients? Why did the engineered lungs not facilitate survival in the rodent models for more than two hours and what can be done to improve surgical outcomes? For these and other reasons we have already discussed in this volume, we believe that the application of a construct composed of a committed primary cell type in combination with a synthetic biomaterial may be adequate to catalyze the latent ability of the adult lung to regenerate. Preliminary data by us and other groups suggest that this may in fact be the case.[13]

6.4 Gastrointestinal tract

Individual components of the gastrointestinal tract (Figure 6.3) represent locally specialized, iterative variations of essentially the same laminarly organized tubular histologic architecture as the bladder, and therefore should be amenable to regenerative therapies based on the same platform technologies successfully applied to the bladder. However, from a commercial perspective, the small intestine represents by far the most pressing current clinical need, with small bowel transplantation representing an unsatisfactory current standard of care for pediatric small bowel syndrome.[20] Additionally, esophageal replacement or augmentation for esophageal cancer may also be commercially viable.

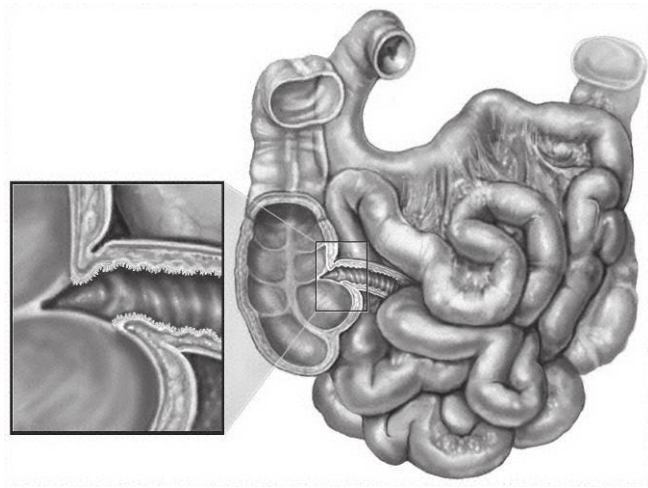

Figure 6.3 Gastrointestinal tract showing internal micro-structure of small intestine

The intestinal epithelium is the most highly regenerative tissue within adult mammals and may therefore be expected to be most amenable towards TE/RM methodologies. Perhaps the best described strategy for regeneration of intestine and other components of the gastrointestinal tract involves the use of *in vivo* derived organoid units, formed from incompletely disassembled clusters of epithelial and mesenchymal cells generated through partial digestion of intestinal epithelium and therefore probably incorporating resident intestinal stem cells.

In one study, autologous organoids were derived from the intestine of six-week-old mini-pigs and used to seed PLLA (poly-L-lactic acid) scaffold tubes that were subsequently matured within the peritoneal cavity of the original donor. Seven weeks post-implantation, this tissue-engineered small intestine (TESI) recapitulated the gross overall laminar organization of native small intestine (SI).[21] Significantly, acellular scaffolds did not result in the regeneration of tissue engineered gastrointestinal structures.

These data notwithstanding, anastomosis of these TESI to host SI within a large animal model remains to be demonstrated. Additionally, up to 10 cm of host SI was harvested to derive donor organoids that are not readily expandable *in vitro*. Whether organoid units capable of seeding a scaffold structure may be isolated from diseased human intestine and how much diseased donor material may be needed remain to be elucidated. The requirement to use the peritoneal cavity as a bioreactor for tissue engineering may also impede widespread application of this approach.

The bladder-derived organ regeneration platform of bio-polymeric scaffold seeded with SMC may be applicable for the regeneration of SI. Stomach-derived SMC were used to seed a collagen-based scaffold prior to implantation within surgically isolated ileal loops of dogs for eight weeks, prior to re-anastomosis to the native intestine. Acellular collagen scaffold was used as a control. By 12 weeks post-surgery, macroscopic analysis of the cell-seeded scaffold implantation site demonstrated regeneration of neo-mucosa resembling native mucosa. However, in animals containing an acellular scaffold, the implant site remained ulcerated up to 12 weeks post-implantation. Additional histological data showed significantly enhanced vascularization, epithelialization and organization of the circular muscle layer at the cell-seeded scaffold defect site relative to acellular control.[22]

Increasing the number of SMC seeded onto the scaffold increased the area of regenerated SI tissue, although no concomitant increase in the thickness of the smooth muscle layer was observed.[23] Nevertheless, these data suggest that a simple regenerative platform composed of biodegradable scaffold nucleated with SMC may be adequate to facilitate SI regeneration. Although this approach must be reproduced using a directly anastomosed tubular scaffold and alternate sources of SMC, if successful, this methodology represents the most straightforward, clinically relevant and commercially viable strategy for regeneration of the SI.

This organ regeneration platform technology may also be used for regeneration of the esophagus. In one such example, patch defects were created in the abdominal esophagus of 27 female rats, subsequently implanted with gastric acellular matrix. Of the 24 survivors, none showed evidence of regeneration of the lamina muscularis mucosa even after 18 months post-implantation.[24] In contrast, a study of a canine model of esophageal resection and replacement demonstrated that PGA tubes seeded with a mixture of keratinocytes and fibroblasts triggered regeneration of smooth muscle laminar organization similar to native esophagus within three weeks post-implantation, whereas acellular PGA tubes formed esophageal strictures and were associated with near complete obstruction within two to three weeks.[25]

In another study in dogs, cervical esophageal defects were patched with either small intestinal sub-mucosa (SIS) alone, or SIS seeded with autologous oral mucosal epithelial cells. After four weeks, dogs implanted with cell-seeded SIS showed almost complete re-epithelialization with minimal evidence of inflammation and, by eight weeks post-surgery, regeneration of the underlying smooth muscle layer. Acellular SIS-grafted animals presented only partial re-epithelialization and a more extensive inflammatory response by four weeks, and no muscular regeneration by eight weeks.

Attempts to introduce an acellular SIS tubular construct into the cervical esophagus of piglets were also unsuccessful, demonstrating scarification and a minimal regenerative response.[26]

Progress has also been made in efforts to tissue-engineer the stomach. Stomach-derived organoid units (analogous to the SI organoids used to tissue-engineer the SI), upon seeding of a biopolymeric scaffold, triggered reconstitution of the gastric and muscularis mucosa in stomach tissue engineered within the peritoneal cavities of swine.[21] In another study, circular defects were created in the stomachs of seven dogs and a composite biodegradable scaffold ('New-sheet'), soaked with either autologous peripheral blood or bone marrow aspirate, was sutured over the defect. By 16 weeks post-implantation, the defect site had formed regenerated stomach with evidence of re-epithelialization, formation of villi, vascularization and fibrosis within the sub-mucosal layer. However, minimal regeneration of the smooth muscle layer was observed, as shown by expression of smooth muscle α-actin, but not calponin, a marker of mature SMC.[27]

Although not strictly a tubular organ, the anal sphincter is a component of the gastrointestinal tract and is critical in regulating patency of the large intestine. Recent efforts to engineer the anal sphincter utilized the same general platform used to catalyze bladder regeneration. SMC isolated from human internal anal sphincter were seeded onto fibrin gels poured around a central mold. Cell-mediated contraction of the gel around the mold resulted in the formation of a three-dimensional cylindrical tube of sphincteric smooth muscle tissue. Although *in vivo* anastomosis remains to be demonstrated, this bio-engineered anal sphincter demonstrated contractile properties and response to defined neurotransmitters consistent with the functionality of native anal sphincter.[28,29] Use of alternatively sourced SMC may facilitate the transition of engineered sphincter towards commercial production.

6.5 Genito-urinary system

The recent reports of functional regenerated neo-phallus and neo-vagina within a rabbit model illustrate how the foundational principles of tubular organ regeneration pioneered for bladder may be extrapolated to facilitate organogenesis of functionally distinct tubular organs.[30] Decellularized corpora cavernosa was used as a collagen-based scaffold matrix for seeding autologous corporal smooth muscle and endothelial cells in a rabbit model

of penile replacement. Implantation of decellularized matrix alone led to formation of a non-functional, fibrotic phallus. However, cell-seeded scaffolds regenerated corporal tissue organization histologically similar to native controls within 3–6 months post-implantation. Tissue engineered penis was functional, as demonstrated by the ability of recipient animals to copulate normally.[31]

For the neo-vagina, autologous vaginal epithelial and SMC were seeded onto the luminal and abluminal surfaces of PGA tubular scaffolds, preconfigured to resemble native rabbit vagina. Seeded composites were implanted in place of the native vagina of nine rabbits, with unseeded controls introduced into six other animals. As has been observed for multiple organs, unseeded scaffold failed to trigger a regenerative response, whereas cell-seeded scaffolds generated stage-specific histogenesis, vascularization, innervation and regeneration of a patent neo-vagina by six months post-implantation with a defined muscular layer and a luminal invaginated epithelium. Organ functionality was confirmed by a graded contractile response of the musculature to electrical stimulation in a manner paralleling native tissue.[30]

A recent report presented long-term post-implantation outcomes from five pediatric patients with urethral defects that had been treated with tissue engineered urethra.[32] Primary cultures of bladder-derived smooth muscle and urothelial cells were established from autologous bladder biopsies. A PGA-based tubular scaffold was constructed by hand for each patient, with sizes ranging from 4 to 6 cm in length. Epithelial cells were seeded onto the luminal surface of the tubular construct and SMC were seeded into the external surface of the tubular construct. Seeded scaffolds were matured for seven days. Overall construction of neo-urethras took 4–7 weeks. Subsequent to implantation, indices of urologic functionality and tissue biopsies were taken for each patient over a period of time up to 72 months post-implantation.

This methodology exactly parallels early approaches towards assembly of neo-bladder,[1] and represents one of the best examples of the extension of a foundational platform technology from its organ of origin (bladder) to a related yet functionally distinct alternative (urethra). We speculate that as has been observed for bladder,[33] application of two distinct cell types may not be required to achieve complete regeneration of native-like urinary structures from regenerated neo-urethra; constructs composed of biomaterial scaffold seeded with SMC (including those derived from alternate cell sources such as adipose) will probably be adequate.

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7

Tissue engineering of solid organs

Abstract. Regeneration of solid organs *in vivo* remains an outstanding goal of tissue engineering and regenerative medicine. In this chapter, we critically review recent progress towards regeneration of heart, kidney, liver, pancreas, spleen and central nervous system with the aim of identifying commonalities of methodology that may underlie the development of platform technologies broadly applicable towards the regeneration of multiple solid organ systems. We focus in particular on highlighting features of organ regeneration platforms conducive to the commercial viability of such technologies. Salient characteristics include design and application of synthetic biomaterials matching critical aspects of the organ parenchyma as well as defined populations of committed cell types capable of triggering a host-specific regenerative response. We illustrate these principles using Tengion's Neo-Kidney Augment™ product, a cell/biomaterial construct currently under pre-clinical development to help ameliorate symptoms secondary to chronic kidney disease.

Keywords: regeneration, extra-cellular matrix, decellularization, decell/recell, heart, kidney, hydrogel, biomaterial, scaffold, hepatocyte, biopolymer, platform technology

7.1 Introduction

It is well established that mammalian fetuses are capable of spontaneous regeneration of damaged organs and tissues until the third trimester. However, adult mammals typically respond to trauma by initiating reparative healing mechanisms. These are characterized by wound contraction, extensive fibrosis and scar tissue formation mediated by the migration and re-organization of myo-fibroblasts, as demonstrated during the response of the heart to ischemic injury.[1] True organ regeneration as noted in certain species of fish and amphibians is associated with an absence of the fibrotic response and concomitant reconstitution of the

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three-dimensional laminar or parenchymal organization of the regenerating organ. One example of such a regenerative response is that of adult mammalian liver to lobectomy.

The objective of *de novo* organ regeneration through TE/RM is to use defined combinations of cells and biomaterials to catalyze a principally regenerative response towards organ trauma while simultaneously ameliorating any tendency towards fibrotic repair. The *de novo* regeneration of a complete organ in mammals has been demonstrated for laminarly organized, tubular organs including bladder, trachea and vessels of the vasculature.[2] However, the regeneration of solid organs presents a unique challenge, requiring the organization of highly specialized cell types into complex three-dimensional micro-architectures within a parenchymal matrix (see Figure 7.1).

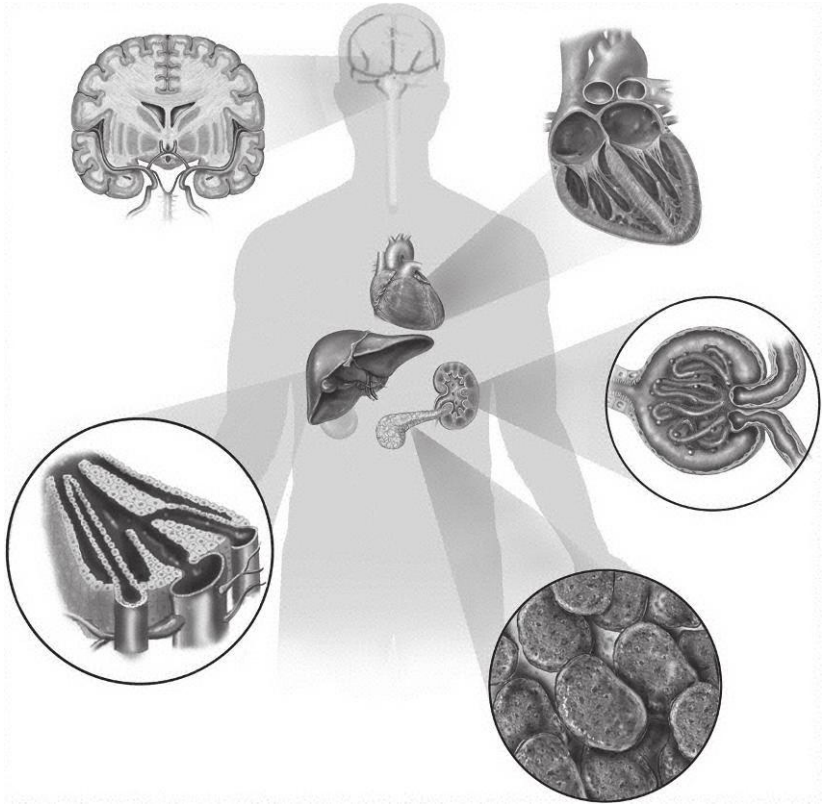

Figure 7.1 Solid organs amenable to TE/RM methodologies. From top right, clockwise: heart, kidney, pancreas, liver and central nervous system

Additionally, the regenerating solid neo-organ must synergize with developing elements of the vasculature, lymphatic and nervous systems throughout neo-organ morphogenesis. In this chapter, we highlight the latest progress towards regeneration of solid organs in mammals, with an emphasis on identifying commonalities of methodology that may drive the creation of an organ regeneration platform broadly applicable towards multiple solid organ systems. We focus on identifying aspects of the regenerative methodology and platforms most conducive towards translation into commercial process development and manufacture (Table 7.1).

7.2 Kidney

Kidney exemplifies the numerous technical difficulties associated with solid organ regeneration. Multiple specialized cell types including podocytes, mesangial cells, endothelial cells, fibroblasts, epithelial cells, and numerous stem and progenitor cell populations are organized across the renal parenchyma into discrete, specialized functional units or nephrons, which serve to selectively filter electrolytes from the vasculature.[3,4] Efforts to trigger regeneration within animal models of ischemic or chronic renal disease have typically centered around the isolation, expansion and re-introduction of defined populations of mesenchymal, embryonic or renal stem cells, potentially capable of site-specific engraftment and directed differentiation along multiple renal lineages as well as facilitating the creation of a regenerative micro-environment through paracrine signaling mechanisms (reviewed in refs 5 and 6).

Cell therapy methodologies have had generally mixed results, with little if any evidence supporting site-specific engraftment and directed differentiation as a mechanism of action by exogenously applied stem or progenitor populations. The introduction of stem cells within the kidney by direct injection into the renal vasculature or renal parenchyma leads to apoptosis or efflux of the majority of applied cells from the target organ within days of implantation.[7] Uncontrolled differentiation of stem cells that actually do engraft may also represent a significant technical challenge and a potential regulatory obstacle against successful commercialization.[8]

Reconstitution of kidney mass, a central prerequisite for true *de novo* organ regeneration, will probably require a cell/biomaterial scaffold composite facilitating the morphogenesis of glomeruli and tubules derived from seeded primary renal cell populations. Migration and directed differentiation of host-derived renal stem and progenitor cells within the scaffold framework, followed by progressive vascularization and innervation

of the developing neo-organ, deposition of extracellular matrix (ECM), and the reconstitution of a renal parenchyma, are also needed.

It may be reasoned that biomaterials derived from the native kidney are best suited to retain renal-specific elements of the ECM that are potentially capable of modulating renal morphogenesis as well as the three-dimensional parenchymal micro-architecture unique to the kidney. To this end, the application of sodium dodecyl sulfate (SDS) detergent to whole rat kidneys in one recent report was shown to facilitate the removal of renal cells while maintaining the functional integrity of the ECM as well as the overall three-dimensional parenchymal organization of the kidney. Murine pluripotent embryonic stem (ES) cells were applied to this renal scaffold via the renal artery or ureter, and the cell-seeded biomaterial composite matured under static or pulsatile culture *without* the application of inductive differentiation agents.

This approach permitted an evaluation of the specific effect of native renal-derived ECM in modulating the directed differentiation of the ES cell population. After arterial seeding, it was observed that ES cells localized within the Bowman's capsule by four days post-seeding and within the associated vasculature and renal cortex by day 10. Niche-specific localization of ES cells was accompanied by concomitant acquisition of the renal markers Pax-2 and Ksp-cadherin, as evidenced by histochemical and RT-PCR approaches.[9] The effectiveness of SDS relative to other detergents in preserving components of the ECM as well as details of the renal micro-architecture was confirmed independently in comparative studies of the effect of decellularization within monkey kidneys with multiple detergent agents.[10]

Although it is not unreasonable to assume that the organ-specific ECM and three-dimensional histo-architecture associated with scaffolds procured by decellularization of native organs may be ideally suited to direct the potential regeneration of that specific organ type, this strategy may not be appropriate for a commercially viable solid organ regeneration platform. Apart from difficulties associated with the procurement of cadaveric organs for scaffold assembly, decellularization is a generally uncontrolled process not easily subject to scale-up, process development and quality assurance. Monitoring and verification of the extent of cell loss will substantially increase cost of goods associated with manufacture.

Furthermore, native scaffolds retain the potential for immunogenicity despite the presumed absence of cells.[11] Reliable and reproducible manufacturability of solid neo-organs will probably require the application of synthetic biomaterials with defined physical and chemical properties (see Text-Box 7.1). Progress towards the creation of biosynthetic scaffolds appropriate for the regeneration of renal architecture was provided by the

Box 7.1 Summary of key features of a commercially viable solid organ regeneration platform

- Synthetic biomaterials, readily manufactured with defined physical and chemical properties, approved by FDA for implantation within the human body
- Avoidance of decellularized scaffolds that require cadaveric organ sources and may be potentially immunogenic
- Use of committed cell types, no stem or progenitor cell populations that require extensive monitoring of stem cell potential and directed differentiation
- Autologous cell sources where possible facilitate FDA acceptance of introduction of cellular material
- Emphasis on a platform approach: cell/biomaterial composites that upon delivery trigger a broad regenerative response within multiple solid organs

demonstration that tubular and glomerular structures spontaneously self-assembled from primary rat renal cell populations within one week of growth in collagen I gels.[12]

The ability to introduce specific bio-mimetic peptides and defined proteolytic cleavage sites within the context of gel-based biomaterials raises the intriguing possibility of controlling the morphogenesis of tubules, glomeruli or other renal structural units to modulate defined functional outcomes. For example, polyethylene glycol (PEG)-based hydrogels engineered with protease degradation sites and controlled densities of RGD peptide or laminin bioligands have been found to regulate epithelial morphogenesis of cysts derived from MDCK (Madin Darby Canine Kidney) cells, such that cysts grown within ligand functionalized gels demonstrated an increased frequency of lumen formation and unambiguous baso-lateral polarization compared with those grown in unmodified hydrogels.[13]

A true renal augment designed to trigger regeneration of glomeruli, tubules, erythropoietin-secreting fibroblasts[14] or other key renal cell populations may potentially be envisioned as an injectable hydrogel containing functionalized matrix optimized to catalyze this defined regenerative outcome. Such methodologies alleviate potential concerns regarding the requirement for major surgical intervention within the diseased organ. Alternatively, a neo-kidney augment may be contemplated as a semi-permanent, implantable,

cell/biomaterial composite that upon introduction within or adjacent to the renal parenchyma of a diseased organ provides a foundational framework for regeneration of tubular or other renal superstructures as well as potentially establishing a regenerative microenvironment through paracrine-mediated recruitment of native stem and progenitor populations as well as amelioration of inflammatory, fibrotic and apoptotic cascades.[15]

To this end, certain species of polyester fleeces have demonstrated the capacity to facilitate the growth of renal tubules within the context of a fibrous artificial interstitium. In this system, a heterogeneous primary renal cell population incorporating stem and progenitor cells was extracted from the sub-capsular space of embryonic rabbit kidneys. The isolated cell population was sandwiched between two layers of polyester fleece and maintained within a perfusion culture system in the presence of aldosterone, a hormone involved in the renin/angiotensin axis.[4] Spontaneous generation of tubular structures was observed with concomitant expression of key functional markers, including cingulin and Na^+/K^+ ATPase. The regenerated tubules appeared to interact with the polyester fibers within the context of the artificial interstitium. The authors speculate that these cell-seeded polyester scaffolds may be multiplexed by horizontal 'tiling' or 'paving' as well as vertical 'piling' to create renal superstructures supporting the continued morphogenesis of renal tubules *in vitro*. The authors suggest that these compounded renal units could potentially form the basis of a true neo-kidney augment upon implantation within the sub-capsular space between the renal capsule and the renal parenchyma,[16,17] although our observations of the mechanical properties of the renal capsule associated with diseased, fibrotic human kidneys suggest that this approach may not be feasible.

Nevertheless, this methodology illustrates one key criterion for commercial viability (see Text-Box 7.1); synthetic polyester fleeces are used for regeneration of renal tubular superstructures, without the requirement for ECM components derived from decellularized kidney or from other naturally occurring sources. In addition, the application of a defined, serum/BPE (bovine pituitary extract)-free medium for tubule growth and maintenance additionally serves to facilitate large-scale process development. Conversely, it remains to be ascertained whether cells derived from human kidney tissue in general and from diseased human organs in particular are capable of supporting the spontaneous assembly of tubular structures *de novo* within the context of polyester or other synthetic polymer-based biomaterial.

Taking these principles into consideration, Tengion is currently developing the Neo-Kidney Augment™ (NKA), a cell/biomaterial combination construct product for treatment of chronic kidney disease (CKD). The bioactive cellular component of the NKA is composed of tubular cell-enriched

populations of primary renal cells. These selected regenerative renal cells are obtained through enzymatic digestion of a kidney biopsy derived from the patient, followed by density gradient separation of the isolated, expanded but otherwise unmanipulated cells. Such cell populations have been shown to positively impact aspects of disease phenotype in rodent models of CKD.[18,19]

The NKA product is prepared by formulating the autologous selected regenerative renal cells secured from the patient's kidney with a gelatin-based biomaterial. As we discussed in the Chapter 3 (Biomaterials for TE/RM), we extensively screened both synthetic and naturally derived biomaterials for biocompatibility within renal parenchyma and identified gelatin-based hydrogels as well-tolerated biomaterials presenting minimal inflammatory and fibrotic responses while simultaneously facilitating tissue infiltration and vascularization within the biomaterial.[20] The gelatin formulation is presumed to provide cell stability, extended shelf life, targeted delivery and retention of cells at the target location. In addition, the biomaterial provides architecture for cell–cell interactions, displacement of fibrous tissue in a diseased kidney and space for increased functional kidney tissue mass. Importantly, interaction with the biomaterial was shown not to significantly impact the tubular epithelial phenotype of the selected regenerative renal cells.[20]

7.3 Heart

Mammalian heart provides one of the clearest demonstrations of the inability of most mammalian solid organs to regenerate. Cardiac ischemia typically results in extensive fibrosis, scarification and loss of function.[1] In contrast, zebrafish and other non-mammalian vertebrates are capable of complete regeneration and reconstitution of function upon removal of up to 20% of the ventricle, leading to speculation that an understanding of the mechanism of action underlying regeneration within model organisms such as zebrafish may identify analogous mechanisms that may be used within mammals to trigger cardiac regeneration. As such, dedifferentiation and proliferation of existing cardiomyocytes was shown to be the principal mechanism of regeneration following ventricular amputation within zebrafish.[21] Proliferation of cardiomyocytes at the wound location was preceded by expression of certain key cell cycle regulators, including the gene *polo-like kinase*, an observation that may open the possibility of triggering cardiomyocyte expansion within damaged mammalian heart by therapeutic agents that specifically target the cell cycle.[22]

This methodology notwithstanding, tissue engineering approaches towards construction of functional mammalian heart have generally focused on decellularization of cadaveric organs to provide scaffold structures for reseeding and implantation. For example, neonatal rat cardiac or aortic endothelial cells were used to seed a decellularized cadaveric rat heart. Upon growth within customized bioreactors for eight days, evidence of spontaneous contractility was observed. Pump functionality of up to 2% of adult was successfully reconstituted.[23] However, although of interest as proof of concept, the requirement for cadaveric organs as a source of biomaterials may ultimately limit the usefulness of this methodology for commercial development and application within the clinic. As we have seen with the kidney, decellularization is a difficult procedure to monitor during quality assurance regimens, and there can be no guarantee that the resultant tissue engineered composite will lack immunogenicity upon implantation.[11] Furthermore, it remains to be demonstrated whether cardiac cells derived from adult human tissue are capable of repopulating a scaffold to regenerate a functional organ. Finally, the requirement for tissue maturation within pulsatile bioreactors will probably substantially increase cost of goods for tissue engineered cardiac neo-organs.

These criticisms aside, the engineering of synthetic scaffolds that recapitulate defined cardiac structures such as valves and chambers remains technically challenging.[24] We speculate that triggering the dedifferentiation and subsequent proliferation of existing cardiomyocyte populations by synthetic biomaterial composites, containing defined biomimetic peptides and/or autologously derived lineage committed cell populations with smooth muscle cell-like properties, may ultimately prove to be the more commercially feasible approach for regeneration of cardiac substructure and ultimately neo-organ regeneration. Evidence supporting the idea that biomaterial composites seeded with lineage-committed cell populations may represent a potential solid organ regeneration platform comes from studies of biodegradable scaffolds nucleated with human ES cell-derived cardiomyocytes alone or cardiomyocytes, endothelial cells and embryonic fibroblasts. Upon implantation within rat heart, more extensive vascularization was observed from tri-culture-seeded constructs when compared with scaffolds seeded with ES cell-derived cardiomyocytes alone.[25]

In addition, acellular or mesenchymal stem cell (MSC)-seeded SIS (small intestinal submucosa) grafts have been implanted on the epicardial surface of a rabbit model of myocardial infarct. Resultant ventricular functionality and histopathology were significantly more improved in MSC-seeded grafts relative to acellular grafts. The use of injectable gels derived from decellularized human or porcine pericardium to trigger migration and

proliferation of cardiomyocytes and cardiac progenitors has also been explored.[26] Such synthetic hydrogels incorporating biomimetic peptides or containing committed smooth muscle, endothelial or other fully differentiated cell types may serve as a commercially feasible organ regeneration platform to trigger cardiac regeneration. These regenerative platforms most likely utilize paracrine and ECM-based signaling to recreate a regenerative microenvironment and thereby facilitate the regenerative response.

7.4 Liver

The regenerative potential of the liver is unparalleled among mammalian organs. Adult liver progenitors are thought to be defined by the population of 'oval cells' capable of reconstituting both hepatocytes and biliary epithelium upon mobilization by an appropriate regenerative signal.[27] However, as with the heart, liver regeneration does not appear to use discrete, organ-specific pools of stem and progenitor cells, operating rather through the increased proliferation of existing, mature hepatocytes. Unfortunately, this magnified proliferative capacity has not translated into the ready expandability of hepatocyte populations *in vitro*. Mammalian hepatocytes remain notoriously difficult to maintain and expand in culture.[28] Regardless, TE/RM approaches to reconstitute the liver have typically focused on the isolation and expansion of mature, adult hepatocytes as a cell source for biomaterials seeding or, alternatively, on the directed differentiation of ES cells, iPS (induced pluripotent stem), adipose or bone marrow-derived MSC towards a hepatic lineage followed by three-dimensional culturing.

Morphogenesis of the mammalian liver is triggered by induction of the embryonic endodermal epithelium by adjacent mesodermal populations (reviewed in ref. 27). Mimicking these early developmental signaling events through co-culture of hepatocytes with mesenchymal cell populations such as bone marrow-derived stem cells (BMSC) or even 3T3 fibroblasts results in significant enhancement of hepatic functionality, as evidenced by prolonged maintenance of hepatocyte-specific morphology and enhanced secretion of albumin.[29] This observation raises the possibility that biomaterials seeded with mesenchymal cell populations may function as a potential solid organ regeneration platform, acting to facilitate the proliferation and functionality of hepatocytes *in vivo*.

Evidence to this effect was provided by studies of Nagase analbuminea rats implanted with corraline hyaluronic acid (HA) ceramic disks seeded either with freshly isolated rat hepatocytes alone, or with rat hepatocytes together

with bone marrow-derived mononuclear cells.[30] Hepatocytes cultured in the presence of BMSC secreted significantly more albumin into the media during *in vitro* culture relative to hepatocytes in monoculture. These effects were recapitulated *in vivo* within four weeks in analbuminic rats, within which HA-based biomaterials seeded with both BMSC and hepatocytes were associated with significantly greater blood serum albumin levels relative to monoculture controls. Finally, implantation of co-cultured biomaterials within mice presenting with chemically induced liver damage resulted in complementation of blood serum albumin as well as increased levels of serum interleukin-6.

This reconstitution of function notwithstanding, no evidence was provided regarding tissue regeneration *in vivo* within the context of the biomaterial, or whether the presence of hepatocytes is in fact essential. A clear demonstration of hepatic tissue regeneration and functional complementation using synthetic biomaterials seeded with BMSC or some other mesenchymal cell population would be of significant commercial interest. As with heart and kidney, we speculate that paracrine action by factors released from the seeded mesenchymal cells may be adequate to trigger angiogenesis and vascularization of the biomaterials as well as to stimulate the proliferation of the resident hepatocyte population. In keeping with the suggestion of paracrine mechanisms, it may additionally be possible to use the resident pluripotent progenitor populations normally found in the liver by implantation of a biomaterial within the canals of Hering to provide additional space for the development of regenerated tissue.[31]

Alternate sources of hepatocyte-like cells may offer a potential substitute for mediating the catalysis of liver regeneration. Hepatocyte-like cells may be derived from bone marrow or adipose primary cells through a multi-step directed differentiation regimen attempting to phenocopy key signaling events during hepatic morphogenesis through the controlled application of recombinant bioactive factors and small molecules, including hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and oncostatin-M (OMS).[32] These pseudo-hepatocytes display a characteristic polygonal morphology, demonstrate expression of key hepatocyte-associated transcriptional and protein markers, and secrete albumin into the media.

Similarly, cells derived from the stromal vascular fraction of adipose can be driven to acquire hepatocyte-like characteristics by application of analogous multi-stage differentiation protocols. Importantly, both adipose-derived hepatocytes as well as undifferentiated adipose stromal vascular fraction-derived cells are capable of engraftment within chemically damaged liver *in vivo*, where they appear to form cords of tissue within the hepatic parenchyma and acquire hepatocyte-specific functionality, as demonstrated by the *in situ* expression of albumin.[33] In this study, engraftment by human

adipose-derived stromal cells within the liver of an SCID (severe combined immunodeficient) mouse recipient was examined, with functionality being monitored through the use of antibodies specifically recognizing human albumin.

The differentiation of bone marrow-derived primary cells into hepatocyte-like cell types appears to be facilitated by growth in three dimensions, as shown by the enhanced secretion of albumin, urea, transferrin, serum glutamic pyruvic transaminase and serum oxaloacetate aminotransferase from hepatocyte-like cells grown in three dimensions on polycaprolactate (PCL) scaffolds relative to similar populations maintained in two-dimensional monoculture.[33] The trans-differentiation of bone marrow cells towards a hepatic phenotype may also be accomplished by using paracrine and ECM-mediated signaling mechanisms between existing hepatic cells and bone marrow cells.

For example, human bone marrow MSC may be incubated in plates containing HepG2 (liver hepatocellular carcinoma cells)-derived ECM and HepG2-derived conditioned media for up to 30 days to facilitate the acquisition of a hepatocyte-like phenotype.[34] These hepatocyte-like cells were seeded onto an RGD modified chitosan alginate polyelectrolyte, fibrous, non-woven scaffold prior to implantation within a rat liver lobectomy model presenting with 70% removal of liver mass. Post-implantation analysis of the cell-seeded biomaterials within 1–2 weeks confirmed the expression of key hepatic markers. Detection of human albumin within rat serum was also demonstrated. No histological evidence for regeneration was provided, although this outcome is unlikely over such a short time period. Taken together, these results provide evidence for functional complementation *without* clear demonstration of *de novo* hepatic tissue regeneration.

An alternative, biomaterials-free methodology for regeneration of hepatic tissue is based on the engineering of contiguous monolayers of primary hepatocytes through culturing on temperature-responsive surfaces.[35] The temperature-sensitive polymer $PIPAA_m$ [poly(N-isopropylacrylamide)] upon decrease in temperature to below 32 °C rapidly hydrates, triggering the spontaneous detachment of cultured hepatocytes in the form of discrete, uniform sheets. Stacking of multiple hepatic tissue sheet monolayers within the subcutaneous space of mice resulted in the formation of a significant hepatic tissue mass, with histologically meaningful micro-architecture, vascularization and functionality as assayed by the presence of glycogen. Importantly, ectopic engineered hepatic tissue was capable of reacting to a regenerative stimulus (two-thirds liver resection) by significantly increased levels of proliferative activity. Furthermore, hepatocytes organized as

stacked monolayer sheets generated significantly greater functional neo-organ volume compared with the same number of hepatocytes introduced into the subcutaneous cavity within an injectable Matrigel™ (an ECM gelatin commonly used to coat tissue culture plasticware) matrix. Such injectable hepatocyte matrices have been proposed as alternate regenerative stimuli for liver.[36]

7.5 Pancreas

Strategies for regeneration of pancreas have focused almost exclusively on the *de novo* regeneration of pancreatic β-cell populations. Morphogenesis of the pancreas broadly resembles hepatogenesis, with induction of the endodermal epithelium of embryonic foregut triggered by adjacent mesenchymal cell populations.[27,37] As a result, methodologies developed to mediate the directed differentiation of ES cells or adult derived stem and progenitor cells towards a pancreatic lineage use many of the same key developmental morphogens as those formulated to mediate acquisition of hepatic phenotype. The forced expression of certain pancreatic transcription factors and/or treatment with defined growth factors is sufficient to trigger acquisition of insulin transcription within non-pancreatic cell populations as well as within pancreatic ductal and acinar cells, although typically not at levels comparable with true β-cells. Lineage trans-differentiation and dedifferentiation towards a progenitor phenotype have been proposed as mechanisms of action underlying the observed *de novo* presentation of β-cell-specific phenotypes *in vitro*. Evidence is also accumulating that these mechanisms may operate *in vivo* to modulate true pancreatic regeneration (reviewed in ref. 37).

Strategies to specifically target pancreatic ductal and acinar cells for delivery of defined transcription factors selected to mediate dedifferentiation and reacquisition of a β-cell-specific phenotype, while useful as proof of concept, may not represent a commercially viable solid organ regeneration platform. Similarly, methodologies oriented around the directed differentiation of ES or adult stem cells will considerably increase cost of goods, with process development and quality assurance issues focused on the evaluation of stem cell potential and the extent and completeness of the directed differentiation process (see Table 7.2 below). To this end, it may be worth exploring whether tissue engineering approaches for the regeneration of pancreas may be designed to use the innate regenerative potential inherent within the organ itself. Although not as dramatic as the liver,

regeneration of mammalian pancreas has been observed under certain circumstances.[38]

Autologously derived pancreatic islet cells or alternate cell sources presenting a β-cell-like phenotype may be engineered within an appropriate gelatinous matrix or other biosynthetic scaffold. Considerable effort is currently being invested in developing pancreatic islet cell encapsulation technology, which may be manifested as a vascular device, microcapsule, tubular or planar membrane chamber, or sheet architecture (reviewed in ref. 39). Encapsulation techniques facilitate the delivery of allogeneic or cadaveric islets cells by modulation of the host immune response. Recent developments in encapsulation methodologies include the application of bioactive hydrogels with functionalized moieties designed to improve β-cell survival and secretion of insulin[40] as well as to modulate inflammation.[41] Implantation of PEG tubes containing rat islet cultures maintained on an acellular pancreatic matrix was observed to lead to partial rescue of insulin secretory activity.[42]

Alternatively, pancreatic islet cells may be expanded through growth over poly-glycolic acid (PGA) scaffolds. Scaffold-seeded cells may then be further matured into tissue engineered islets within a thermo-responsive gel prior to harvesting and implantation beneath the kidney capsule of streptozotocin-induced diabetic mice, triggering a subsequent return to the normo-glycemic condition.[43] Similarly, transplantation of pancreatic islets grown over a biodegradable scaffold composed of a vicryl fleece with polydioxanone backing and implanted within a canine total pancreatectomy model resulted in normo-glycemia without the requirement for exogenous insulin injection ($n = 4$), in one case up to five months post-implantation. In contrast, dogs receiving an equivalent mass of islets without scaffold did not become normo-glycemic at any time.[44]

The sheet architecture solid organ regeneration platform pioneered for application in liver regeneration[35] has also been applied towards ectopic regeneration of pancreatic tissue.[45] In this study, isolated rat pancreatic islets were expanded over laminin-5-coated $PIPAA_m$ plates. Implantation of the tissue engineered pancreatic sheets within the subcutaneous space of rats led to reconstitution of pancreatic-like tissue structures within seven days post-implantation. As with the liver, the authors speculate that stacking of pancreatic islet cell sheets may lead to regeneration of an increased mass of pancreatic tissue. Note that none of these studies have examined the regeneration of native pancreas *in situ*. This may be a function of the additional challenges inherent in modulating regeneration *in situ*, including the requirement for vascularization and oxygenation

specific to the internal volume of regenerating solid organs located deep within the peritoneal cavity. It will be of significant interest to evaluate whether islet cells implanted within biomaterials and ligated to lobectomized pancreas are capable of catalyzing partial *de novo* organ regeneration.

7.6 Spleen

Although the regeneration of spleen may not be clinically or commercially relevant, the principles developed for engineering these organs may have broader implications for solid organ regeneration. In this regard, progress towards regeneration of spleen has been reported through the use of platform technologies successfully applied towards engineering of small intestine and stomach, these representing examples of tubular, laminarly organized organs with fundamentally distinct micro- and macro-architecture compared with the spleen or other solid organs.[46]

In this approach, organoid units generated by the incomplete digestion of rat splenic tissue were seeded within tubular PGA/PLA (poly-lactic acid) scaffolds, and the resultant composites implanted within the omentum of the peritoneal cavity of splenectomized rats. This ectopic tissue engineered spleen demonstrated splenic tissue organization as well as providing protection against pneumococcal-induced septicemia. Interestingly, spleen slices cultured ectopically within omentum also mediated formation of quasi-spleen-like structures capable of providing protection against induced septicemia.

7.7 Central nervous system

Potentially the best defined examples of a solid organ regeneration platform demonstrating evidence of cellular regeneration *in situ* within a damaged organ *and* catalyzed by implantation of a cell/biomaterial composite at the injury site comes from brain. Tissue engineering of brain and spinal cord typically involves the introduction of gel-based biomaterials within the brain that may be nucleated with neuronal stem cells and may additionally be supplemented with paracrine signaling factors such as vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF) or components of the ECM, including laminin and fibronectin. For example, a biomimetic hydrogel incorporating matrix metalloproteinase (MMP) degradation sites, a laminin-derived peptide and the neurotrophic

factor BDNF was shown to direct the *in vitro* differentiation of seeded mesenchymal stem cells towards a neuronal lineage. Subsequently, this biomimetic hydrogel was introduced into the intrathecal space within a rat model of spinal cord injury. Although no histological evidence for neuronal regeneration was presented, rats treated with the biomimetic hydrogel showed the greatest improvement in tests of locomotion compared with rats treated with non-biomimetic control hydrogels.[47]

In another approach, defined brain defects were created within rat models and subsequently implanted with a gelatin-based scaffold impregnated with VEGF. Histological evidence suggested migration of endothelial, astroglial and microglial cells within the periphery of the scaffold at 30 days post-implantation.[48] This observed regenerative effect was dependent on the presence of VEGF. A demonstration of the application of a non-gel-based scaffold for tissue engineering of brain is the use of poly-lactic-*co*-glycolic acid (PLGA) micro-particles in the 50- to 200-μm size range. These neuro-scaffolds were seeded with neural stem cells and introduced into the brain cavity of stroke-induced rats by directed injection under magnetic resonance imaging guidance. Within seven days post-implantation, the neural stem cells had dispersed within the scaffold, and presented as a tightly packed mass at the center of the biomaterial, but with a broader distribution resembling a honeycomb-like structure towards the periphery. The graft displayed a mixed population of neuronal, astrocytic and stem cell-specific markers, together with evidence of inflammation, but little if any vascularization within the body of the biomaterial. No evidence was provided that the tissue engineered brain tissue had any functional significance in terms of impact to the rat stroke model.[49]

7.8 Summary

When examined together, platform technologies for regeneration of solid organs remain largely as academic proof of concept (see Text-Box 7.2 for a synopsis of factors needing to be addressed for successful commercialization). This is in marked contrast to analogous platform systems developed for tubular organs such as bladder and bladder derivatives, which are currently undergoing Phase I/II clinical trials.[2] As we have seen, there are few published reports documenting the *in situ* regeneration of a solid organ in response to damage as a function of the implantation of a cell/biomaterial composite. The majority of published reports have focused on tissue engineering approaches towards solid organ regeneration using the

Box 7.2 What needs to be addressed to develop a broadly applicable solid organ regeneration platform?

- Identification of a synthetic scaffold structure that is broadly relevant to solid organ parenchyma, regardless of organ type.
- Committed cell types that may be capable of triggering organ regeneration within the context of an implanted scaffold.
- Alternatively, common approaches for the isolation of organ-specific cell populations capable of mediating regeneration within the context of an implanted scaffold. For example, cell populations may be isolated from multiple solid organs by density centrifugation under standard conditions prior to scaffold seeding.
- Standardized methodologies for combining organ-derived or organ-independent cell populations with biosynthetic scaffolds to create implantable composites, for example stacking or piling of cell sheets.
- A focus on methodologies that avoid prolonged maturation periods within bioreactors, but rely instead on triggering the body's innate regenerative potential to stimulate neo-organogenesis.
- Identification of common mechanisms of action being used across multiple organ types through common regeneration platforms.

peritoneal cavity or the subcutaneous space as a living bioreactor to facilitate the vascularization of the regenerating composite that is a prerequisite for organogenesis. However, it is our position that such a methodology may not be conducive to large-scale clinical application, nor does it represent a commercially viable developmental strategy.

In this regard, a comparison with organ regeneration platforms developed for application towards the regeneration of laminarly organized tubular organs may be helpful. The use of committed smooth muscle cells seeded onto a biodegradable scaffold of synthetic polymer such as PGA, PLGA or PCL provides an example of a commercially viable organ regeneration platform that has been successfully applied towards regeneration of bladder and bladder derivatives. The serial stacking and piling of polyester fleeces nucleated with renal primary cell populations[16,17] may represent a viable

solid organ regeneration platform amenable to process development, large-scale manufacture and industrial quality assurance regimens, provided the caveats discussed earlier are addressed. Similarly, serial stacking of tissue sheets engineered by monolayer formation over temperature-responsive $PIPAA_m$ surfaces, as has been demonstrated for liver and pancreas, may be viable for commercial development beyond proof of concept.

Regenerative platforms that focus on committed cell populations such as hepatocytes, pancreatic islet cells and cells derived from the stromal vascular fraction of adipose *instead* of stem and progenitor cells have the potential to substantially reduce cost of goods by avoiding technical challenges associated with the isolation and expansion of stem cell populations, maintenance and monitoring of stem cell potential, monitoring and characterization of directed differentiation protocols, and the costs associated with recombinant cytokines required to drive directed differentiation (Table 7.1). Furthermore, introduction of MSC within the renal parenchyma of rat models of glomerulonephritis has resulted in abnormal differentiation towards glomerular adipocytes, raising the potential for additional regulatory headaches. It remains unclear how the potential for mis-directed differentiation within the solid organ parenchyma may be definitively eliminated.[8] A more detailed summary of these issues is presented in Table 7.2.

The observation that adipose-derived stromal vascular fraction cells are capable of engraftment *in vivo* within liver and may acquire hepatic functionality raises the possibility that readily isolatable adipose cells implanted within the context of synthetic biomaterials or as serially stacked tissue sheets may be capable of stimulating the innate regenerative potential latent within liver and, potentially, other solid organs. To this end, it has been reported that conditioned media from MSC enhance the survival and functionality of pancreatic islet cells following transplantation in diabetic rats.[50] There is no evidence to indicate that the stem cell potential of these cell types is directly connected to their secretomic profiles: it is likely that fibroblasts or smooth muscle cells derived from these 'MSC'-like populations may function just as effectively for paracrine signaling during the regenerative process. Such a combination of readily isolatable, committed cell types complexed with a biomaterial that may be reliably manufactured, has defined physical and chemical properties, and is already acceptable to FDA and other regulatory agencies for implantation within the human body would represent the ideal platform technology for the commercially viable regeneration of solid organs.

Table 7.1 Comparison of solid organ regeneration platforms

Study	Solid organ	Scaffold	Native/ synthetic	Cell type	Regenerative outcome
Ross *et al.*, 2009. Embryonic stem cells proliferate and differentiate when seeded onto kidney scaffolds. *J Am Soc Nephrol* 20: 2338–47	Kidney	Decellularized kidney	Native	ES cells	*In vitro* only
Joraku *et al.*, 2009. *In vitro* generation of three dimensional renal structures. *Methods* 47: 129–33	Kidney	Collagen I gel	Native	Primary rat renal cells	*In vitro* only, induction of tubule/glomeruli within collagen gel
Roessger *et al.*, 2009. Potential of stem/progenitor cell cultures within polyester fleeces to regenerate renal tubules. *Biomaterials* 30: 3723–32	Kidney	Polyester fleeces	Synthetic	Primary rabbit renal cells	*In vitro* only, generation of tubular structures within fleece
Ott *et al.*, 2008. Perfusion decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nature Med* 14: 213–21	Heart	Decellularized heart	Native	Neonatal rat cardiac or aortic endothelial cells	Pump functionality, 2% of adult heart
Lesman *et al.*, 2010. Transplantation of a tissue engineered human vascularized cardiac muscle. *Tissue Eng Part A* 16: 115–25	Heart	Biodegradable, porous	Synthetic	Human ES cell-derived cardio-myocytes + endothelial cells + fibroblasts	Increased vasculari-zation *in vivo* from tri-cell seeded constructs

Table 7.1 *continued*

Study	Solid organ	Scaffold	Native/ synthetic	Cell type	Regenerative outcome
Seif-Naraghi *et al.*, 2010. Design and characterization of an injectable pericardial matrix gel: a potentially autologous scaffold for cardiac tissue engineering. *Tissue Eng Part A* 16: 2017–27	Heart	Pericardial ECM-based gel	Native	N/A	Neo-vascularization *in vivo*
Takeda *et al.*, 2005. Availability of bone marrow stromal cells in three dimensional coculture with hepatocytes and transplantation into liver damaged mice. *J Biosci Bioeng* 100: 77–81	Liver	Corraline HA ceramic disk	Synthetic	Rat hepatocytes/ bone marrow cells	HA scaffolds with both cell types trigger significantly improved blood serum albumin levels within 4 weeks in anabuminic rats compared with monoculture controls
Tai *et al.*, 2009. The use of a polyelectrolyte fibrous scaffold to deliver differentiated hMSCs to the liver. *Biomaterials* 31: 48–57	Liver	Chitosan alginate	Natural	Differentiated human MSC	Implantation within 70% rat lobectomy model expression of hepatic markers within 1–2 weeks
Ohashi *et al.*, 2007. Engineering functional 2 and 3 dimensional liver systems in vivo using hepatic tissue sheets. *Nat Med* 13: 880–5	Liver	$PIPAA_m$	Synthetic	Hepatocytes	Stacking of hepatic monolayers within subQ space in mice resulted in formation of hepatic-like tissue
DeCarlo *et al.*, 2010. Pancreatic acellular matrix supports islet survival and function in a synthetic tubular device: in vitro and in vivo studies. *Int J Mol Med* 25: 195–202	Pancreas	PEG tubes	Synthetic	Rat islet cultures	Partial rescue of insulin secretory activity upon implantation in diabetic rats

Table 7.1 *continued*

Study	Solid organ	Scaffold	Native/ synthetic	Cell type	Regenerative outcome
Kodama *et al.*, 2009. Engineering functional islets from cultured cells. *Tissue Eng Part A* 15: 3321–9	Pancreas	PGA	Synthetic	Pancreatic islet cells	Implantation within renal capsule of diabetic mice results in normo-glycemia
Kin *et al.*, 2008. The use of an approved biodegradable polymer scaffold as a solid support system for improvement of islet engraftment. *Artif Organs* 32: 990–3	Pancreas	Vicryl/ polydioxanone	Synthetic	Pancreatic islet cells	Implantation of islet cells/ scaffolds within canine pancreatectomy model resulted in normo-glycemia w/o insulin. Cells without scaffold did not become normo-glycemic
Shimizu *et al.*, 2009. Bioengineering of a functional sheet of islet cells for treatment. *Biomaterials* 30: 5943–9	Pancreas	Laminin 5-coated $PIPAA_m$	Synthetic	Rat pancreatic islets	Implantation of pancreatic sheets within subQ space of rats generated pancreatic-like structures within 7 days post-implantation
Grikscheit *et al.*, 2008. Tissue engineered spleen protects against overwhelming pneumococcal sepsis in a rodent model. *Surg Res* 149: 214–18	Spleen	PGA/PLA scaffolds	Synthetic	Rat spleen-derived organoid units	Implantation within splenectomized rat peritoneal cavity led to regeneration of spleen-like structures
Bible *et al.*, 2009. The support of neural stem cells transplanted into stroke induced brain cavities by PLGA particles. *Biomaterials* 30: 2985–94	CNS	PLGA microparticles	Synthetic	Rat neural stem cells	Implantation of neural stem cell seeded microparticles within brain cavity of stroke-induced rats resulted in neural cell infiltration within 7 days

Table 7.2 Potential of stem and committed cell populations for application in commercially viable solid organ regeneration platforms

Stem cells	Committed cells
Considerable initial variability in proliferative capacity and multi-lineage differentiation potential (donor effects, passage number in culture, etc)	Straightforward, readily isolatable cell sources. Phenotype not subject to donor variability
Expansion in culture leads to loss of differentiation potential. Monitoring multi-lineage differentiation potential is lengthy and expensive	Readily expanded in culture without loss of lineage-specific characteristics
Directed differentiation is an uncontrolled, inefficient process. Only a proportion of cell population acquires lineage-specific characteristics	No requirement to monitor stem cell potential. No requirement for monitoring directed differentiation
Requirement for inductive cytokines to direct lineage-specific differentiation leads to significant increase in cost of goods	No requirement for recombinant cytokines, substantially decreasing cost of goods
Multiple molecular, proteomic and functional tests required to evaluate stem and differentiation potential. Substantial increase in time, cost and labor. Tests may be misleading and unreliable	No regulatory concerns regarding effects of recombinant cytokines on cell transformation
Long-term effects of inductive cytokines on cells implanted *in vivo* not known. Potential for transformation	Significant reduction in time required to expand committed cell population, as no requirement for extended differentiation period without cell growth
Differentiation protocol significantly increases time frames for generation of differentiated cells (e.g. up to 6 weeks differentiation in 1% FBS-based media). Cells not expandable during differentiation	Application of committed smooth muscle cells for organ regeneration demonstrated across multiple organ systems within *in vivo* models
Potential for abnormal differentiation *in vivo*, teratoma formation	No possibility of abnormal *in vivo* differentiation or teratoma formation

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8

Regulatory and quality control

Abstract. Medicinal product manufacturing requires regulatory agency oversight to ensure that the products are safe for patients. Regulations require that laboratories and production facilities be constructed and maintained according to strict requirements for continued cleanliness. Employees in these facilities must be specially trained and their work monitored during the manufacturing process. The documentation required to be compliant with these regulations helps to ensure that the product is made the same way every time. If there is a problem with the performance of the product, this same documentation can be used during an investigation to find the cause and take any necessary corrective actions. This chapter will cover the general requirements for manufacturing TE/RM products in a regulatory compliant manner.

Keywords: good manufacturing practices, compliance training, quality, investigational new drug, standard operating procedure, Food and Drug Administration, good tissue practices

8.1 Good manufacturing practice

Good manufacturing practices (GMP) are regulations disseminated by the US Food and Drug Administration (FDA) under the authority of the Food, Drug, and Cosmetic Act.[1] As these regulations have the force of law, failure to comply may result in serious consequences, including product recall, company fines and incarceration of those in the company responsible for the failure to comply. GMP is part of a quality system requiring manufacturing and testing of active pharmaceutical ingredients, diagnostics, products and medical devices. GMP is also sometimes referred to as cGMP, for current good manufacturing practices.[2] This is to remind manufacturers that they must employ technologies and systems that are up to date in order to comply with the regulation. The idea behind this is to keep awareness

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among manufacturers that systems and equipment used in previous years to safeguard production may be less than adequate by today's standards.

The purpose of GMP is to enable companies to minimize or eliminate instances of product contamination, confusion and mistakes. In so doing, the consumer is protected from purchasing and using a product which is not effective or perhaps is dangerous. It is important to note that GMP guidelines are not detailed instructions on how to manufacture products, but instead are general guiding principles to observe during manufacturing. Many countries have laws in place governing the manufacturing of pharmaceutical and medical products, and have created their own GMP guidelines corresponding to these laws. In general, these guidelines follow some basic, and arguably common-sense, principles:

- Manufacturing instructions and procedures must be clearly written in language that is not vague; there must be no doubt as to how the steps are to be implemented.
- Processes for the manufacture of a product must be controlled. All critical steps are to be validated to ensure product consistency and compliance with specifications. Any changes to the process which impact the quality of the product must be validated.
- Manufacturing efforts and equipment must be segregated from research and development efforts and equipment.
- Manufacturing personnel, or operators, must be trained to perform and accurately document the procedure.
- Records must be made during manufacturing to show that all of the required steps were performed as defined by the procedures and instructions.
- Any deviations from the defined procedures must be documented.

Key components to the GMP regulation include guidance on record keeping, personnel qualifications, sanitation, cleanliness, equipment verification, process validation and complaint handling. As the intent of GMP guidelines is not to detail instructions on how a product should be manufactured, the majority of the requirements are general, thereby affording each manufacturer the opportunity to decide how to best implement the necessary controls for their own product. While providing appropriate flexibility, this places the responsibility on the manufacturer to interpret the requirements in a manner which makes sense, from a compliance perspective, for their particular product.

Prolific throughout GMP regulations is the concept of quality management.[3] This management is generally divided into two elements, the

first being quality assurance (QA).[4] QA focuses on the systematic monitoring and evaluation of the various components comprising a specific manufacturing process. In so doing, quality personnel are charged with making sure that the product is appropriate and suitable for its intended use or purpose, and that mistakes are not made during production which can negatively impact product performance. QA may also identify areas of improvement during the production and associated processes, further reducing the risk of product failure. In general, items under QA purview include:

- Raw material quality review
- Manufacturing facility
- Product manufacture
- Outside services relating to or impacting production
- Product inspection

The second element of quality management is quality control (QC).[5] QC is concerned with product testing for integrity, with an emphasis on:

- Process and production controls
- Process management
- Product performance criteria
- Record keeping
- Personnel competence for the assigned tasks

Quality management is an integral part of any regulated facility that manufactures medical products under GMP regulations. A generalized flow diagram for a GMP process is illustrated in Figure 8.1.

The version of GMP used by regulators outside the US includes guidelines by the World Health Organization (WHO), used primarily in developing countries, and the European Union's GMP (EU-GMP).[6] Rigorous GMP guidelines have also been developed by, and are followed in, Australia, Canada, Japan and Singapore.[7] In the UK, most aspects of GMP are covered in the Medicines Act of 1968.[8] Regulatory agencies have the authority to conduct unannounced inspections of medical product manufacturing facilities as part of their enforcement policy. These inspections must be performed at a 'reasonable time', which for all intents and purposes means that inspections may occur at any time during the hours of operation for the business. These inspections are conducted to ensure that the facility is in compliance. Using the US FDA as an example, the representative conducting the inspection issues a list of observations documented on form FDA 483.[9] The items on the list are ones which the representative believes

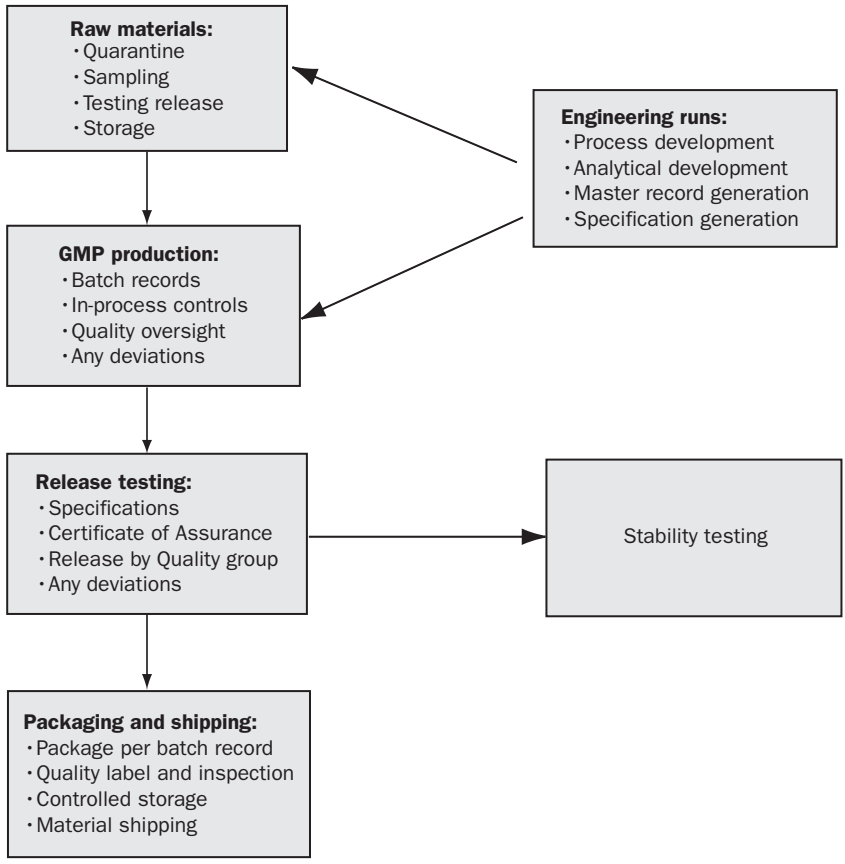

Figure 8.1 Flow diagram illustrating linear progression of processing steps (left-hand boxes) and the timing of the quality functions (right-hand boxes)

deviate from current GMP, and they are communicated to facility officials at the conclusion of the inspection. It should be emphasized here that this list of observations does not represent a final decision by the FDA on whether the facility is in compliance. The following are examples of some of the more common violations cited:

- Procedures in a regulated area failing to conform to current regulations.
- Procedures in a regulated area conforming to current regulations, but not being followed.
- Procedures in a regulated area conforming to current regulations and being followed, but inadequate documentation to support that they are being followed.

It needs to be appreciated that the observations detailed by the investigator represent opinions based on their interpretation of policy and guidelines. While it is not a formal requirement to provide a written response to the items listed on the 483 form, as the majority of companies do provide one, it has become an expectation. Failure to provide a rebuttal to the cited items will allow for a potentially one-sided and biased opinion of the manufacturing organization to the regulatory agency. Actions taken by the manufacturing site can range from none to making significant changes to processes and policies. Facility management may best be served by reviewing each observation cited and deciding whether it represents an unacceptable practice. If the decision is yes, then a corrective action plan needs to be developed. Once completed, the plan needs to be communicated to the FDA, preferably in written form, as soon as reasonably possible.

The FDA wants to see an accurate, clear and concise description of how the observation will be corrected. It is prudent to avoid needless detail and any historical narrative in the description, as this does little to help either the regulatory agency or the manufacturing site to resolve the issue. For observations which, in the opinion of the facility management, need further clarification with respect to regulatory agency policy, others within the FDA may be contacted. Having the original investigator be a part of any discussions between facility management and others within the FDA is advisable. Documentation of discussions, including questions asked, identity of persons taking part and response to questions, is also advisable.

In the spirit of international cooperation and harmony, the European Medicines Agency (EMA) and the FDA have embarked on a joint GMP inspection pilot program.[10] The objective of this joint effort is to better serve the global medical products manufacturing community by optimizing inspection capacity. The goal is to increase the number of facilities monitored while reducing unnecessary duplication of efforts.

There are other good-practice programs that are closely aligned with GMP. For example, good laboratory practice (GLP) ensures consistency and reliability of non-clinical laboratory and research organization results by instituting a quality system of controls.[11] GLP guidelines focus on the processes undertaken for non-clinical safety study planning, performance, monitoring, recording, achievement and reporting. GLP certification was established by regulatory agencies to garner a degree of confidence that the work submitted to them is properly conducted and adequately documented to allow for anyone skilled in the art to replicate the results. GLP study costs are approximately ten times the cost of conducting a non-GLP study, and are not necessary until an organization needs to obtain regulatory approval for further product development. A GLP study requires:

- Staff documentation, including responsibilities for all personnel involved
- An approved and valid study design
- A quality assurance program
- Personnel training documents
- Standard operating procedures (SOP)
- Study performance, including study plan and how the study will be conducted
- Statistical analysis of study data, where applicable
- Results reporting
- Records and reports storage

Good clinical practice (GCP) is another example of good-practice programs.[12] The guidelines contained herein focus on the conduct of human clinical trials, including defining the responsibilities of trial sponsors, investigators and monitors. The purpose of GCP is to protect the human rights of the clinical trial subjects, while at the same time providing assurance of product safety and efficacy.

8.2 Good tissue practices

Good tissue practices (GTP) is a set of regulations covering human cells, tissues or tissue-based cellular products (HCT/Ps) meant for delivery to a human subject.[13,14] These regulations pertain to both the facilities and the manufacturing process by which medicinal products are made from these materials. While many of the GTP requirements are patterned after those found in GMP regulations (i.e. quality programs, controls, process validation), GTP does have some unique requirements focused primarily on preventing the introduction, transmission and spread of communicable diseases. Aspects of these unique requirements include:[15]

1. *Donor eligibility* – medical records must be reviewed for relevant risk factors for communicable disease (i.e. intravenous drug use, tattoos, incarceration). In addition, screening and donor testing is required for a continually growing list of relevant communicable diseases.[16–20]
2. *Tissue and recovery* – the procurement method must not contribute to contamination or risk of communicable disease transmission.
3. *Tissue and cell receipt* – incoming materials must be evaluated for the presence of micro-organisms, damage and contamination. It is well recognized that such materials may contain a high bio-burden upon receipt.

4. *Supplies and reagents* – specifications must be established as they pertain to preventing the transmission of communicable diseases.
5. *Facilities* – while similar to those in GMP facility requirements, additional provisions must be afforded to allow for a means to prevent tissue and cell mislabeling, contamination, and exposure to communicable disease agents.
6. *Environmental control and monitoring* – requirements may be more than for a GMP facility, given that aseptic conditions must be strictly adhered to because HCT/Ps cannot be terminally sterilized.
7. *Equipment* – cleaning procedures must be established and verified that they prevent the introduction and transmission of communicable disease agents.
8. *Process controls* – must establish that products are not contaminated during processing, and that introduction and spread of communicable disease agents are controlled. As part of the controlled process, pooling of human tissues or cells is not permitted.
9. *Labeling* – proper identification of HTC/Ps must be controlled. Unlike GMP requirements, donor eligibility documentation must be a part of this labeling.
10. *Pre-distribution shipping* – transport from donor collection site to processing site must include documentation that measures have been taken to prevent communicable disease transmission during shipping.
11. *Distribution* – packaging and shipping containers must protect against contamination, and there are requirements for completing donor eligibility determination.

In cases where an HCT/P is the raw material for a TE/RM product, both GTP and GMP regulatory requirements must be followed.

8.3 GMP-compliant laboratories and manufacturing facilities

Laboratories that are GMP-compliant are used for a number of applications, the most common being support of pre-clinical or translational research, production of limited numbers of test articles for clinical trials, and further safety development of products already in use for patient treatment. Any facility making a medicinal product for commercialization or test articles for clinical trials must be GMP-compliant (more details are provided in Chapter 10 on manufacturing). This includes laboratory facilities performing fee-for-service manufacturing of clinical trial material or pre-clinical research.

The cost of building a GMP-compliant facility compared with a non-GMP facility can be very high, approaching 30% higher capital expense. This increased cost is due to the premium in fees paid to validation and compliance experts, as well as process engineers. This is in addition to the costs for architectural firms and building engineers. Facility designers must also take into account the need for dedicated and compartmentalized heating, air conditioning and ventilation systems, which will require multiple access points for maintenance and validation personnel. A company wishing to build a facility should have a clear business strategy for optimum utilization to justify and recover the capital costs, as well as the recurring costs of maintaining a compliant operation.

It is advisable for a company wishing to build a GMP facility that architects having experience in designing such facilities be enlisted, and if possible visit a facility they have designed. This will provide a measure of confidence in the skills of the designer, in addition to providing a sense for the company of the finished product. There are a number of items the company needs to consider before the planning and designing phase can begin. For one, the company's manufacturing process should be clearly defined. This will allow for an accurate space map drawing to be made, to ensure accommodation of the work flow, procedural documentation, equipment and utility loads.

It is also vital that the company has its own validation and quality staff to work closely with the contractors developing the design, and the builder constructing the facility. Qualification and validation of the new facility should then be straightforward with a minimum number of issues requiring attention. However, even in the best cases of detailed planning, errors in design and construction can occur. In such cases, modifications made during construction and after completion will further increase the total cost of the facility, perhaps significantly. It is suggested that a company contacts its local design and building firms to discuss their specialized needs for a GMP facility.

8.4 Standard operating procedures and batch records

A SOP is an approved, written procedure describing how a task or operation is to be performed. It ensures that operations are performed over time in as close to an identical manner as possible, and that this performance is in compliance with applicable regulations. A SOP may be general in nature or highly specific. For example, a procedure on how to disinfect a bio-safety cabinet before use may generally be followed regardless of the

manufacturer, model or size of the cabinet. A highly specific SOP needs to be followed for the culturing and growth of a particular cell type for use in a therapeutic product. More than one SOP is usually required to cover all of the various phases of a manufacturing process and its control, listing steps which must be carried out, how they are to be carried out and who will carry them out. Such documents are a requirement for those operating in a regulated industry, such as manufacturing of TE/RM products.

Just as there are various departments and subsequent divisions of responsibility within a company, each department needs to develop their own SOP. While a variety of different formats are deemed acceptable, the following is a list of information essential to each SOP:

- Heading
- Approval or effective date
- Version control
- Introduction
- Scope
- Purpose
- Approval signature
- Document history

The heading should include the company name, document title, date of creation and specific document number assigned by document control. Any changes made to the document after its creation should also be briefly summarized and signed by the owner, or writer, of the document. Responsibilities of the personnel involved in the procedure and references to documents the SOP relied upon are also included. The procedure itself should be described in short steps. An example of a SOP template is shown in Figure 8.2.

When developing a SOP, it may be useful to consider the following groupings to ensure adequate coverage in relevant areas:

- General policies
- Manufacturing procedures
- Clean room operations, procedures and forms
- Quality control procedures and forms
- Quality assurance procedures and forms
- Facilities management procedures and forms
- Warehouse procedures and forms
- Computer systems procedures and forms
- Validation procedures and forms

(Company Name)		Page 1 of 2	
Doc. TYPE	STANDARD OPERATING PROCEDURE	Doc. No.	*(assigned by document control)*
		Eff. Date	*(day-month-year)*
TITLE:	*(operation covered)*		

SUPERCEDES:	*(document numbers of earlier distributed versions of this SOP)*	DISTRIBUTION DATE:	*(when document was sent out for general use)*
DISTRIBUTION AREAS:	*(department using the SOP)*		

DOCUMENT OWNER	Sign & Date
(SOP writer)	
APPROVERS	Sign & Date
(technical approver)	
(quality)	

(Company Name)		Page 2 of 2	
DOC. TYPE	STANDARD OPERATING PROCEDURE	Doc. No.	*(assigned by document control)*
		Eff. Date	*(day-month-year)*
TITLE:	*(operation covered)*		

1.0 PURPOSE: 2.0 SCOPE: 3.0 REFERENCES: 4.0 MATERIALS:

5.0 REAGENTS: 6.0 EQUIPMENT: 7.0 PROCEDURE: 8.0 ATTACHMENTS:

Figure 8.2 Example of a SOP layout detailing the contents of the cover page (top three panels) and the signatures required. Information headings for the contents of the remainder of the document are shown in the bottom panel.

Batch records are documents that extend from a SOP. A batch record accompanies a product during its manufacture, and directs the personnel assigned to making the product exactly how to make it. Batch records must be followed as written and each time a product is made a fresh copy of the

current version of the batch record is issued to the operator. Blank spaces in the document are filled in as the operator performs each task as a means of documenting what was carried out. In cases where a step is critical to the success of the manufacturing process, a witness may be required to observe the operator and then sign off upon completion of that step. Like a SOP, although a variety of different formats are deemed acceptable, the following is a list of batch record features:

- Title or subject of the batch record – this information should be included on each page.
- Identification number – a unique identifier for each record.
- Page number – included on each page.
- Hazard communication – informs the operator of any hazards associated with the procedure and any required safety precautions.
- Procedure – details in a step-wise, chronological manner what the operator will do.
- Label information – instructions for how to label manufactured products so that mix-ups do not occur.
- Quality assurance review – to ensure that the record was properly filled out and completed.

Key information contained in the batch record often includes:

- Date the activities take place
- List of materials used
- Any measurements taken
- All equipment used
- In-process and control results
- Names of individuals performing and/or witnessing the tasks, and anyone who may have filled out the record
- Yield information
- Labeling and packaging verification
- Any sampling taken for analysis
- Any investigations performed

Quality review of batch records generally focuses on:

- The raw materials used in the manufacturing process
- Any changes in raw materials used
- The manufacturing process itself
- Results of any sample analysis

- Any deviations from the process steps
- The results of any investigations

There are a variety of web-based resources and services to guide a newcomer in drafting these types of documents. Examples are provided in references 21–26.

8.5 Personnel training and documentation

As should be evident from section 8.1, GMP regulations are essential directives for the production and testing of medicinal products. But like any mandate handed down by regulatory authorities, their effectiveness is only as good as the training of the people performing the task. It is therefore crucial that employers provide opportunities for employees to become directly involved in assuring compliance with current practices. Regulations handed down by the US FDA and Health Canada are explicit in their language regarding the use of qualified individuals for personnel training in GMP.[27,28] Qualified individuals include experienced employees, subject matter experts and qualified trainers. In all cases, proper procedures and processes must be in place and documented to qualify these individuals. One approach to qualifying trainers is referred to as 'train the trainer'. In this type of program, there is formalized instruction, demonstration of proficiency and assessment of performance. One advantage of this approach is consistent informational content and delivery, thus reducing variation in employee training.

It should also be recognized that formalized training can be costly and time-consuming. An internet search will reveal a number of fee-for-service consulting groups and online courses which can provide such formalized teaching. For some examples, see references 29–31. Another approach to trainer qualification is based on an individual's experience. In this instance, company management makes a judgment as to whether a person is qualified to be a trainer. This judgment should be based on unambiguously defined parameters and documentation. This type of training can be less time-consuming and much less costly than formalized instruction if the trainer also happens to be a company employee.

GMP regulations also require that training be conducted on a continuing basis. In this case, the regulations do not specify the frequency of the training. In general, most companies will have training at a minimum of once per year. Points to consider when developing a continuous training

program include the method of training, volume and type of information to be presented, and number of employees to be trained. For example, classroom-style instruction may work best if there are a reasonably limited number of employees, whereas a web-based approach may provide greater benefit when employee numbers are large. If the type of information to be imparted is technical rather than procedural, individual or small-group training may be in order. The frequency of training is largely dependent upon the volume of information to be covered. It may be advisable to conduct training more at multiple times during the calendar year, thereby covering more manageable amounts of information per unit of time. Each session should be documented with the type of instruction given, the time, and list and signatures of those in attendance.

The types of GMP training may be broadly categorized as:

- Introductory
- Foundational
- Refresher
- Documentation
- Laboratory

Introductory training should be designed as the first exposure to GMP regulations, often including a general overview of GMP principles, terms and language used in the regulatory field. In some cases, a written assessment of employees' understanding of the material may be requested. Foundational training focuses on the importance of following agency regulations and is often taken by all employees at a medical product manufacturing facility. The goal here should be to increase awareness and an ability to promote compliance in the workplace.

Refresher training is, as the name suggests, a program designed for individuals having experience working in a GMP-regulated environment. This includes employees in Quality, Production, Warehouse, Facilities and Senior Management groups. Training is specialized for the unique duties and responsibilities of each group. Documentation practices are critical for compliance with agency regulations, particularly when an audit is performed or when investigating SOP process deviations. Just like refresher training, the information covered is specialized for the unique duties and responsibilities of the various groups within a manufacturing organization. Laboratory GMP training is targeted to Quality group members and focuses on the essentials of a quality control laboratory.

In addition to GMP training, the US Occupational Safety and Health Administration (OSHA) also publishes training requirements.[32] Generally,

every facility will have a safety officer to function as the point of contact for information and compliance questions. Based on the type of products manufactured by a facility, the training requirements will be variable. Fire safety training, however, is the one constant required for all. Flammable and combustible materials are commonplace in most facilities. For this reason, all employees should be aware of the risks in their work-spaces and understand how to respond appropriately should a fire occur. Employees who take the time to familiarize themselves with the location of safety devices (fire extinguishers, pull alarms, safety showers, fire blankets, etc.) and proper route of egress before an accident occurs are more likely to respond to an emergency situation in a calm and efficient manner. Generally, all personnel should be encouraged to implement the RACE acronym in the case of a fire:

- Remove all individuals from the affected area
- Activate the pull alarm and call emergency services
- Close all doors and windows
- Extinguish the fire

For companies engaging in the development and production of TE/RM products, where use of chemicals, mammalian tissue and mammalian cells occurs, both a blood-borne pathogens exposure control plan[33–35] and a chemical hygiene plan[36,37] need to be in place.

The blood-borne pathogens exposure control plan should contain the information necessary to protect workers and the surrounding environment from hazards associated with the use of biological materials. The plan should include guidelines to provide a means for evaluating the risks of work involving biological materials and introduce the proper handling practices that will minimize the risk of an occupationally acquired infection. History has shown that if not handled appropriately, infectious agents can be transmitted to laboratory employees and, rarely, to people outside of the laboratory.

Bio-hazardous materials are those which are either known to cause or may cause human disease. Blood-borne pathogens are defined as any micro-organism capable of causing human disease, which may be present in human blood, human blood components and products made from human blood. Pathogens include, but are not limited to, bacteria, fungi, viruses, parasites and toxins. Hepatitis B virus and human immunodeficiency virus are of major concern. A thorough exposure control plan will consist of:

- Introduction to include scope, program administration, accessibility and review
- Definitions of words and terms used throughout the plan

- Exposure determination, based on job function, to assess the likelihood of exposure
- Compliance methods including engineering and work practice controls, such as personal protective equipment (PPE)
- Employee training
- Record keeping
- Appendices such as the OSHA's blood-borne pathogen standard 29 CFR 1910.1030 and an explanation of different bio-safety levels

The chemical hygiene plan should define the policies and procedures for protecting employees from any potential health hazards associated with the chemicals with which they work. Employees should know the properties of individual chemicals with which they work. The more familiar one becomes with a chemical, the more likely one is to handle the chemical in a prudent manner. There are numerous safety resources available to all employees who may have questions regarding a particular chemical. Material safety data sheets (MSDS) must be provided by any chemical manufacturer or vendor for every chemical purchased by a laboratory or manufacturing facility. These sheets exist in hard copies accompanying each purchase, and in electronic format through the manufacturer and vendor websites. A chemical hygiene plan should include:

- Responsibilities of management and staff
- MSDS explanation
- Explanation of chemical labels on containers
- Safety operating procedures to provide guidance for the safe handling of hazardous chemicals
- Handling practices including procurement, transportation and storage
- Potential physical hazards such as flammability, corrosiveness and explosiveness
- Particularly hazardous substances requiring additional control measures, including carcinogens, reproductive toxins and acute toxins
- Exposure control measures such as chemical fume hoods, emergency showers, eyewash stations and PPE
- Communication hazards related to chemicals produced by the facility

Manufacturers of TE/RM products also need to be aware of the proper ways to ship patient specimens and infectious substances. The purpose here is to protect anyone who comes into contact with the package during shipment, as well as the general public, in the event of a breach in the containment properties of the package. Classification of the shipment is key

in determining the level of containment required for the package,[38] proper package labeling, whether a dangerous goods declaration form is required, and whether the shipment requires a permit or export license.[39] Regarding permits and licensing within the US, further details may be obtained by contacting the Centers for Disease Control Etiologic Agent Import Permit Program,[40] the US Department of Agriculture Animal and Plant Health Inspection Service, National Center for Import and Export,[41] and the Department of Commerce Bureau of Industry and Securities.[42]

8.6 Best practices and the need for harmonization

Best practices are techniques or methodologies which have reliably been proven to lead to a desired result. Such generally accepted, informally standardized procedures may be based on experience, research or common sense. The rationale here is that with proper attention to detail, the desired outcome can be achieved most often, reliably and effectively. It is also important to recognize that a 'best' practice can change for the better as improvements are discovered and successfully implemented. While a best practice generally spreads throughout an industry or scientific discipline after a success has been demonstrated, this spread is often slow. Reasons for this relatively slow adoption may include a lack of knowledge about current best practices, a lack of motivation to make the required changes for successful adoption and a lack of the required skills necessary for adoption. A good place to begin assembling best practices information for a discipline of interest is a portal for online resources.[43]

'Harmonization', for the purposes of this discussion, will refer to efforts aimed towards replacing the variety of product standards and other regulatory policies adopted by different regions with a set of uniform standards which are applied globally. Prior to 1990, medicinal product development varied across international borders. Regulatory requirements were not harmonious; there was duplicate quality, safety and efficacy testing. In short, best practices were not defined across international regulatory agencies and there were often redundant local requirements. Taken together, this lack of defined best practices across locales often resulted in considerable, and it may be argued needless, expenditure of time and resources which ultimately delayed patient access to innovative products.

Scientists, clinicians and pharmaceutical manufacturers began proposing that development of medicinal products be harmonized based on good science and best practices. From these informal discussions came the first International Conference on Harmonization of Technical Requirements for

Registration of Pharmaceuticals for Human Use (ICH) in 1990.[44] The purpose of this meeting was to initiate the objective of increasing international harmonization of technical requirements for medicinal products. Representatives from Europe, Japan and the US agreed that it is important to have an independent evaluation of medicinal products before they are allowed to be marketed and sold to consumers. The ICH, as an international, science-based organization, focuses on the technical requirements for new medicinal products and may be considered a joint venture involving regulators and industry as partners with equal standing. The organization comprises a steering committee, coordinators, secretariat and expert working groups. The process towards development of a harmonized guideline generally includes:

- Building a consensus
- Regulatory action initiation
- Regulatory consultation
- Harmonized text adoption
- Implementation

The areas of focus during this process include *in vitro* data and *in vivo* preclinical safety studies, the quality of medicinal product manufacturing and controls, and the conduct and reporting of efficacy resulting from clinical investigational studies in human subjects. The ICH operates under the assumption that best practices exist, that they are identifiable and that implementation can best be achieved following a joint regulatory agency/industrial manufacturer initiative. To do so will require regulatory agency support for guideline development and adoption of harmonized guidelines across geographic locations. Industry must also agree to support such guideline development and ultimately implement completed guidelines in their respective medicinal product programs. The ICH has produced over 50 international harmonized guidelines, including:

- E6 – Guideline for good clinical practices[45]
- E5 – Ethnic factors in the acceptability of foreign clinical data[46]
- Q7 – Good manufacturing practice guidance for active pharmaceutical ingredients[47]
- Q6A/B – Specifications: test procedures and acceptance criteria for new drug substances and new drug products; Chemical substances;[48] Specifications: test procedures and acceptance for biotechnological/biological products[49]
- Q3A/B/C – Impurities in new drug substances;[50] Impurities in new drug products;[51] Impurities: guideline for residual solvents[52]

- Q1A/B – Stability testing of new drug substances;[53] Photostability testing of new active substances and medicinal products[54]
- M4 – Common technical documents for the registration of pharmaceuticals for human use[55]
- MedDRA – The medical dictionary for regulatory activities[56]

In 1999 a Global Cooperative Group (GCG) was formed within the ICH in response to a growing interest in harmonization beyond the original three regions of Europe, Japan and the US.[57] Representatives from other regional harmonization initiatives were invited to participate in the GCG. They included:

- APEC – Asian Pacific Economic Cooperation[58]
- GCC – Gulf Cooperative Council[59]
- PANDRH – Pan American Network for Drug Regulatory Harmonization[60]
- SADC – South African Development Community[61]
- ASEAN – Association of Southeast Asian Nations[62]

In 2007, this group was further expanded to include regulators from countries supportive of ICH guideline implementation as well as countries with a global presence in medicinal product manufacture and research. These countries included Australia, Brazil, China, Taiwan, India, South Korea, Russia and Singapore. There are other groups also working on harmonization. They include the USP Pharmacopias Group,[63] WHO, the Pan American Network for Drug Regulatory Harmonization and the International Pharmaceutical Excipients Council.[64] Critics of the ICH state or infer that regulators often focus on harmonizing around their local regulations rather than best practices, often not addressing fundamental and therefore key issues. The global harmonization process is also time-consuming and very resource-intensive. Cultural issues, with respect to the review and regulatory systems, can stalemate this process. Nonetheless, the ICH can point to substantial gains towards global harmonization for which they have been largely responsible.[45–56]

More recently, the field of TE/RM has come to the forefront of harmonization efforts. The driver for such efforts is the use of human cells for these types of therapeutic approaches. International regulatory agencies are keen to promote these efforts as a move towards addressing what may be interpreted as unrealistic expectations and misconceptions within the general public regarding the use of human cells, particularly stem cells, as a therapeutic. The ICH may consider the following areas as directly relevant to evaluate the efficacy of therapies using human cells:

- A standard of reporting and publishing criteria so that comparisons across investigators can be simpler to make
- Standardize the technology to enable comparison of validation assays
- Relevant animal species/models should be universally accepted
- Separate professional standards of defined success for a cell therapy from regulation standards
- Better management of conflicts of interest
- Technology to assess bio-distribution and cell fate
- Data for optimal timing of stem cell delivery

The last two bullet points above are critical areas of contention within the scientific community and the regulatory agencies. Tracking cells after delivery and monitoring cell stability and fate are parameters which need to be addressed. Precisely where the cells ultimately reside following delivery can have adverse effects on patient health and well-being. For example, cells delivered to the heart muscle may, if they enter the bloodstream, take up residency within the lung and interfere with pulmonary function. Such an effect on the lung may be further complicated if the fate of these cells is to become cancerous, a demonstrable outcome for some stem cells. In fact, controlling differentiation of stem cells is of sufficient concern from a regulatory perspective that committed cells may be a more logical starting point for therapies using cells in their formulation (see Chapter 1, section 5, for a more detailed discussion on this subject). The lifespan of cells delivered to the body can also influence the bio-distribution, fate and optimal delivery timing. If implanted cells exist in the body for 48 hours or less then they may be considered transient, and the regulatory bar to enter the clinic is lower for transient cell therapies.

8.7 Investigational New Drug application

The US FDA requires a medicinal product sponsor, usually a manufacturer, to submit an Investigational New Drug (IND) application to support safely beginning clinical trials of new pharmaceuticals or biological products in human subjects. The application serves two major functions. First, this document is the means by which the sponsor legally obtains exemption from FDA rulings governing the interstate transportation and distribution of drugs or biologics during clinical investigation. Second, an IND application is a critical first step towards drug approval for commercialization. Prior to IND submission, it is expected that the sponsor will have approached the FDA to engage in a pre-IND consultation. This meeting will help the sponsor understand the types of data necessary, and

what the FDA is expecting, to warrant an IND submission. Early establishment of a good rapport with FDA division personnel who will be reviewing your submission helps to make the path towards IND allowance much smoother. There are three types of IND applications considered by the FDA (Investigator, Emergency, Treatment), and two IND categories (Commercial, Research (non-commercial)), but we concentrate here on commercial IND only.

As per guidance by the FDA,[65] an IND must contain information in the following three areas:

1. Animal pharmacology and toxicology – preclinical animal data used to determine whether the test article is reasonably safe to begin human testing.
2. Chemistry and manufacturing – data relevant to the manufactured product composition and stability, and controls used for producing the product. The FDA needs to be confident that the company can reliably produce and supply consistent batches of the product.
3. Clinical protocols and investigator information – protocol details will be scrutinized to determine the degree of risk the test subjects will be exposed to. The clinical investigators will be evaluated as to their qualifications to administer the test article and to fulfill their clinical trial duties. In addition, information relevant to the commitment by the sponsor to obtain informed research subject consent, study review by the institutional review board of the test site and adherence to investigational new drug regulations must be provided.

Detailed requirements for a new IND include:

- Chemistry/manufacturing data to ensure identification and quality of the product, in addition to information regarding sterility.
- Pharmacology/toxicology data from acute studies conducted in two species, such as rodents and canines, with histopathology in at least one of the two species.
- Pharmacokinetic and toxicity studies employing the same parameters as proposed in the clinical trial.
- Clinical trial design which defines the therapeutic area, patient population and efficacy endpoints.

An Investigator's Brochure (IB)[66] must also be submitted as part of the IND package. In this document, any information relevant to product testing in humans needs to be compiled and presented. The purpose is to provide

investigators with sufficient information so they can understand the rationale for the study design and the importance of complying with the protocol, particularly with respect to the dose, dosing frequency, administration methods and safety monitoring procedures. It is the sponsor's responsibility to keep the information contained in the IB current. It is suggested that the brochure be reviewed at least annually, and updated when any new information which can affect the trial becomes available. The brochure should contain the following information:

- Sponsor and product names
- Date of release and revision number
- A table of contents
- Confidentiality statement and signature page
- Document summary
- Introduction to cover the product and target treatment population
- Physical, chemical and pharmaceutical properties and formulation
- Non-clinical studies and pharmacology
- Pharmacokinetics and metabolism in animals
- Toxicology
- Effects in humans
- Pharmacokinetics and metabolism in humans
- Safety and efficacy
- References
- Reports
- Summary data and guidance for the investigator

Once submitted, the FDA has 30 days to respond. During this time, the FDA will be reviewing the document for safety to make sure that the human test subjects will not be placed in an unreasonable risk situation. If the agency does not respond to the sponsor with sufficient concerns and place the application on hold, the sponsor is free to begin the clinical trial. Investigators, manufacturers and regulators outside the US are encouraged to follow ICH guideline E6 for good clinical practices.[45]

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9

Pre-clinical and clinical evaluation of TE/RM products

Abstract. There is an established pathway for pharmaceutical compound development and review by the FDA. Developers of TE/RM products have to improvise certain parts of this established pathway, especially regarding ADME Tox testing. Both *in vitro* and *in vivo* animal testing is required before the FDA will allow any medicinal product to be tested in humans. These studies need to be well defined, controlled and monitored. Development and execution of a clinical trial rely heavily on the experience of the clinical investigator, who works in close collaboration with the study sponsor. For small companies who may not have the depth of experience required to monitor the study within their own employees, there are opportunities within the commercial sector to hire these individuals on a fee-for-service basis. This chapter will cover key aspects of the product evaluation process.

Keywords: ADMET, ADME Tox, combination product, device, biomaterial, biocompatibility, animal models, scaffold

9.1 Regulation of TE/RM products

TE/RM products represent a relatively new therapeutic area for regulatory agency oversight. This has posed challenges not only for the US Food and Drug Administration (FDA) in how these products are categorized, as they may contain a combination of cells, biomaterials and devices, but also in how manufacturers need to think about testing and characterizing their product. A recent publication by Lee *et al*. provides an excellent overview of the FDA process and some of the key scientific considerations which may be applicable to developers of TE/RM products.[1] These authors emphasize several key points to consider when planning clinical trials with TE/RM products:

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1. The appropriate regulatory pathways need to be identified as early as possible during product development.
2. Engage regulatory agencies, the example used being the FDA, early during product development. The FDA encourages such informal exchanges so that they can have a preview of what they will be seeing during the anticipated formal filings. At the same time, the product developer can gain valuable insight into what the agency will be looking to see in the filing.
3. Be up to date and well versed in all relevant agency guidance documents and regulations.
4. Try to anticipate questions that the agency will be asking at the clinical phase of product development.
5. Plan for changes in product design and manufacturing.

The remaining sections of this chapter will focus on specific components of TE/RM product development pertinent to regulatory agency evaluation. A complete list of guidance documents from the three FDA centers in charge of regulating medicinal products [Center for Biologics Evaluation and Research (CBER), Center for Drug Evaluation and Research (CDRH), and Center for Devices and Radiological Health (CDER)] can be found on their respective websites.[2–4]

9.2 Preclinical studies

Preclinical studies, sometimes referred to as preclinical development or non-clinical studies, focus on obtaining safety data prior to testing in humans during a clinical trial. Data from iterative and feasibility testing are also collected and analyzed during the preclinical studies. During the 1990s, there was a dramatic rise in technological advances relevant to *in vitro* preclinical testing. Specifically, techniques were developed to assess the disposition of pharmaceutical compounds within isolated cells and tissues. Referred to by the acronym ADMET or ADME Tox,[5] this collection of tests has become the standard by which all medicinal compounds are judged:

1. *Adsorption* – how efficiently the compound is taken up by the target cells. Assays include cell permeability and transport, by measuring the rate at which compounds of interest can be found inside the cell after being delivered to the cell culture media. Compound solubility and pH stability are also examined because during *in vivo* oral administration a drug will be exposed to the low pH of the digestive system.

2. *Distribution* – defined as the reversible transfer of the compound from one compartment to another. *In vitro*, this is measured by the ability and tenacity with which the compound reversibly binds to proteins in the blood plasma and target tissue proteins. The idea is to mimic as much as possible a scenario whereby the compound enters the bloodstream, is distributed throughout the body and is presented to the target tissue.
3. *Metabolism* – the rate at which the compound is broken down by the cells. The majority of small molecule compounds can be metabolized by the cytochrome P450 family of enzymes in the liver. *In vitro*, test compounds are added to cultures of hepatocytes and the rate of metabolite formation and production is measured.
4. *Excretion* – compounds and their metabolites need to be removed from cells. Assays include p-glycoprotein (P-gp)-mediated efflux by canine kidney cells stably transfected with the human multi-drug resistance gene. What is measured is the ability of the compound to serve as a substrate for P-gp, which is a membrane-bound transport protein. The rate and amount of compound appearing in the culture medium after dosing of the cells may also be measured.
5. *Toxicity* – the degree to which a compound causes damage to a cell. This is sometimes measured and reported as the amount of compound required to kill 50% of the cultured cell population (LD_{50}) after a defined duration of exposure. Other *in vitro* tests include cell mitochondrial toxicity, as measured by disrupted energy metabolism, increased free-radical generation, and altered apoptosis and genotoxicity, as revealed by chromosomal alterations and gene mutation analysis.

These advances in testing have provided an opportunity to assess pharmaceutical compounds on human and other mammalian cells *in vitro*, thereby complementing *in vivo* animal data by the addition of information predictive of human cell metabolism and toxicity. However, TE/RM products rarely, if ever, lend themselves to such pharmacologic and toxicologic studies as the 'small molecule' drug compound development programs have. The major reason is that these products contain live cells either alone or in combination with natural or synthetic materials. In regulatory agency language, the material component of this combination product (cells + materials) is referred to as the device portion, or the biomaterial.

Using the ADMET paradigm, cells alone or in combination with a biomaterial are not generally adsorbed by other cultured cells in a manner comparable with pharmaceutical compound adsorption. As they are not adsorbed, they cannot be metabolized or excreted in the classical sense of

these *in vitro* assays either. As such, products containing cells require substantial additional information to be provided to regulatory agencies in order to assess safety of the proposed regenerative medicine product. Data may be obtained through numerous different types of studies, with the expectation of combining various experimental systems to assemble a data set of sufficient comprehensiveness to permit sound decisions regarding the conduct of human clinical trial studies. TE/RM product parameters to be tested *in vitro* often include:

- *Cell viability* – the percentage of cells within a population that are alive. This may be measured by a variety of methods, including colorimetric, fluorometric and enzymatic. The simplest method is trypan blue dye exclusion.
- *Cell morphology* – shape and appearance as determined by microscopy.
- *Cell phenotype* – physical characteristics, often assessed by expression of specific proteins using immunohistochemical approaches.
- *Cell function* – may be measured by specific enzyme synthesis and/or activity, cytokine production, or secretome analysis.
- *Cryopreservation* – how well the cells tolerate the freeze–thaw cycle. May be investigated by determining the number of cells recovered, their viability and the attachment efficiency to tissue culture plastic or biomaterials post-thaw.
- *Biocompatibility of cells with biomaterial* – will the cells attach to, remain viable, grow and remain metabolically active when combined with a biomaterial, whether it be natural or synthetic.

As TE/RM products cannot be evaluated in the same way as pharmaceutical compounds with respect to ADMET, what is needed for these products is a quantitative analysis of biomarker expression to define the mechanism of action and assessment of safety and efficacy. A biomarker is an indicator of some biologic, pathologic or pharmacologic pathway, and is typically used to predict a disease state or treatment response. Biomarker discovery generally involves cataloging differences between treatment groups that respond favorably to a therapeutic from those that respond poorly or have no response at all. Understanding the pathophysiology of the disease state being addressed will help in developing a short list of biomarkers to be examined. Types of biomarkers include those for disease (i.e. increased blood levels of glucose in patients with diabetes), efficacy or outcome (i.e. lower blood glucose levels associated with diabetes treatment), surrogate (i.e. elevated blood levels of low-density lipoprotein in patients with

insulin-resistant diabetes) and mechanism (i.e. increased free-fatty acid concentration inhibits the anti-lipolytic action of insulin, contributing to insulin-resistant diabetes). Some biomarkers may also be used as prognostic or predictive indicators of disease [i.e. elevated blood glycated hemoglobin (A1C) in patients developing diabetes].

Biomarker analysis of TE/RM products is the best correlation to the established regulatory path of ADMET testing which pharmaceutical compounds go through. The technology available to perform biomarker discovery and analysis includes:

- Multiplex cytokine array and secretome analysis to address function, potency and fitness of use
- Flow cytometry and immunohistochemical staining to assess protein expression and phenotype
- Automated biochemical analyzers for metabolite analysis
- Real-time polymerase chain reaction to quantitate gene expression

These technologies may be used in combination to analyze TE/RM products consisting of cells alone (cytokine array, flow cytometry, polymerase chain reaction), spent culture medium from cells alone or cell/scaffold combinations (cytokine array, biochemical analyzer) and cell/scaffolds (immunohistochemical staining, polymerase chain reaction). These same technologies can also be used for *in vivo* biomarker analysis of urine and blood following TE/RM product treatment of the patient, for the purpose of correlating outcomes with the *in vitro* analysis, a key component to any ADMET testing for product development.

When pursuing biomarker development, rigorous study of the identified biomarkers should be carried out to avoid a situation where a TE/RM therapy may change a biomarker, but is irrelevant to the disease state. TE/RM products may also work by affecting multiple mechanisms involved in the regenerative process, so it is important that the biomarker represents a clinically important effect. Caution must be exercised when proposing surrogates. If accepted as a substitute for a desired clinical outcome, because the results may be measured in a shorter time frame compared with waiting for a clinical trial to be completed, it needs to be recognized that the surrogate may not accurately predict the final clinical outcome. It is therefore prudent to integrate the results of biomarker analysis with the relevant biology of the system examined. It may also be better to qualify a biomarker for its intended use, rather than validate a biomarker using statistical correlation. Finally, a panel of biomarkers, or a combinatorial approach, may be more accurate than relying on a single biomarker to make decisions.

Perhaps the most basic requirement in any medicinal product development program is data to provide reasonable assurance of the product's safety. Unlike pharmaceutical compounds, TE/RM products which contain cells need to be evaluated for the potential to go through harmful changes in their characteristics, such as malignant transformation. This is especially important for products containing cultured stem cells.[6] As a consequence, the model design needs to address this potential and should include a sufficient number of cells to detect rare events with reasonable statistical confidence. It is also advisable to use models which approximate the human condition, so that the investigator can assess the potential for any adverse events. With a thorough study design, the proof-of-concept studies, which are used to confirm the scientific merit and medical approach to the unmet medical need, will often reveal potential adverse effects.

Regardless of whether a disease model or healthy animals are used, studies intended primarily to provide safety data are termed pivotal toxicology studies. Proof-of-concept studies should also provide data regarding the potential duration of the clinical effect following test article delivery. Here again, unlike traditional small molecule pharmaceutical products, these data are of considerable importance owing to the inherent risks of any therapy involving a product which contains live cells. If such a therapy fails following a short time interval of benefit, this may be viewed as a late-occurring toxicity event or outright failure of the treatment. An additional point to consider is whether to use autologous or allogeneic cells in the product. An adverse immune response by the recipient of an allogeneic cell-containing product is almost a certainty,[7] so animal studies designed to evaluate immunosuppressive approaches may be required.

Small animal models for medicinal product testing are the more cost-effective approach towards establishing proof-of-concept. Purchasing, housing and per diem care costs are multiple-fold less expensive than large animal studies. Once proof-of-concept is established, however, regulatory agencies usually expect to see large animal good laboratory practice (GLP) studies in Investigational New Drug documents filed by a sponsor. The rationale here is that larger species such as dog and pig are generally more similar to humans in specific organs or organ system physiology. For TE/RM products, particularly those involving an implantable scaffold or device, these larger animals will also permit testing of items similar in size to those proposed for people. In rare instances, the sponsor may persuade the FDA that a small animal study is sufficient, given that a large animal model with which to perform the required studies is not available or appropriate. Protocol development for animal studies follows the same scientific principles as would be expected for any *in vitro* experimental investigation;

an objective is defined and supported by a description of the materials and methods used to address the objective. One difference, however, is that compliance with applicable animal welfare regulations must also be stated in the protocol and be supported by the appropriate documentation. A final report is issued upon completion of the study, which includes the results and conclusions. In the case of GLP studies, this final report is quality assured.

9.3 Clinical protocol development

The protocol for a clinical trial contains the study plan on which the clinical trial is based. This document allows for investigators at multiple sites to conduct the study in an identical manner, which is critical for the data to be combined and analyzed together. The protocol serves as a reference tool for study managers and administrators, in addition to providing the investigator with a description of their duties and responsibilities. In general, the protocol explains the objective of the study, its design and methodology, and the trial organization. Also included are descriptions of who may participate as a study volunteer, the level of participation which is expected for the volunteer to remain in the trial, as well as the length of the study. The US, European Union and Japan have made efforts to standardize the format and content of clinical trial protocols according to guidance documents produced by the International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use regarding good clinical practice.[8] The following sections are intended to provide additional detail on the organization and content of a generic clinical trial protocol.

- *Title page* – contains the phase of the study (Phase I, II, III, etc.), study design (i.e. randomized, non-randomized), multi- or single-trial site, the name of the medicinal product being studied, the disease target, name of the study sponsor, funding institution and unique identifying number of the protocol.
- *Table of contents*
- *List of abbreviations used throughout the document*
- *Study summary* – includes information found on the title page (title, study phase, protocol number, study design, disease target, study centers) in addition to a brief statement of the primary objective of the study, number of anticipated study volunteers, main inclusion criteria for study volunteers, reference therapy (i.e. current standard of care) and a brief description of the statistical methodology used for analysis.

- *Introduction* – includes an opening statement that the study will be conducted in compliance with international standards of good clinical practice. After this statement, provides a background discussion on the target disease, a description of the medicinal product to be investigated, a summary of all available pre-clinical and clinical data, a rationale for the dose, and a risk/benefit discussion.
- *Study objectives* – includes both primary and secondary objectives as they relate to the study purpose.
- *Study design* – generalized description to include the study type (i.e. randomized, non-randomized), a flow diagram illustrating the procedures, duration of study volunteer participation, and a description of the sequence and duration of trial periods including follow-up. Primary (and secondary, if any) endpoints of the study also need to be described. This is dependent upon the study's main objective (i.e. safety, efficacy). Also included is a description of the primary safety endpoints to be measured, if different from the primary study endpoint.
- *Test subject selection* – creates a list of criteria which volunteers must meet to be considered for enrollment into the study (i.e. target disease, age), as well as criteria for study exclusion (i.e. drug use). Also includes a section describing how volunteers will be recruited (i.e. referring clinicians) and screened (i.e. clinical laboratory testing).
- *Test subject withdrawal* – in some situations a volunteer may be asked by the investigator to withdraw from a study (i.e. failure to keep scheduled follow-up appointments for data collection), or they may choose to withdraw on their own (i.e. the volunteer withdraws their consent to participate in the study). It is important that the investigator makes every effort to obtain, at a minimum, survival data on anyone leaving the trial before its completion.
- *Study article* – brief description of the medicinal product, including the treatment regimen, how study volunteers were assigned to study groups, the steps required to prepare the study material and how it is administered. Also include how the study monitors will assess and track study volunteer compliance with the treatment regimen, and any prior or concomitant therapy that the volunteer has or is undergoing. Packaging and shipping of the test article also need to be described, along with receipt and storage conditions (i.e. temperature, humidity).
- *Study procedures* – divided by visit, describe the procedures and treatments that the study volunteer will be subjected to.
- *Statistics* – a description of the statistical methods used for determining the sample size for the trial. Include the overall statistical approach used for analysis and a definition of the subject population.

- *Safety and adverse effects* – defines what an unanticipated adverse event (i.e. any experience that develops or worsens over the course of the study) and a serious adverse event (i.e. prolonged hospital stay, disability, incapacity, death) are, defines the reporting period for such an event, how such events will be recorded and reported to the trial sponsor, institutional review board (IRB) and the FDA, and any pre-existing condition which may result in an adverse event, abnormal laboratory values, hospitalization or surgery.
- *Data handling and record keeping* – includes information on how confidentiality will be maintained and managed, how source data, which are contained in source documents, will be kept secure, the required information to be recorded in patient case report forms, as well as the policy for retention of these records.
- *Study monitoring* – describes the frequency and duration of monitoring visits, and what will be expected to be provided by the clinical site to the monitor for auditing.
- *Ethical considerations* – a statement attesting to the study being carried out in applicable regulatory compliance.
- *Finances* – provides information as to how the study will be financed (monetary amounts are not required), any conflicts of interest on the part of the clinical investigator and any payments to study volunteers.
- *Publications* – a clear description of publication policy (i.e. who owns the trial data, who needs to be consulted as to if or when the data can be published, and who is responsible for publications).
- *References* – contains information cited in the protocol.
- *Attachments* – all relevant documents associated with the trial (i.e. investigator agreement, volunteer consent, study flow chart).

9.4 Clinical trial

Clinical trials are human research studies designed to assess the effect of new medicinal products or treatments in study volunteers. The treatment may be a brand new one for a previously untreated or untreatable ailment, or the trial may be a comparative one to a treatment that is currently in use. Clinical trials are the process by which new treatments and products are tested for safety and effectiveness, a prerequisite for regulatory agency approval. These trials generally involve volunteer patients with specific health issues which the investigator believes will benefit from this new treatment. Based on results from the preclinical studies, the investigator identifies the product which will be tested. It is also prudent to involve a

panel of expert investigators or key opinion leaders during the development of the study design, especially if the new product to be tested is proposed to replace current standards of care. This panel can also advise on the types of patients who are most apt to benefit from the new treatment. In the US, the National Institutes of Health classifies clinical trials based on their purpose.[9] For example:

1. A compassionate use trial is a means to provide experimental medicinal products before the FDA has given final approval for use in humans. The FDA must approve compassionate use, usually only after a case-by-case analysis. This use is granted only for individuals who are very ill and have no other treatment options available to them.
2. Diagnostic trials are conducted to define tests or procedures that have better accuracy, sensitivity or turnaround time for diagnosing a particular disease or condition than is currently available. This type of trial usually includes volunteers who exhibit symptoms of the disease or condition being studied.
3. Expanded access trials allow for the distribution of experimental drugs to participants who are not responding to currently available treatments for their illness and who are also unable to participate in ongoing conventional clinical trials.
4. Prevention trials are designed to discover better ways of disease prevention in people who have never had the disease or to prevent recurrence of a particular disease. These types of trials may include the use of medicines, vaccinations, dietary supplements such as vitamins and minerals, or changes in lifestyle.
5. Quality of life trials are sometimes referred to as supportive care trials. During these trials, ways to improve comfort and quality of life for individuals with a chronic illness are investigated.
6. Treatment trials test novel treatments, which may include new combinations of drug therapies, new approaches to surgery or radiation therapy, or application of TE/RM products.
7. Screening trials are designed to address the best and most practical way to detect diseases or health conditions.

Great care needs to go into choosing a study design. As TE/RM products most often fall into the treatment trial category, the remaining part of this section will focus on designs for this type of clinical trial. It needs to be acknowledged that these design criteria were initially produced for pharmaceutical compound drug testing, and modifications to the approach will have to be made when testing cells and devices containing TE/RM products.

There are two main types of treatment studies: randomized and non-randomized. In a randomized controlled trial (RCT), study volunteers are allocated at random to receive one treatment type over another. One of these treatments is the control, which is usually the standard of care. In some trials the control may be no treatment at all. The randomization is designed to prevent any bias that the investigator has from affecting the study results and their subsequent interpretation. Randomization procedures should include equal treatment group sizes to allow for adequate statistical powering; they should have low selection bias to prevent the investigator from inferring the next group which the next study volunteer will go to, and there should be a balance in covariates across the groups. In a double-blind randomized trial, neither the study volunteers nor the clinical investigators know who belongs to the control group and the test group. Only upon completion of the study is this information revealed.

Double-blind randomized studies have proven to be extremely successful towards eliminating subjective bias of both the study subjects and the clinical investigators, as neither knows to which group the study volunteers belong. In contrast, single-blind studies, in which the investigator knows but the study volunteer does not, runs the risk of the volunteer becoming biased as a result of their interaction with the investigator. When both investigators and study volunteers know to which group the volunteers are in, the RCT is called unblinded or open. To alleviate as much bias as possible from these types of studies, it may be desirable to blind people assessing the data. As the clinical outcomes are measured after the study participants receive the full course of the treatments, RCTs are quantitative studies, which make them one of the simpler and more rigorous ways of determining a cause-and-effect relationship between treatment and outcome in clinical research. Despite the widespread acceptance of the scientific merits of randomization, there may be reluctance on the part of clinicians to participate in RCTs.

The main reason for this reluctance seems to be that physicians feel that the patient–physician relationship is compromised if the physician must explain to the patient that the treatment for their cancer, for example, would be chosen by a 'coin toss' or 'computer'.[10] The United States Code of Federal Regulations governing human experimentation has been interpreted to imply that a physician must tell the patient about the use of randomization. Thus, as a result of the non-participation by subpopulations with disease, results of an RCT may not necessarily apply to the entire patient population. Caution must be exercised when extrapolating the inference from a clinical trial to the entire population with disease.

A non-randomized clinical trial is one in which the study volunteers are not assigned by chance to different treatment groups; they may choose which group they want to belong to, or they may be assigned to the groups by the clinical investigator. The reporting of a non-randomized trial requires special care, especially when claims are made about efficacy. Reporting should address the potential biases that could affect the conclusions.[10] This is especially true for trials which use a comparison with a historical control group. Nonetheless, a non-randomized study is valid for evaluating pilot studies of novel therapies, provided that the potential biases are recognized. Non-randomized studies are also compelling when the treatment outcome is so remarkable that it cannot be explained by the combination of the potential biases.

Clinical trials are typically divided into four phases, each of which is treated as a separate entity requiring its own clinical investigation. A description of each is provided below:

- Phase I – Initial studies on a small group of volunteers to determine if there are any adverse effects of the treatment. These studies may include patients who do not have the disease for which the new medicinal product is designed to treat. At this phase, the overall safety of the treatment is unknown.
- Phase II – This is a controlled clinical study to evaluate the effectiveness of the product for a particular illness in volunteers with the illness. Phase II can commence only after the safety of the treatment has been demonstrated during Phase I. The treatment group is generally larger than in a Phase I trial. The purpose is to determine if there are any common short-term side effects and risks.
- Phase III – Controlled and uncontrolled trials, often randomized and involving multiple centers, include a greater number of volunteers than in Phase II. The goal is to gather additional information to evaluate the overall effectiveness and benefit–risk relationship of the medicinal product, including any side effects, overall safety and quality of life.
- Phase IV – After regulatory agency approval, this phase involves post-marketing studies to gather additional information regarding the product's risks, benefits and optimal use. The purpose is to allow for detection of any rare or long-term adverse effects among a larger patient population that may not have been evident during the previous phase trials.

It needs to be appreciated that clinical trials are very costly and take years to complete. The costs are most often borne in their entirety by the

sponsoring company developing the medicinal product. These costs include manufacturing of the product, compensation for the personnel at the clinical site and monitoring. The length of time the trial takes to complete is dependent at first upon the rate at which study volunteers can be enrolled. As one would expect, only certain people will have the target illness. Of these, only a subset will meet other selection criteria, such as the absence of other diseases or health-related issues, which may interfere with the ability of the new treatment to be effective. The sooner volunteers can be enrolled, the sooner the product may reach the market. It is therefore in the sponsor's best interest to accelerate enrollment as this will translate into a reduction in overall trial costs. Because there may be a conflict of interest here, it is important that the utmost ethical attention be paid to all volunteer recruitment proposals. Finally, with respect to the time it takes to conduct a clinical trial, it may take years to determine if the new therapy has the desired effect in patients with a chronic illness.

9.5 Clinical trial site monitoring

Clinical trial sites are monitored to ensure oversight of the clinical study by the sponsor. The monitor may be a qualified employee of the sponsor or a contract employee, representing the sponsor, from a contract or clinical research organization (CRO). Regulatory agency guidelines specify that all clinical trials have a process in place for appropriate oversight and monitoring to ensure the safety of the participants and the soundness of the outcomes data. Site monitoring visits are usually divided into four types, described below.

1. *Pre-study visits.* Sometimes referred to as site selection or site qualification visits, the goal is for the sponsor to determine if the clinical study investigator and the clinical facility have the ability and capacity, respectively, to conduct the study. This visit usually involves a facility tour and a discussion of the basic fundamentals of the study, especially as they relate to patient recruitment. Also discussed are qualifications and responsibilities of the clinical investigator, informed consent requirements given by the IRB of the site, documentation, test article and study record security, and adverse event reporting.
2. *Site-initiation visit (SIV).* Once a site is selected, a team from the sponsor will meet with essential staff at the site to provide training directly relevant to the trial protocol. This includes study overview and

patient eligibility requirements, requirements for test article shipping, receiving and handling, good clinical practice guidelines,[11] and case report forms (CRFs). At this point, the sponsor or CRO will make sure that the clinical investigator fully understands their responsibilities as outlined in 21 CFR 312.[12] This visit usually takes place after all regulatory requirements have been fulfilled and IRB approval has been granted for the study to begin. The SIV is the last step before the clinical trial site can begin enrolling patients for the study. Generally, those in attendance from the sponsor include a monitor, sometimes referred to as the clinical research associate, a medical monitor, often a person holding a medical doctorate with or without practical experience in the area of the treatment to be studied, and a project manager. From the site, the principal investigator, the clinical trial coordinator and the data manager should be in attendance.

3. *Monitoring visits.* A monitoring schedule will be developed by the sponsor or CRO which defines the frequency and duration of these visits. The purpose of these visits is to ensure that the trial site is compliant with all applicable regulations, guidelines and the study protocol. The monitors will have access to all source documentation and CRFs. These visits provide an opportunity to assess site accuracy in reporting, as well as making sure the investigator continues to carry out the agreed-upon activities, and has not delegated them to previously unspecified personnel.
4. *Close-out visits.* Upon completion of the study, a monitor will make a visit to bring formal closure to the study at the site. The monitor will ensure that all data have been collected and reported, that there is a final accounting of all test articles, and that the clinical investigator's files and records are complete. This visit often occurs upon submission of the draft final study report by the investigator to the sponsor, but before the final version of the report is submitted. This allows for clarification and resolution of deficiencies or ambiguities by the investigator which may be discovered during the close-out visits.

Review of regulatory and source documents are often the focus of these site visits. It is the responsibility of the study site to ensure that the binder(s) containing the regulatory documents is (are) complete and up to date with respect to protocol and investigator brochure versions and approvals, any and all IRB and sponsor correspondence, adverse events, and statement of the investigator (FDA Form 1572, see ref. 13). There should not be any erasures or changing of dates on any of the documents. The use of white-out

or pencil is discouraged. Source documents need to contain the medical records of the study subjects, including laboratory reports, physician notes and procedures documenting the study. Document signatures are also required. Making sure that the study documentation is in compliance is crucial for addressing regulatory agency inquiries which can arise during a routine or random inspection.

9.6 Contract research organization

A contract research organization, sometimes called a clinical research organization (CRO), is an entity that offers fee-for-services support primarily to manufacturers of medicinal products and biotechnology companies. Foundations, research institutions, colleges and universities, as well as government organizations may also enlist the services of a CRO. CRO clients are referred to as sponsors. As an industry, CROs emerged during the late 1990s in response to the regulatory complexities encountered by companies during their product research and development stages. The Code of Federal Regulations (CFR), specifically 21 CFR 312.3b, of the US FDA defines a CRO as 'a person that assumes, as an independent contractor with the sponsor, one or more of the obligations of a sponsor, e.g., design of a protocol, selection or monitoring of investigations, evaluation of reports, and preparation of materials to be submitted to FDA'.[14] Depending on the organization, services provided may include, but are not necessarily limited to:

- Laboratory analysis
- Experimental design
- Product development
- Manufacturing
- Product formulation
- Pre-clinical studies
- Clinical trial management
- Project management
- Data entry and statistical analysis
- IRB approval
- Document preparation and submission to regulatory agencies

In looking at the list above, it should be appreciated that a CRO can assist at all stages during the life cycle of a medicinal product. Outsourcing such

services offers a competitive advantage for a biotechnology company; it can be very cost-effective to pay a contractor compared with having in-house staff. This may be especially true for smaller companies that do not have the financial resources to build, equip and staff a research or analytical laboratory. A CRO offers the advantage of providing an existing laboratory facility and trained personnel ready to work on the project, thereby reducing the timeline for company milestones.

By the very nature of the business and the clients they serve, CROs must be up to date on the most current regulatory agency requirements for medicinal product testing, manufacturing and agency filing. This will provide a heightened level of confidence in the sponsor that the project will be executed and reported in a compliant manner, which is essential for regulatory agency approval for the manufacture, distribution and sale of any medicinal product. Clinical trial site monitoring, data management and outcomes reporting are specialized areas which are needed during very defined portions of a product life cycle. Contracting these services only when needed can help contain costs compared with a company having to hire dedicated staff who may not be utilized on a continuous basis. There are websites dedicated to locating a CRO for a variety of particular needs (for example, see ref. 15).

Arguably the largest growth in CRO business has come from, and will most likely continue to be, the areas of *in vivo* animal testing and clinical trial management. The reasons for this include the substantial upfront costs of building and maintaining an animal vivarium. As a point of reference, in 2006 the Medical College of Wisconsin, Children's Research Institute, built a 75,000 sq.ft. vivarium at an average cost of $363/sq.ft. ($27.2 million total).[16] Adding this to capital equipment, staffing and supply costs, building and maintaining a vivarium for a small or start-up company's own private use is out of reach. Clinical trial management often requires the services of data collectors, statisticians, regulatory auditors and project coordinators. As mentioned above, for a small company with one clinical trial being performed at a time, it is generally not cost effective to have this number of people with these specific skill sets dedicated full time to the study. When selecting a CRO, it is sensible to have a detailed list of the services needed before approaching them and being presented with what may be a confusing menu of options. Having an outside consultant perform an audit of the organization, paying particular attention to regulatory compliance updating, personnel training and experience, reporting, and familiarity with the type of medicinal product being tested, should provide a degree of confidence in choosing the right CRO for the right project.

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Manufacturing

Abstract. Manufacturing of TE/RM products needs to take place in a tightly controlled and qualified environment. As with any activity under regulatory agency scrutiny, building and maintaining such an environment requires a substantial investment in construction, personnel and infrastructure. Proper planning and development are critical to containing costs. In this chapter, key aspects of the planning and development stages, as well as regulatory requirements for maintaining a GMP-compliant facility, are considered.

Keywords: GMP manufacturing, HVAC system, ISO classifications, critical areas, validation, qualification, raw materials, bill of materials

10.1 Facility considerations

The regulatory requirements placed upon the biotechnology industry for production of medicinal products are demanding. These requirements are not only for the manufacturing process itself, but also include the facilities in which the manufacturing process takes place. Conceptual planning of the facility in its entirety, taking into consideration the technology used for the manufacturing process, the equipment requirements and the workflow, is crucial as these factors will affect the facility layout.[1] Making every effort to get the blueprints correct, whether it is for a new building or retrofitting of an existing structure, will save time, money and effort later on. A team consisting of architects, engineers and regulatory compliance experts should be involved. Any building used for the manufacture, processing, packaging or holding of TE/RM products needs to be of sufficient size, construction and location to facilitate cleaning, maintenance and proper operation.

The first general area which must be taken into consideration when building good manufacturing practice (GMP)-compliant facilities is the external environment. This environment must be sufficient to support the

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building of a well-designed structure and refers to much more than the ground upon which the building sits. If the land, air or water resources around the building provide an opportunity for water damage, insect or vermin infestation, bacteria, mold, or vegetation contamination, then production in a GMP-compliant manner within the facility may be compromised. Points to consider prior to new construction or retrofitting existing structures include:[2]

- Is there sufficient area available for facility expansion?
- Are the local water, sewage and power service adequate?
- Is there a suitable local labor force available?
- Can adequate security for the facility be arranged?
- Are there undesirable activities taking place in the area which may pollute the facility with noise, odor, vermin or micro-organisms?

Once a suitable location for the facility has been identified, a site development plan is prepared in accordance with all applicable local laws and regulations. Any company standards, provided they do not conflict with local regulations, are also included in this plan. The basics of any GMP facility design include:

- Clean rooms
- Climate control within the facility
- Personnel control
- Control and flow patterns for materials, products and waste

10.2 Clean rooms

Clean rooms and climate control must be fully integrated for the facility to be GMP-compliant. Clean rooms are used so that any possible contamination of the product may be controlled. Sources of contamination include micro-organisms and particles found in the air and on people and their clothing, equipment, and materials used to construct and furnish the room. In some cases, the manufacturing process may also generate particulate contamination. While arguably the most important tool in controlling contamination, the design, installation and validation of the heating, ventilation and air conditioning (HVAC) system is often the greatest consideration in designing or retrofitting a GMP manufacturing clean room facility. This is due to the high energy costs of running the system in addition to the potential negative effect on operator safety and product quality if the system is not sufficient.

The design of the system is dependent not only on the number of clean rooms to be serviced and their total area, but also on the layout and equipment configuration within the rooms. While other services for the facility can be moved relatively easily after structural completion of the building, the HVAC system is an integral part of the structural framework, making any changes to the system post-construction both time-consuming and costly. It cannot be emphasized enough the importance of having this component of the facility well planned and designed. The air handling unit within the facility will help to maintain the clean environment by providing an appropriate volume of clean air at the proper temperature and humidity. Cleanliness is achieved by passing the air through high-efficiency particulate air (HEPA) filters. The level of filtration depends on the classification of the room being served.

Global standards and classifications for clean rooms have been established by the International Standardization Organization (ISO).[3] As a reference point, ordinary or 'non-clean room' air found in a typical building is ISO Class 9, or Class 1,000,000 (no more than 1,000,000 particles larger than 0.5 microns in 1 cubic foot of air). An ISO Class 5 room,[4] often referred to as a Class 100 room, has no more than 100 particles larger than 0.5 microns in 1 cubic foot of air. These rooms often utilize terminal HEPA filters, meaning that the filter is located at the end of the air duct right before the air enters the room. In contrast, an ISO Class 8, or Class 100,000 (100K) room (no more than 100,000 particles larger than 0.5 microns in 1 cubic foot of air) often has non-HEPA filters within the air handling unit.

For the manufacture of tissue engineered products, operations such as tissue culture under a Class 100 bio-safety cabinet, culture growth in enclosed incubators and microscopic examination of cells in culture vessels may be carried out in a Class 10,000 (10K) clean room (no more than 10,000 particles larger than 0.5 microns in 1 cubic foot of air). In contrast, activities such as biomaterial scaffold preparation, bioreactor assembly and storage of manufacturing process supplies are permitted in a Class 100,000 (100K) clean room. When planning a clean room layout, personnel, product, materials and waste flows should be considered. Generally, it is best to have personnel flow in a single direction from entering the facility in a lower classified area (i.e. a vestibule area) then moving to a higher classified area (i.e. Class 10K gowning room). Controlled access to production areas is also recommended. This same holds true for the product flow. Materials flows should use material airlocks or pass-through into classified areas. Waste flows need to be controlled and separate from product. Figure 10.1 shows an example of a clean room layout, whereby personnel would enter a classified gowning room through an unclassified vestibule area, before entering the classified production areas.

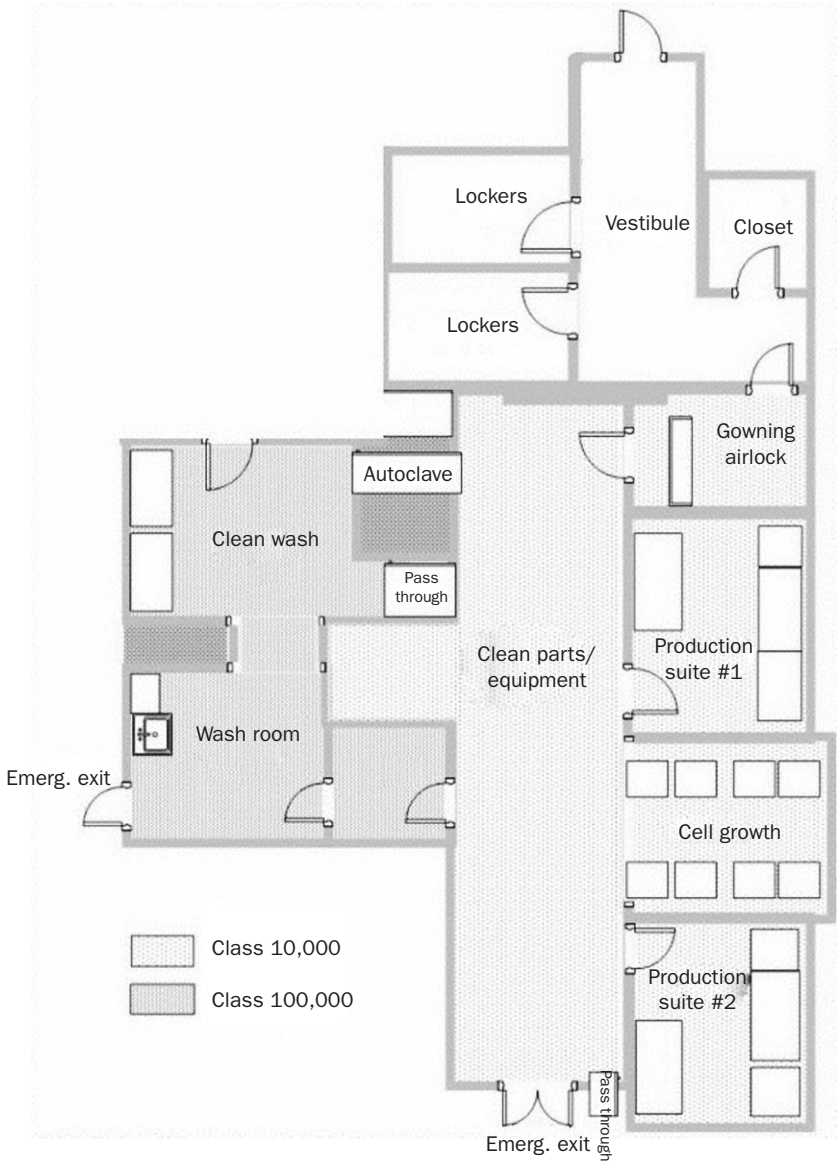

Figure 10.1 Example of a clean room layout illustrating the class 10,000 and class 100,000 air rooms. Open blocks in the various rooms show equipment and lab bench positions. Rooms at the top of the diagram (vestibule, closet, lockers) are non-clean or class 1,000,000.

Particle concentrations in a clean room must be continually monitored, as they invariably change over time, depending on whether the clean room is being actively used for product manufacturing. As part of the systems validation, operating tolerances must be determined for GMP-critical parameters, including air change rates, room differential pressures, temperature and humidity. Clean rooms for TE/RM product manufacturing will have positive air pressure compared with connecting rooms and hallways, so that when operators enter and leave, the positive pressure will cause the air to flow out from 'clean' (filtered air) to 'dirty' (unfiltered air) rooms, and not the reverse. Placement of air inlet and outlet ports within the clean rooms to minimize entry of airborne particulates and odors must also be carefully considered. For example, it is unwise to place outlets right next to inlets, which explains why many designs have air entering the room through the ceiling and exiting the room from the opposite side through a duct in the wall located near the floor. In some instances re-circulated air may be acceptable, so additional precautions may need to be taken to ensure that particles are removed from the manufacturing area. An example of some typical specifications for clean room areas are as follows:

- Temperature: 72 ± 2.5 °F
- Humidity: 45 ± 5%
- Air velocity: 60 ft/min (±2)%
- Air pressure: positive pressure, 0.05 inches (or 0.01 cm) of water between aseptic processing and other areas.

In addition to proper air handling to control potential contamination, clean room surfaces should be non-porous, chemically resistant, and easy to clean and disinfect. This includes flooring, walls, ceiling and bench tops. Popular flooring materials include vinyl and epoxy, with smooth, welded seams and rolled corners where the floor meets the wall. This is to help ensure that there are no rough surfaces or corners where contaminating materials may settle. Walls may be constructed with standard wall board or sheet rock, provided that the surface interior to the clean room is sealed using epoxy paint. The ceiling must also be sealed; drop ceilings are not acceptable. Any penetration through the ceiling (i.e. for light fixtures) and walls (i.e. electrical outlets) requires gasketing and sealing. Bench top surfaces include stainless steel, chemically resistant laminate, and polyester, vinyl-ester and epoxy resin. The surface should also have rounded corners and edges to facilitate cleaning. As surface cleaning may require spraying or aerosolization of cleaning solutions, equipment having surfaces which may be sensitive or

easily damaged by the cleaner (i.e. microscopes) should be protected by some type of cleanable, perhaps disposable, covering.

All clean room furniture and equipment should also be easy to clean and produce a minimum of particles which could potentially contaminate the manufacturing process. Items such as pencils, paper and materials comprising natural fibers are often not allowed inside a clean room. Plumbing poses special concerns within a clean room, as sink traps and floor drains can be rich sources of microbial and odor contamination. If drains and sinks are required in a clean room for the manufacturing process or for cleaning, it is best to have the ability to close and seal their openings when not in use.

A clean room may be divided into 'critical' and 'general' areas. In this vernacular, a critical clean room area is the vicinity around the point of product manufacture where contamination may directly access the process. Critical areas often contain bio-safety cabinets and laminar flow hoods to help protect against product contamination. As mentioned above, these items are often located in Class 10K rooms. General clean room areas are those where contamination has less of an opportunity to directly access the process, but nonetheless should be kept cleaner than a non-clean room area. This is because of its close proximity to the critical area, thereby increasing the possibility of contamination transfer. A Class 100K room may be considered a 'general' clean room area.

Rigorous cleansing is essential to controlling any clean room contamination. Facility managers and operators need to establish a code of practices corresponding to the standard of cleanliness required for the manufactured product. Although clean rooms are not sterile, i.e. totally free of uncontrolled micro-organisms, with proper cleaning the level of these micro-organisms can be rendered so low as to pose a minimal threat to product contamination. Factors to consider when defining cleanliness standards include the amount and types of equipment in the room, the materials worn and used for the cleaning, the types of cleanser, and the frequency with which the rooms will be cleaned. Cleaning of a clean room should be performed using only products which have a proven track record in successfully keeping these rooms clean in accordance with regulatory guidelines. Tasks to be completed include cleaning of:

- All work surfaces (i.e. bench tops and table tops)
- Furniture
- Equipment
- Floors
- Walls

- Ceilings
- Doors, including jambs and frames
- Lockers in the gowning areas

Trash receptacles and waste containers also need to be emptied. The frequency with which these items need to be cleaned will be established during development of the aforementioned code of practices. Generally speaking, the higher the class of the room, the greater the frequency with which cleaning is performed. For example, a Class 10K room may have the work surfaces and floors cleaned daily, the walls every other day and the ceiling once a week. In contrast, a Class 1K room may have the work surfaces and floors cleaned twice a day, with the walls and ceiling being cleaned once a day. There are also general regulations for the personnel cleaning the clean room. The following list is recommended as a minimum for successful clean room operation:

- Only approved garments are allowed inside
- Personal items (i.e. jewelry, keys, wallet, cash) must never be removed from beneath the clean room garments
- No eating, including gum chewing, or smoking
- Cosmetics (i.e. lipstick, mascara, hair spray, cologne) should not be worn
- Any items taken into the clean room should be cleaned to the same level as the clean room surfaces
- No items should be allowed to rest directly on the bench or table top surface
- Do not enter the room if you are ill (i.e. respiratory or gastrointestinal disorders)

10.3 Environmental monitoring

An environmental monitoring (E/M) program is established to document the level of control over microbial and particle contamination. The E/M program describes the type and frequency of microbiological testing in order to detect changing trends of microbial counts and microflora growth within the controlled environment of the clean room. Aerosol particle counters are used to determine air quality by counting and sizing the number of particles in the air. The results provide information about the physical construction of the room, the performance of the HVAC system, personnel cleanliness, gowning practices, the equipment and cleaning operations.

A well-developed E/M program will help to ensure that the standard of cleanliness for the manufacturing process is upheld. Analogous to development of cleanliness standards, the E/M program needs to define the types of monitoring (i.e. microbial identification and limits testing; aerosolized particles and microbes), the methods used (i.e. contact plates, air sampler with filter trap) and the frequency of the sampling. Frequency determination may be approached by first establishing how critical certain steps are in the manufacturing process. For example, the risk of contaminating cells during passaging from one open vessel to another in a bio-safety cabinet is greater than keeping the closed vessels in an incubator. The greater risk places the open passaging as being more critical than closed incubation, so sampling of the bio-safety cabinet should be more frequent than sampling of the incubator. Also included in this monitoring program is a sampling plan, which describes how sampling sites were identified and the number of samples which need to be taken. The areas of a clean room which are sampled include:

Air. Methods for microbial testing include active air sampling, in which a predetermined volume of air is drawn over a sterile media plate, which is then incubated to determine the number of microbes per cubic area. An alternative method, called passive air sampling, involves exposing plates containing sterile microbial growth media for specific lengths of time, generally 30–60 minutes, before incubating and quantitating microbial growth. This method is passive because it relies on the random chance that micro-organisms will settle onto the surface of the media. Of the two methods, this one is least preferred owing to an inability to accurately measure the volume of air from which the microbes came. Particle testing usually requires specialized pieces of equipment, employing electronic means to measure particles by either light scattering or light blocking. The particle passes through the light source (typically a laser) and if light scattering is used, then the redirected light is detected by a photo detector. If light blocking (obscuration) is used the loss of light is detected. The amplitude of the light scattered or light blocked is measured and the particle is counted and tabulated.[5]

Surfaces. This includes walls, floor, ceiling, work bench and table tops, and equipment. The two primary methods of sampling surfaces are contact plates (sometimes referred to as 'touch' plates) and swabs. Contact plates contain specialized growth media, depending on whether the purpose is to test for bacteria (i.e. trypticase soy agar) or mold and yeast (Sabouraud dextrose agar). These plates are poured in such a manner that the medium rises and sets just slightly above the top edge of the rim of the plate, thereby forming a slight protrusion. The plate is then pressed against any flat

surface to be sampled, or 'touched', thus picking up any microbes present. Following appropriate incubation (i.e. 37 °C for bacteria, 25 °C for molds and yeast), the number of viable micro-organisms per unit of surface area can be calculated. Swabs are used when contact plates are not appropriate, such as when surfaces are non-flat and for areas which are difficult to reach (i.e. some areas of equipment). The sterile swabs are rubbed over the surface and then used to inoculate specialized growth medium either in liquid broth (tube) or solid agar (plates) forms. As the surface area swabbed cannot be accurately measured, this testing method is more qualitative than contact plating.

Personnel. The most widely used method is contact plating. Sampling areas include gloved hands and fingers, wrist and forearm. These are the areas most likely to come into contact with sterile fields, the product and areas immediately around the product during the manufacturing process. Personnel sampling is one way of monitoring how well they are gowning prior to entering the clean room.

In the event that microbial contamination is detected in the area, identification of the culprit should be pursued. This information will provide a means to develop more effective cleaning and monitoring, so that future contamination is minimized. Typical specifications for clean room areas are the following:

- Airborne particles: <100,000 measurable particles ≥ 0.5 μm diameter/ft^3
- Airborne microbial contamination: <1 colony-forming unit (c.f.u.)/9 cm settling plate/6 min exposure
- Surface microbial contamination: <2 c.f.u./contact plate
- Personnel gowns: <40 c.f.u./contact plate
- Personnel gloves: <10 c.f.u./contact plate

10.4 Process controls

Quality systems must be in place to control the manufacturing environment, validation of equipment, material and operational controls. Overall, the first step in establishing quality systems is to have a quality assurance department that exists separately from the manufacturing operations. This department is responsible for making sure that the product works as it is intended to. Towards this goal, a standard operating procedure (SOP) and training records must be in place to ensure that processes are performed in an identical manner each time they are carried out. Control of the

manufacturing facility includes how the air is measured to ensure that it is clean, how pests are controlled and how the facility is cleaned. Any equipment used in the manufacturing process must be controlled with respect to cleaning and storage, as well as calibration and qualification procedures. This is necessary to provide evidence that the equipment performs its intended function. Materials need to be properly received, sampled and tested to demonstrate that they are not incorrectly labeled and that they are of the proper effectiveness. Operational controls focus on validation and in-process testing. Validation is a means by which documentation is provided as evidence that the procedures to manufacture the product perform their functions as intended.

Process controls and validation are critical to meeting regulatory agency standards for product approval. Careful attention to the design of both the process and the process controls can reduce the dependence on finished product testing for release, although some finished product testing will most certainly be required. For a process to be validated, the manufacturer must provide a written validation protocol identifying all procedures and tests performed, the data which will be collected, what the data will be used for, the specified number of replicate processes which will be run to demonstrate reproducibility of the process, and how variability between runs will be measured. This last-named measurement must accommodate parameters which may result in the greatest chance of product failure (i.e. equipment failures, environmental changes). Validation data to document the effectiveness of a process may be obtained by verifying successful implementation of a procedure which was validated previously. Alternatively, literature which demonstrates that the implemented processes are known to be effective is also acceptable. The FDA process validation guidelines[6] include the following:

- Validation must account for changes in the manufacturing process that can affect product characterization.
- Testing must be designed to address product consistency with respect to its intended performance.
- Testing procedures must be validated.
- Product testing must not adversely affect the product.
- Approved product specifications must be reviewed and compared with the actual product.
- The change-control program must be adequate.
- The validation program must be sufficiently documented and maintained.

10.5 Raw material qualification

As any TE/RM product manufactured in a GMP-compliant manner is done in such a way that the process and final product are controlled, there is also the expectation that the raw materials will not be changed without a determination of the impact on the final product. As such, a qualification and control program for raw materials is considered a key factor in assuring that regenerative medicine products are well defined, consistent, safe and suitable for their intended use. This being said, it is surprising how often raw materials qualification is viewed as secondary to other manufacturing concerns. Without such qualification, the supply chain of materials needed for production is placed at risk. A single supply chain failure may result in loss of product batches, production schedule delays and compromising of patient health. Qualification is a means of providing documented assurance regarding the appropriateness of the raw material for its intended use in the manufacturing process. These documents will aid the company in their risk analysis when it accepts the regulatory responsibility of producing a medicinal product.

Elements of raw material qualification include vendor selection, material handling and material test specifications. It is desirable to qualify both the raw material and the vendor at the same time. Potential vendors should be audited to ensure that they are in compliance with relevant industry standards. Information regarding the vendor's compliance history and any past or present problems with the raw material in question is critical to the vendor selection process. Obtaining a sample of the raw material for in-house or third-party testing to verify the vendor's certificate of analysis (COA) is also commonly done. Critical raw materials (defined here as those whose quality deficit may negatively impact the final product's identity, purity, potency, efficacy or toxicity) may necessitate testing of multiple vendor lots and perhaps testing for additional characteristics. For raw materials which are readily available, multiple vendors may be qualified, thus providing an alternative source in the event that one vendor fails to supply the desired material in a timely fashion. For custom raw materials there may not be multiple vendor options. Smaller, less well-established vendors also pose a risk to the supply chain, in the event that they are not able to stay in business.

In handling of raw materials, quarantining upon receipt is an important step in controlling the process. This enforced isolation is so that proper sampling and testing can be performed. The quality control group will first verify the physical integrity of the shipment container; obvious signs of surface contamination or integrity breach need to be documented. Material samples will then be taken for analysis, and acceptance or rejection will be

based on conformance of the testing results to previously established specifications. Accepted materials will then be released for use in the manufacturing process, while rejected materials will be moved to a separate area for eventual return to the vendor or destruction. Accepted materials are generally used on a 'first-in-first-out' basis to avoid using, and accumulating, materials which have expired or are past their shelf life. A periodic audit should be conducted to verify that the raw materials received can be accounted for as quarantined, rejected, accepted and used during the manufacturing process. Raw material test specifications include:

- Safety – sterility or bio-burden, adventitious agents and endotoxin.
- Identity – US Pharmacopeia tests for most chemicals, while complex materials may require a unique profile of predetermined characteristics and appropriate analytical methods, such as sequencing or liquid chromatography.
- Ancillary materials – includes those materials used during the manufacturing process but are not intended to be present in the final product. Examples include trypsin used during cell passage and tissue culture media.

A COA may be acceptable for the following raw material test specifications if identity testing and the vendor's test results are validated:

- Purity – testing for impurities in chemicals, and deleterious substances such as proteases and nucleic acids in biological and related materials.
- Strength – test for concentration, specific activity and potency.
- Performance – for complex raw materials including those used for tissue culture (i.e. serum, cytokines, growth factors) and scaffold production (i.e. biomaterials), test for cell growth and tensile strength, respectively. While such tests are material specific, reference standards should be used whenever possible.

The following steps may be useful towards developing raw material qualifications:[7]

1. Collect information on the material regarding available grades, what testing is usually involved, is the material listed in a compendium (i.e. US Pharmacopeia) and is the material critical for the manufacturing process.
2. Set up a qualification strategy and acceptance criteria so that a quality profile can be generated. See if this aligns with the information provided

on the COA provided by the vendor. Include characteristics (i.e. biochemical) which are consistent and required by the process.
3. Determine the nature of the information provided by test results or vendor audits. For example, do the test results directly relate to safety, or do they relate to the stability, of the material.
4. Approve the final selection of tests which will be performed on each material before release, keeping in mind that not all tests on the COA provide useful relevant information, and some tests not listed may need to be performed routinely.
5. Determine the frequency of vendor and material re-qualification.

The manufacturing of any TE/RM product also requires that a list of the raw materials, components, parts, quantities and any assemblies be made. This list, or bill of materials (BOM), provides the details on all of the items needed to manufacture the final product. Accuracy and completeness in the BOM is important from both a regulatory and a commercial perspective to reduce the risk of the product being made inefficiently, incorrectly or not at all. Without a BOM, the final transition from product concept to tangible item cannot be made.

As should be apparent, manufacturing a medicinal product in a regulatory-compliant manner involves a variety of details that span multiple levels of the manufacturing process, not the least of which is the facility and processing controls. Fortunately, various guidance documents and reference materials are available to support these efforts.[8–11]

10.6 Manufacturing process

Manufacturing of a TE/RM product is a multi-step process. Figure 10.2 is meant to serve as a general overview example for the manufacture of a cell/scaffold combination product based on our experiences with a neo-bladder augmentation product.[12]

It needs to be appreciated that each step contained in each box will require an individual SOP and batch records to be in compliance with regulatory requirements. Some steps during this process may be performed concurrently if they are not interdependent (i.e. scaffold preparation is independent of, and can take place during, cell isolation and expansion). In contrast, some in-process testing steps are dependent upon others and may need to be performed in a sequential manner (i.e. cells must be cultured and expanded before cell morphology and confluence can be tested at a specific duration time).

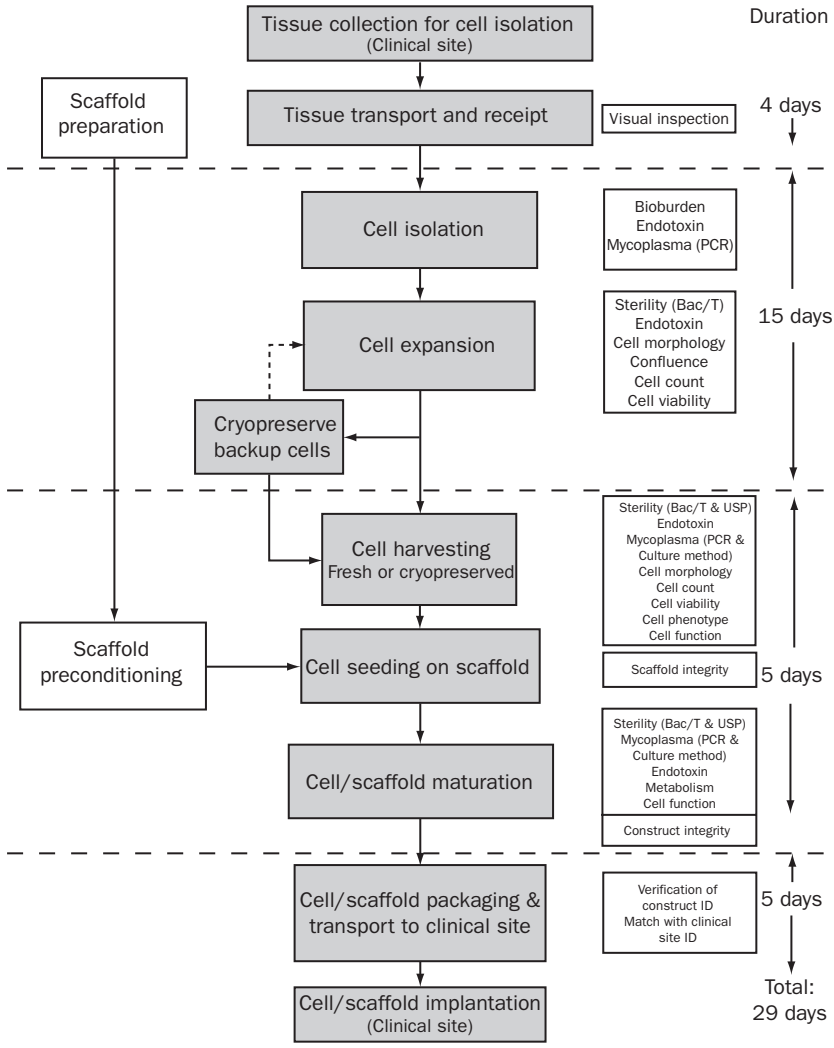

Figure 10.2 Flow diagram illustrating an example process for the manufacture of a TE/RM product consisting of a biomaterial scaffold (left-hand boxes) and cells (middle boxes). The step-wise timing and type of quality functions to be carried out during this process are shown in the right-hand boxes. A timeline for groups of steps, divided by the horizontal dashed lines, is indicated at the far right-hand side of the diagram.

The tissue collected is usually in the form of a biopsy of a pre-determined mass. Once retrieved at the clinical site, it needs to be transported to the manufacturing site for processing. It is strongly recommended that a courier service be used for this transport. Such services generally provide specialized pickup and delivery options for time-sensitive packages. Couriers generally ensure that the package is handled securely (i.e. in access-controlled areas) and safely to prevent damage. If it is determined upon receipt by visual inspection that the shipping container and/or the contents are damaged, it is important that the courier also has a robust chain-of-custody system in place to aid in the investigation as to where and when the damage may have occurred. Samples should be taken during the cell isolation steps to determine the bio-burden load and levels of endotoxin and mycoplasma. The results from this in-process testing step are important for determining if, and to what extent, this starting raw material is contaminated.

This information will help guide subsequent cell expansion steps, in terms of controlling and eradicating any contamination. Following cell expansion, it is advised to cryopreserve some portion of the cells as a back-up for cell expansion in case subsequent steps result in an insufficient number of cells being obtained for the product, or if the final product is found to be contaminated or non-functional. This back-up will also alleviate the need to go back to the clinical site and request additional patient tissue, as most patients do not appreciate the fact that their tissue may have been improperly handled and are less inclined to grant the request. Further in-process testing for cell morphology, confluence, cell number and viability will enable the manufacturer to assess whether the process will result in the number of healthy cells required for the final product.

Scaffold preparation usually takes place in parallel with cell expansion, so that the proper number of cells, obtained either from fresh cultures or cryo-preserved, can be harvested and seeded onto the preconditioned scaffold. Scaffold preconditioning (i.e. wetting, incubating in tissue culture medium) is usually necessary for biocompatibility with the cells. After a specified duration to allow the cells to attach and grow on the scaffold, sometimes referred to as a maturation step, the final product is packaged and shipped to the clinical site for patient implantation. A multitude of in-process testing takes place during the final steps of cell harvesting through packaging and shipping to the clinical site to ensure product integrity, sterility, functionality and identity. Once implanted, clinical monitoring takes place to evaluate the safety and efficacy of the product.

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11

Intellectual property

Abstract. Regardless of size, all biotechnology companies possess some type of intellectual property. While most often used to describe an invention, this property may also include a brand, a design, a process or a service. Intellectual property may be the most valuable asset for a company when trying to raise capital from private investors or the public market. As such, this property is recognized under the law. Securing and protecting it is vital to a company's success. In this chapter, key steps prior to filing a patent will be discussed, along with potential risks and counter measures if not carried out properly. The section on patent filing covers generalized steps in the process, along with references to selected resources, as a guide toward a successful filing. The final sections cover alternative means to protect IP.

Keywords: public disclosure, invention, in-licensing, trademark, provisional filing, utility application, patent, non-compete clause, non-disclosure

11.1 Definition of intellectual property

Intellectual property (IP) is defined as creations of the mind for which property rights are recognized under IP law. IP is often divided in two categories: industrial property, examples of which include inventions (patents), trademarks, industrial design and trade secrets; and copyright, which generally covers literary and artistic works. An important distinction between these two categories is that copyright protects the form of expression, rather than the subject matter. A copyright does not have to be novel, non-obvious, useful or industrially applicable, as does a patent. Patent generally refers to a right granted for an invention or discovery of any new and useful process, machine, manufactured article, composition of matter, or a novel and useful improvement of the same. Owners of IP are granted certain exclusive rights, thereby denying others the right to perform the same action or produce the same product in the same manner. These

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exclusive rights permit the owners to benefit from the property they have created, thereby producing a financial incentive for creating and investing in IP. On a global scale, IP has a demonstrable effect on economic growth.[1] A patent by itself does not grant the inventor the right to commercialize the protected technology; a patent grants the right to exclude others from commercializing it.

11.2 Landscape assessment

A crucial component of any business strategy is to determine the competition. For a company with a goal of establishing a presence in novel technology to be applied in the fields of TE/RM, understanding the depth and breadth of existing IP is the first step towards understanding its competitors. Without such an assessment, defining the features and specifications of a new product are risky, potentially leading to infringement on exiting patents. A block to market entry may result unless the new product is redesigned, at increased cost and delay of market launch. Landscapes are analogous to maps, in which variables such as the types of patent filings, technical, scientific and business information are distilled into a form which is easier to manage and navigate.[2]

A variety of no-cost search engines specifically for patents relevant to a particular company's business strategy have greatly facilitated internet searching the IP landscape. The United States Patent and Trademark Office (USPTO)[3] allows for patent and trademark searches based on key words contained in a variety of fields within the application. Both text and figures may be downloaded and printed for use. Patent Lens[4] enables the user to search for patents filed in the US and other countries, and has an Explore Patent Landscapes feature for volume analysis of data focused on particular topics. Patents.com[5] and FreePatentsOnline[6] are also quite popular for patent searches. For those who may need more guidance, or prefer the comfort and security of having a professional conduct the landscape assessment, a multitude of commercial entities may be found on the web. Regardless of the path chosen to assess the landscape, the following should be kept in mind.

First, issued and filed patents should be categorized as not relevant, low, medium or high relevance. Low relevance may be used for those patents which are about to, or have already, expired. Also included in this category are patents in which the claims or embodiments are very narrow or questionable. Medium relevancies are those around which claims or embodiments may be designed. This will require a more creative effort on

the part of the inventor, but should be within reasonable achievement. High relevancies are the most difficult to deal with, as these patents may cause a substantial barrier to market entry. In some cases, patents in this category may be too costly to overcome, or cannot be overcome no matter how creative the inventor, thus effectively blocking market entry.

Once patents are categorized, a review of the technology that resides in the public domain, which technologies are proprietary and what technologies are unclaimed, can be made. Armed with this information, charting a path forward into space which is aligned with the company goals can then be implemented. A flow diagram, such as shown in Figure 11.1, may be helpful in putting this information into perspective.

In the event that the landscape assessment reveals a block to market entry, or the company does not have the resources to chart an IP path forward into market space that it is committed to enter, an alternative approach is in-licensing the needed technology.[7] In-licensing, also referred to as simply licensing, is authorization by a licensor to a licensee to use a brand name, patent or some other proprietary right in exchange for a fee or royalty. Licensing will protect the licensee from infringement by the

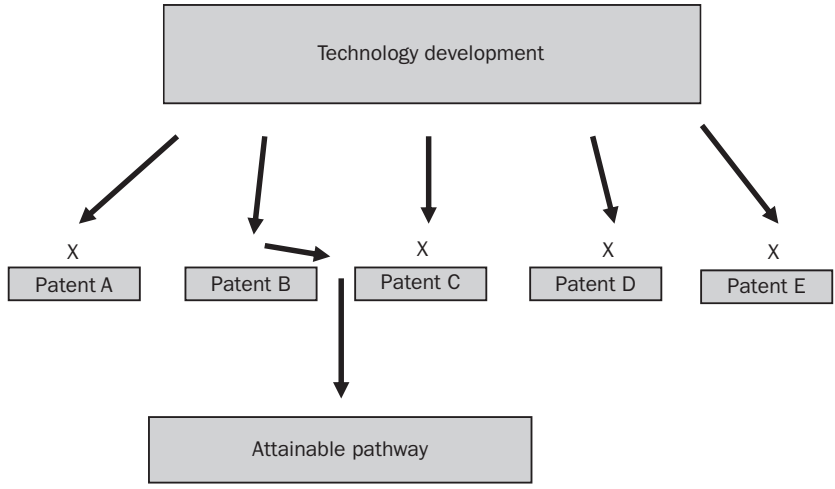

Figure 11.1 Example of a decision tree for developing technology. Once the technology has been identified, the next step is to determine what intellectual property (patent) obstacles are in the way of development. In some cases, filed or existing patents will prevent further development along certain pathways (indicated by the 'x'). It is the responsibility of the developer to plot a course around filed or existing patents (finding an attainable pathway) to avoid legal conflicts.

licensor. In-licensing is often valid for a specified length of time, thus protecting the licensor from value increases of the license, as well as fluctuations in the market. A license may also have limits as to the territories to which the rights pertain, often specified by the terms 'North America', 'Europe' or 'Asia'. The licensor will be able to reap the benefit of the licensee by profiting from the skills or market expansion potential of the licensee. Licensing is a strategy frequently used by companies to enable penetration into foreign markets in which the company has no prior expertise. Fees for licensing vary depending on the negotiation skills of the parties involved, but ranges from 5 to 25% of the wholesale price of the commodity are not uncommon.

11.3 Operational documentation

The purpose of operational documentation, also called record keeping, is to ensure that the records documenting the production and collection of analytical data in laboratory notebooks is maintained in a consistent, detailed, traceable and legally defensible manner. The documentation should contain the orderly presentation, organization and communication of analytical data in a written, physical document that clearly identifies the procedure followed, the name of the individual performing the procedure and the date on which the procedure was performed. All laboratory personnel need to take ownership of, and be responsible for, entering the required information in pertinent notebooks in accordance with a standard operating procedure (SOP) developed by their employer. Laboratory personnel should also be responsible for ensuring that their notebooks are reviewed by a second person who is knowledgeable about the specifics of the information contained in the notebook. Laboratory managers need to ensure that notebook formats include all of the required information, as well as that laboratory personnel record all pertinent information, as defined by the company SOP.

In general, all record keeping in laboratory notebooks should comply with the following:

1. Bound, individually numbered laboratory notebooks with pre-numbered pages should be used by personnel for analytical data record keeping.
2. In the front of each notebook, one or more pages are designated for signatures.
3. In the front of each notebook, the name or title of the notebook.

4. In the front of each notebook, the identification number for the notebook.
5. Each notebook page includes the reviewer signature and date of signing.
6. Before signing or making an entry in a specific notebook, each individual enters his or her name (printed), signature and initials to the signature page(s).
7. All entries must be made in permanent black ink. Other colors are not acceptable.
8. All entries must be legible, dark and suitable for photocopying.
9. Erroneous data shall not be obliterated or rendered unreadable.
10. Corrections must be made by striking a single line through the erroneous data such that it can still be read.
11. No correction fluid (e.g. 'white out') or correction tape to be used in correcting data.
12. All strike-outs must be dated and initialed by the person making them.
13. Unused space on a partially used page or within a dated entry must be crossed through with a single diagonal line, dated and initialed.
14. Unused pages in a notebook must be crossed through with a diagonal line, dated and initialed.
15. Each completed notebook page is subject to review. The reviewer checks the information on each page for completeness, correctness and compliance with SOP and signs and dates the page.
16. If inserted sheets are included as part of the official record of the procedure recorded in the notebook, the sheets must be taped (and/or stapled) onto a numbered page.

While these requirements may appear onerous, failure to document appropriately may jeopardize an otherwise successful IP filing. In the event that a laboratory notebook needs to be inspected during the course of a patent examination, evidence of removed pages, data corrections using white out or obliteration of data will cast a degree of doubt and suspicion on both the information and the recorder of the information. Likewise, failure to appropriately mark, sign and date unused portions and complete pages of an entry opens the possibility of information being added after the entry was reviewed and signed off, thus nullifying the purpose of the review process for information accuracy. Signatures and dating are crucial for determining the timelines of IP development.

11.4 Disclosure

A disclosure in IP terms is often divided into two categories, namely public disclosure or disclosure as part of the patent application process. A public disclosure may be broadly defined as any public communication of information relating to an invention. This communication may be in the form of print (scientific publications, letters to the editor of scientific journals), oral presentation at a meeting open to the public (i.e. international, national or local scientific meetings) or electronic (i.e. internet forum, chat rooms or blogs). This information may be general, such as conceptual ideas or a working framework for application development, or detailed, such as specific steps and materials used to develop a product. From a legal perspective, public disclosure is when an inventor places general or detailed information about an invention in a situation where there is no control over who sees it or has access to it. It is strongly recommended that an invention disclosure be filed with senior management before releasing any information which permits others to duplicate the process of the invention which, in turn, facilitates the manufacture of the product described by the invention.

If an invention becomes public knowledge before a patent application is filed, the inventor is at risk of surrendering rights to the patent. Depending on the country of filing, there may be a grace period between public disclosure and filing date, which will allow the inventor to retain rights to the patent. For filings to the USTPO[3] this grace period is one year from the date of disclosure. After this time, the inventor has lost all rights to the IP of the invention. Japanese patent law will allow for a six-month grace period.[8] Most European countries are under the Patent Cooperation Treaty (PCT),[9,10] and any public disclosure prior to a patent filing will forfeit the patent rights. Inventors need to keep in mind that the location of the public disclosure affects interpretation of patent laws. As an example, US patent law does not count as a public disclosure the presentation of information if it occurred outside of the US. This generally holds true for oral presentations at meetings, but is less clear if the information is presented in print or electronic formats outside of the US. Under the PCT, if information relating to an invention is presented anywhere in the world, the inventor loses the right to file with its members.

Disclosure as part of a patent application process requires the inventor to provide specific details about the invention. While the USTPO no longer accepts disclosure filings,[11] company employees who create inventions may best be served by submitting a disclosure to their senior management and legal council, if available. The disclosure should be sufficient to allow a person skilled in the art to reproduce or use the invention. This requirement

is sometimes referred to as sufficiency of disclosure, or enablement, depending on the country. In Europe, for example, an application must disclose the invention in a manner that is sufficiently clear and complete for it to be carried out by a person skilled in the art.[12] In the US, the application must be sufficiently complete that a person of ordinary skill in the art of the invention can make use of the invention without undue experimentation, even though there is no precise definition of 'undue experimentation'. Additional requirements for the US application are the 'best mode requirement', meaning that the disclosure must also contain the 'best mode' of making or practicing the invention,[13] as well as the 'enablement requirement', which discloses how to make use of the invention.[14] In a broad sense, the disclosure should include:

1. Contributor(s) information including title, citizenship and home contact information.
2. An indication of whether the invention has been reduced to practice, or if any models or prototypes exist.
3. A description of the invention, including a brief summary stating novelty and utility, as well as a detailed description which may include any published or unpublished documents, slides or drawings.
4. Date of the conception of the invention and the location of where this date was documented so that it may be validated.
5. A discussion of what challenge the invention addresses, as well as any potential or expected commercial applications.
6. Identification of any prior art references.
7. Signature and date page for all inventors and a witness.

This disclosure document will often form the foundation of a patent filing. As such, it is in the best interest of the inventor to ensure that the information is presented in a manner that is easily understood by those both skilled and unskilled in the art.

11.5 Filing

A patent application is an intricate legal document. Filing a patent application without the guidance and advice of a patent attorney or patent agent is difficult and should not be undertaken by beginners. While a patent agent is less costly than a patent attorney, both have similar if not identical expertise in patent filing. However, a patent attorney may be a better choice

to defend an application during the patent examination process and defending patent claims in court. The more expertly written the patent, the better protected is the invention. If the document is not written or filed properly, the risk of the application being dismissed, the invention date being lost and the claims not being allowed is great. Even when enlisting the services of a patent attorney or agent, the inventor should still take the time to become familiar with and understand key components of the filing process. The more educated the inventor is with the process, the easier it will be for the patent attorney and inventor to discuss the invention. This often reduces the time the attorney needs to spend on the filing, reducing the cost of filing for the inventor.

A multitude of guides for how to file a patent may be found on the internet. Consistent with the discussion above, virtually all of them strongly encourage enlisting legal council for a proper and accurate filing. It is also possible to enlist a service which will file a patent for a flat fee or provide the applicant with a menu of services to support a filing (i.e. patent search, written opinions, domestic or foreign applications). In keeping with the idea that the more informed the inventor is regarding the application process the more efficient the process can be, a discussion of the general steps to filing a patent is in order.

1. Perform a database search to determine if a patent which covers your invention has been filed or has been issued. This topic is covered in more detail in Landscape Assessment above.
2. Prepare a disclosure document, discussed in section 11.4.
3. After a review of the disclosure document decide how best to proceed with invention development and patent protection. Input by patent council may be critical in this decision-making.
4. Prepare a complete and full written description of the invention, if the invention disclosure is determined to be insufficient.
5. Prepare a patent application.
6. Submit the patent application with all supporting documents and fee payments to the appropriate agencies. Guidance on how and where to submit may be found on the websites for the USTPO, the Asian Patent Alliance and the European Patent Office.[3,8,9]

Inventors also need to be aware that there are different types of applications which may be filed. A provisional application, for example, expires one year after the filing date. This type of application is not examined by the patent office, and it cannot be issued as a patent. A provisional application will enable the inventor to obtain a filing date for the information disclosed in

the application. This date may be important for filing a future non-provisional application, which will be examined by the patent office and can ultimately be issued as a patent. Once filed, additional materials supporting the non-provisional application may not be submitted. A continuation application is a second application for the identical invention described in the non-provisional and is filed before the prior application is abandoned or becomes patented. The purpose of this filing is to pursue additional claims disclosed in the earlier application that has not yet been issued or abandoned. New rules have been put in place by the USTPO which limit the number of continuations filed to two, unless just cause can be demonstrated. Prior to this rule, applicants could file as many continuations as desired, often resulting in a continuing widening of the claims.

Placing a limit on the number of continuation applications is designed to clearly define the breadth of the claims covered in a given application. A divisional application is similar to a continuation, and is filed when aspects of the subject matter claimed are required to be withdrawn, usually in response to decisions handed down by the patent office. A term often used synonymously with non-provisional application is utility patent application. This application requires that a specific, credible and substantial usefulness of the invention be demonstrated. The criteria may vary, however, depending upon the agency to which the utility is being filed. For example, in European patent law, demonstration of utility is not a decisive factor in determining patentability,[15] whereas industrial applicability is.[16]

Once a utility application is filed, the process to issuance of a patent takes at least one year, but often takes much longer. The factors which influence this time include the number of applications received by the agency and the backlog of applications the agency may already have. Once received, the application is assigned to an Examiner who is responsible for evaluating this application with others in the same technical area. The experience of the patent Examiner can also affect the length of time it takes from application to issuance. The inventor also needs to realize that once filing is complete, there is no guarantee that an application will become an issued patent. The Examiner may feel that prior art exists which contains sufficiently similar, or identical, elements of the application being examined.

After the review, the Examiner will issue an Office Action to the inventor, a written communication informing them of whether the application can issue as a patent. If the decision by the Examiner is not to issue, the Office Action will contain the rationale for this decision. It is rare for an application to be allowed to issue without at least one Office Action being communicated. Relatively minor items to be addressed include poor quality of, or incorrect, diagrams and drawings. More substantial items include

finding that the invention under review was found to be contained in prior art described in the public domain or in previously filed applications or issued patents. If the application is found to contain more than a single invention, it will also not issue. The inventor is given the opportunity to reply to the Office Action items in writing.

At this stage, it is advisable to have a patent attorney represent the inventor. The response may include amending claims that the Examiner took issue with, or explaining how the Examiner misinterpreted the prior art or patent law. The inventor needs to keep in mind that in amending claims, the only material that may be included must already be contained in the original application. The patent attorney may also verbally discuss with the Examiner any claim rejections and language for claim amendments which would circumvent the existing prior art.

It is not uncommon for multiple rounds of Office Actions and responses. As long as there are responses by the inventor or their legal representative, the Examiner will continue to issue Office Actions, or will eventually be convinced to issue the patent. Once it is determined that the application will issue as a patent, the Examiner sends a notice of allowance to the applicant. At this point, any government issuance fees must be paid. Once issued, maintenance fees must be paid to maintain the patent as enforceable. Issued utility patents are enforceable for up to 20 years. A timeline is shown in Figure 11.2 to illustrate this process. It is possible that, if no Office Actions are received, the applicant can receive a notice of allowance in as little as 6–18 months after filing. However, often there are multiple Office Actions and responses, thereby pushing this time to patent issue to greater than 18 months. Five years was chosen for illustration here based on our experience and that of our colleagues in the biotechnology field.

Should an application be rejected outright, a continuing application may be filed. This will preserve the invention date while providing additional time

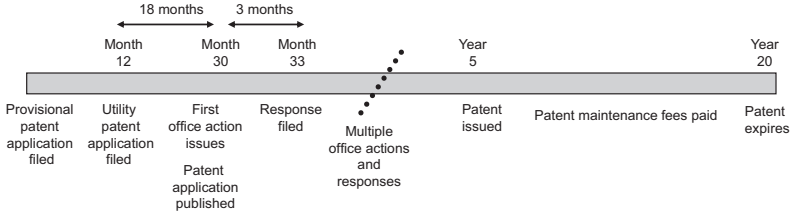

Figure 11.2 Example of a typical timeline and the steps involved during the progression from patent filing to issuance. The greatest unknowns with regard to time are the number and frequency of office actions issued by the agency and the time it takes for the applicant to address them adequately.

for the applicant to present a stronger argument for their invention. There is also an appeals process which the inventor can pursue. This process includes a group of more senior patent Examiners. The hope here is that the applicant's patent attorney can persuade a new Examiner to issue the patent.

11.6 Freedom to operate

Freedom to operate (FTO) means determining whether a particular process or commercialization of a product can be done without infringing on the patent, design or trademark rights of others. From a patent perspective, FTO means that a company has determined with a reasonable degree of certainty that its process or product does not infringe on the IP rights of others. It is acknowledged that absolute certainty cannot be determined given how the patent process works, most notably the length of time it takes for patent examination (see section 11.5 and Figure 11.2).

To establish reasonable certainty, it is useful to enlist the services of a legal or IP analytics firm to conduct a Clearance Search or Infringement Search to determine if there are granted patents or patent applications which, upon granting, your innovation may infringe. Obtaining a written legal opinion on whether your innovation infringes upon the IP of others is helpful should there be any legal challenges which question your company's freedom to operate. As patent rights are specific to different regions, this analysis needs to be related to particular countries where operation is anticipated. In the likely event that this search reveals granted patents or patent applications relating to the innovation for which you are seeking freedom to operate, thereby limiting your freedom to operate, further analysis needs to be performed to determine if the matter claimed in these patents may still be available for use. Some situations to look for in this regard include:

1. Patents in different countries – the claimed matter in your innovation may be upheld in a country where there are no similar patents or applications.
2. Issued patents may not be enforced – maintenance fees for an issued patent may have lapsed, thereby making them unenforceable.
3. All patents expire eventually – check the expiry date.
4. Patents have limitations – they include limits to countries and scope.
5. There may be exceptions – patents in some countries may give freedom to operate for a specific use.

What options are available if it is concluded that there is no freedom to operate? In-licensing, which was discussed in section 11.2, is perhaps the most popular and simplest strategy employed. This will allow the license holder to use the specified technology for a specific purpose, market and length of time. The licensee should recognize that, depending upon the terms and conditions of the agreement, there is the potential of losing a measurable amount of autonomy in how their business may be conducted. In-licensing may also be costly, in terms of up-front lump-sum payments and royalties.

Another approach is called cross-licensing. This strategy involves an exchange of licenses between two companies so that certain patents owned by one company can be used by the other. For this to work effectively, the two companies need to make sure that their patent positions are strong and of value to the other company. A variation of this approach is called patent pooling, in which two or more companies in a related technological area collect their patents to form a pool for the purpose of establishing a centralized collection for patent rights. If possible, from the standpoint of maintaining autonomy, the best strategy is to invent around existing patented technologies, as discussed in section 11.2 and Figure 11.1.

11.7 Trade secrets

A trade secret is generally defined as information, relevant to a process, design or practice, which the owner has taken reasonable measures to keep from the public domain.[17,18] This information must also provide some economic benefit to the holder, and this benefit must be derived from the information not being disclosed. Trade secrets may also be referred to as confidential or classified information, depending upon the country. In the US, trade secrets are protected by both state and federal laws. Most states use a version of the Uniform Trade Secrets Act as guidance for what defines a trade secret and as part of their civil codes should a trade secret be revealed without the owner's consent.[19,20] Under the federal Economic Espionage Act of 1996,[21] which is a criminal statute, it is a felony to sell or disseminate trade secrets without the owner's consent. In determining whether material may be considered a trade secret or not, the following questions are worth considering:

1. To what scope has the information been revealed outside of the owner's business?
2. How much of this information is known by the employees of the owner?

3. How extensive have the steps taken by the owner to keep the information a secret been?
4. How easily may this information be obtained by others?
5. How valuable could this information be to business competitors?

There is no set legal procedure for obtaining trade secret protection. Such protection only becomes relevant when the owner decides that the information is of value and must be kept secret. Although having a trade secret entitles the owner to legal recourse for damages or to stop use of the secret by others, such protection is not as robust as a patent, which requires a full disclosure. As with disclosure for a patent (see section 11.4), it is important from a defensive position that there exists written or electronic proof of when the trade secret was conceived or created. One approach is for the owner to mail a hard copy of this information to themselves, and retain the postmarked and sealed envelope as evidence. A source-code-dated electronic copy of the information, deposited with an entity which is not part of the owner's business, such as an outside electronic storage company, may serve as an electronic proof.

One of the most commonly used ways of protecting a trade secret is to put a contract in place between individuals or companies. A non-disclosure agreement (NDA) is one of the better ways to protect information that a business does not want to become public.[22] A NDA may also be referred to as a confidentiality agreement, confidential disclosure agreement, proprietary information agreement or secrecy agreement. Regardless of the name, this agreement is a legally binding contract in which the parties involved pledge to protect the confidentiality of proprietary or secret information that is disclosed during employment or in the course of engaging in a business transaction. A mutual NDA is one in which both parties are restricted in their use of the information disclosed. This type of NDA is often put into place when businesses are discussing a joint venture or structured merger. Many of these are so-called unilateral or one-way agreements, which restrict the use of information by a single party. This is to help ensure that the party to which the information is disclosed does not take and use the information for their benefit without compensating the disclosing party. A typical NDA will contain the following:

1. A clear definition of what constitutes the confidential information.
2. Any exclusions from what must be kept confidential.
3. The obligations of the party to which the confidential information is disclosed.
4. The time period during which confidentiality must be maintained.
5. The time period during which the agreement is binding.
6. Any types of permissible disclosure.

Exclusions from what must be kept confidential may include materials that are available in the public domain, prior knowledge about the information which the individual had prior to disclosure or information gained from another source. Perhaps the most significant obligation of the party which receives the information is to ensure that anyone who they disclose to, in the course of their business dealings or developmental research, remains bound by the obligations in the NDA restricting use and disclosure. For the most part, permissible disclosure includes those requested by a court of law. As with any legally binding agreement, paying careful attention to the language of the document is important before signing. For example, any language which states or implies that no confidential relationship exists between the parties opens the possibility for the party with the confidential information to waive or lose any claim to a trade secret.

Another commonly used way of protecting a trade secret is to put a non-compete clause, or covenant not to compete, into place. These are terms used in contract law under which one party, usually an employee, agrees not to pursue a similar profession which would compete directly with another party, usually the employer.[21–23] While most states in the US recognize some form of non-compete agreements, the extent to which non-compete clauses are legally endorsed can vary. The state of California is a notable outlier, as non-compete agreements are void, except for specific situations according to the law.[24] In Europe, non-compete clauses are allowed, provided that the employer can demonstrate a reasonable business reason for having one.

Although less commonly used, there are additional restrictive contracts related to a NDA and non-compete clauses which are designed to safeguard trade secrets.[23] For example, a garden leave clause in an employment contract provides for employee remuneration during the time the employee is restricted, but they are not required to perform any service at the workplace. The use of this type of clause is increasing by employers to prevent highly skilled employees, who may have trade secret knowledge, from resigning and then going to work for a competing organization.

Another example, a forfeiture for competition agreement, is one in which an employee surrenders specified benefits, or pays a predetermined amount of money, to engage in activities that are in direct competition with their former employer. In this last example, some employers may require employees, particularly those hired into management and executive positions, to sign an anti-piracy agreement. Should an employee leave an employer for any reason, this agreement will not allow the employee to solicit or hire individuals from their former employer. The term 'cherry picking' is often used in reference to former employees who do try to hire personnel away from their former employer.

11.8 Trademarking

A trademark is a distinctive mark which is placed onto goods by a manufacturer to identify the goods as being made by that particular manufacturer.[25] Trademarking can also be applied to services that an individual or company provides. The words 'brand', 'logo' and 'mark' are sometimes used when referring to a trademark. Using a trademark indicates that the manufacturer believes the quality of their goods or services will elevate their standing in the marketplace. As such, a known trademark indicates to the buyer that a reputation is staked on the product. From a consumer standpoint, trademarking is designed to prevent the buyer from being misled as to the origin of the product. Trademark imitations can potentially harm both the manufacturer and the buyer, who may be misled on the source of the goods. Infringements of a trademark are offenses which are generally punishable under the law. The following symbols are associated with trademarks in the US:

1. An unregistered trademark is designated by ™
2. A registered trademark is indicated by ®.

A trademark may be registered provided it sufficiently distinguishes one manufacturer's goods and services from another. Registration in some countries is necessary for the exclusive right to use it, as the law considers a trademark to be a legitimate form of property. While use of a trademark not previously used allows exclusive right to its use in the US, Canada and Great Britain, it is prudent to register the trademark to support that right. Registration within the US is handled by USPTO.[3] The process involves submitting an application and a review of the application, and assuming that any questions raised by the reviewer are sufficiently addressed, the application is then published for opposition. This gives any person or organization that may be impacted by the trademark registration an opportunity to file an Opposition Proceeding to stop registration of the mark.

If this occurs, a hearing is held to determine the validity of the opposition. In the event that there is no opposition to the mark, or that the hearing to determine the validity of the opposition decides in favor of the applicant, a Certificate of Registration will be issued. The World Intellectual Property Organization[26] facilitates trademark registration internationally. There is, however, one substantial difference from the US process in that international registration of the mark takes place before any opposition proceeding occurs. Opposing parties are given an opportunity to challenge the registration for a defined length of time from the registration date. A flow diagram is shown in Figure 11.3 to illustrate the general process, which for

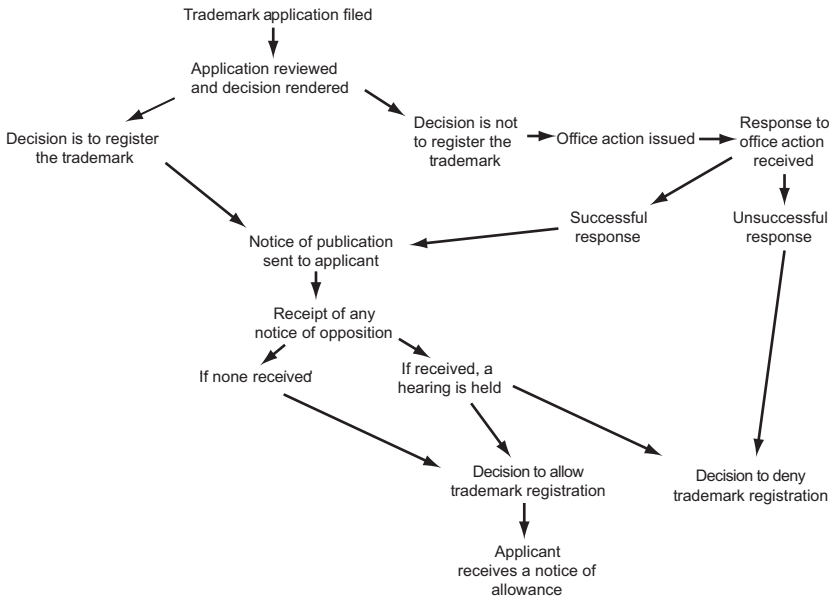

Figure 11.3 General steps for registering a trademark. Similar to the patent issuing process, the number and frequency of office actions issued by the agency and the time it takes for the applicant to address them adequately are the greatest unknowns with regard to time. Unlike the patent issuing process, however, the public is given an opportunity to oppose trademark registration. If a notice of opposition is received, successful resolution may increase the time it takes for trademark registration.

the USPTO takes 10–18 months for a successful application.[27,28] The reasons for a decision to deny a trademark registration most often include that the mark was a generic term, geographically descriptive, misdescriptive, scandalous or immoral. In rare cases where a mark is opposed, it is usually because the applicant's mark is confusingly similar to the opposer's mark.

Trademark rights must be maintained through actual lawful use of the trademark; if not actively used for a defined length of time, determined by individual countries, rights of use will stop. Although most countries allow trademarks to be licensed to third parties, it is the responsibility of the trademark owner to ensure the quality of goods produced by the license holder or run the risk of the trademark being considered abandoned by the judiciary system. Sale and transfer of a trademark with or without the underlying goodwill or asset supporting the mark depends upon the individual country allowing the trademark.

11.9 The Leahy–Smith America Invents Act

The Leahy–Smith America Invents Act (AIA) was signed into law by the President of the United States on 26 September 2011.[29] This is arguably the most substantial change in the US patent system since 1952, and changes the system from a 'first to invent' to a 'first to file' system. This change in the law places the US patent system more in harmony with the existing 'first to file' patent systems of most other countries. The AIA also does away with interference proceedings, in which a panel of judges belonging to the US Patent Office determine which of two applicants is not entitled to the patent if both claim the same invention. This new law also puts a system in place for opposition of the patent after it is awarded. These changes go into effect on 16 March 2013, 18 months from enactment.

The 'first to file' system, which is sometimes called the 'first-inventor-to-file system', secures the rights to a patent for a given invention to the first person (the inventor) to file an application regardless of the date of the actual invention. Consistent with information provided in earlier sections of this chapter, patent examiners will continue to evaluate prior art in determining the novelty of an invention. The AIA does modify the definition of prior art, which may bar patent issuance to include public disclosures prior to the filing data, with the exception of publications by the inventor within one year of filing. This one-year grace period does not extend to inventors who do not publish their inventions prior to filing. The following scenarios should help to illustrate this point.[30]

Scenario 1:

- Person A invents and immediately publishes
- Person B independently publishes subject matter of the invention published by Person A
- Person A files patent application within 1 year of the invention they published

Result:

- Person B publication is not prior art even though it was published before Person A filed an application

Scenario 2:

- Person A invents and immediately publishes
- Person B independently invents and files a patent application
- Person A files patent application within 1 year of the invention they published

Result:

- Person B application (or patent) is not prior art even though it was filed before Person A filed an application

Scenario 3:

- Person A invents
- Person B invents and publishes
- Person A files a patent application
- Person B files a patent application

Result:

- Person B is entitled to patent even though Person A was first to invent and first to file
- Person B publication is prior art to Person A application
- Person B publication removes Person A application as prior art to Person B application

These changes in the law also reduce the significance of laboratory notebooks and invention disclosures as a means to preserve the date of an invention, although these two methods of documentation are still valid as evidence of the invention and recordkeeping.

What will be the impact of these new changes to the US patent system? For small companies, defined as a micro-entity in the AIA, the cost of filing an application is dramatically reduced.[31] This may help to level the playing field for securing protected rights between start-up and well-established companies. In contrast, large well-funded companies, perhaps with in-house legal teams, may be in a better position to file as soon as an invention is conceived, thus placing the small companies at a competitive disadvantage. The effect may be a reduction in the number of start-ups trying to enter the market, as intellectual property, in the form of patents, is a tremendous value generator for a small business trying to raise capital. When considering a strategy for a patent portfolio, the new pressure to be the first-inventor-to-file must be balanced with the disclosure requirements for enablement and the written description to support the broader claims. It may be strategically beneficial to consider more frequent and narrower filings, in addition to paying an additional fee to have a prioritized examination of the application.

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