Review Article

CMJ

www.cmj.ac.kr

Tissue Engineering: Current Strategies and Future Directions

Jennifer L. Olson, Anthony Atala and James J. Yoo*

Wake Forest Institute for Regenerative Medicine, Wake Forest University School of Medicine, NC, USA

Novel therapies resulting from regenerative medicine and tissue engineering technology may offer new hope for patients with injuries, end-stage organ failure, or other clinical issues. Currently, patients with diseased and injured organs are often treated with transplanted organs. However, there is a shortage of donor organs that is worsening yearly as the population ages and as the number of new cases of organ failure increases. Scientists in the field of regenerative medicine and tissue engineering are now applying the principles of cell transplantation, material science, and bioengineering to construct biological substitutes that can restore and maintain normal function in diseased and injured tissues. In addition, the stem cell field is a rapidly advancing part of regenerative medicine, and new discoveries in this field create new options for this type of therapy. For example, new types of stem cells, such as amniotic fluid and placental stem cells that can circumvent the ethical issues associated with embryonic stem cells, have been discovered. The process of therapeutic cloning and the creation of induced pluripotent cells provide still other potential sources of stem cells for cell-based tissue engineering applications. Although stem cells are still in the research phase, some therapies arising from tissue engineering endeavors that make use of autologous, adult cells have already entered the clinical setting, indicating that regenerative medicine holds much promise for the future.

Key Words: *Biomaterials; Cell transplantation; Regenerative medicine; Stem cell; Tissue engineering*

Article History:
received 31 March, 2011
accepted 8 April, 2011

Corresponding Author:
James J. Yoo
Wake Forest Institute for Regenerative Medicine, Wake Forest University School of Medicine, Medical Center Boulevard, 1834, Wake Forest Road, Winston-Salem, NC 27109, USA
TEL: +1-336-713-7294
FAX: +1-336-713-7290
E-mail: jyoo@wfubmc.edu

INTRODUCTION

Patients suffering from diseased and injured organs are often treated with transplanted organs, and this treatment has been in use for over 50 years. In 1955, the kidney became the first entire organ to be replaced in a human, when Murray transplanted this organ between identical twins. Several years later, Murray performed an allogeneic kidney transplant from a non-genetically identical patient into another. This transplant, which overcame the immunologic barrier, marked a new era in medicine and opened the door for use of transplantation as a means of therapy for different organ systems.

As modern medicine increases the human lifespan, the aging population grows, and the need for donor organs grows with it, because aging organs are generally more prone to failure. However, there is now a critical shortage of donor organs, and many patients in need of organs will die while waiting for transplants. In addition, even if an organ becomes available, rejection of organs is still a major problem in transplant patients despite improvements in the methods used for immunosuppression following the transplant procedure. Even if rejection does not occur, the need for lifelong use of immunosuppressive medications leads to a number of complications in these patients.

These problems have led physicians and scientists to look to new fields for alternatives to organ transplantation. In the 1960s, a natural evolution occurred in which researchers began to combine new devices and materials sciences with cell biology, and a new field that is now termed *tissue engineering* was born. As more scientists from different fields came together with the common goal of tissue replacement, the field of tissue engineering became more formally established. Tissue engineering is now defined as "an interdisciplinary field which applies the principles of engineering and life sciences towards the development of bio-

DOI: 10.4068/cmj.2011.47.1.1

logical substitutes that aim to maintain, restore or improve tissue function."[1] Then, after the discovery of human stem cells by Thomson's group in the early 1980s,[2] the field of stem cell biology took shape and suggested that it may one day be possible to obtain and use donor stem cells in tissue engineering strategies, or perhaps even reactivate endogenous stem cells and use them to regenerate failing organs in adult patients.

The fields of stem cells, cell transplantation, and tissue engineering all have one unifying concept-the regeneration of living tissues and organs. Thus, in 1999, William Haseltine, then the Scientific Founder and Chief Executive Officer of Human Genome Sciences, coined the term *regenerative medicine*, in effect bringing all these areas under one defining field.[3]

In the past two decades, scientists have attempted to engineer virtually every tissue of the human body. This article will review the basic techniques used in tissue engineering and discuss some of the progress that has been achieved in this field.

THE BASIC COMPONENTS OF REGENERATIVE MEDICINE STRATEGIES

The field of regenerative medicine encompasses various areas of technology, such as tissue engineering, stem cells, and cloning. Tissue engineering, one of the major areas of regenerative medicine, follows the principles of cell transplantation, materials science, and engineering toward the development of biological substitutes that can restore and maintain normal function. Tissue engineering strategies generally fall into two categories: the use of acellular scaffolds, which depend on the body's natural ability to regenerate for proper orientation and direction of new tissue growth, and the use of scaffolds seeded with cells. Acellular scaffolds are usually prepared by manufacturing artificial scaffolds or by removing cellular components from tissues via mechanical and chemical manipulation to produce acellular, collagen-rich matrices.[4-7] These matrices tend to slowly degrade on implantation and are generally replaced by the extracellular matrix (ECM) proteins that are secreted by the in-growing cells. Cells can also be used for therapy via injection, either with carriers such as hydrogels or alone.

1. Biomaterials for use in regenerative medicine

In the past, synthetic materials were introduced to replace or to rebuild diseased tissues or parts in the human body. The manufacture of new materials, such as tetrafluoroethylene (Teflon) and silicone, opened a new field of research that led to the development of a wide array of devices that could be applied for human use. Although these devices could provide structural support or replacement, the functional component of the original tissue was not restored. However, studies in cell biology, molecular biology, and biochemistry allowed a better understanding of the ECM and its interaction with cells in the tissues of the body, as well as interactions with growth factors and their ligands, and as a result, new biomaterials were designed with these interactions in mind.

In tissue engineering, biomaterials replicate the biological and mechanical function of the native ECM found in tissues in the body. Biomaterials provide a three-dimensional space in which cells can attach, grow, and form new tissues with appropriate structure and function. They also allow for the delivery of cells and appropriate bioactive factors (e.g., cell adhesion peptides, growth factors) to desired sites in the body.[8] Because most mammalian cell types are anchorage-dependent and will die if no cell-adhesion substrate is available, biomaterials provide this substrate while allowing delivery of cells with high loading efficiency. Biomaterials can also provide mechanical support against *in vivo* forces so that the predefined three-dimensional structure of a tissue-engineered organ is maintained during tissue development.

The ideal biomaterial should be biodegradable and bioresorbable to support the replacement of normal tissue without inducing inflammation. Incompatible materials are destined for an inflammatory or foreign-body response that eventually leads to rejection or necrosis. Because biomaterials provide temporary mechanical support while the cells undergo spatial reorganization into tissue, a properly chosen biomaterial should allow the engineered tissue to maintain sufficient mechanical integrity to support itself in early development, while in late development, it should have begun degradation such that it does not hinder further tissue growth.[8] The degradation products, if produced, should be removed from the body via metabolic pathways at an adequate rate to ensure that the concentration of these degradation products in the tissues remains at a tolerable level.[9]

Generally, three classes of biomaterials have been utilized for engineering tissues: naturally derived materials (e.g., collagen and alginate),[10-14] acellular tissue matrices (e.g., bladder submucosa and small intestinal submucosa),[4-7] and synthetic polymers such as polyglycolic acid (PGA), polylactic acid (PLA), and poly (lactic-co-glycolic acid) (PLGA).[15-18] These classes of biomaterials have been tested with respect to their biocompatibility.[19,20] Naturally derived materials and acellular tissue matrices have the potential advantage of biological recognition. However, synthetic polymers can be produced reproducibly on a large scale with controlled properties such as strength, degradation rate, and microstructure.

2. Cells for use in cell therapy and tissue engineering

1) Native cells: When native cells are used for tissue engineering, a small piece of donor tissue is dissociated into individual cells. These cells are expanded in culture and either injected directly back into the host or attached to a support matrix and then reimplanted. The source of donor tissue can be heterologous (such as bovine), allogeneic (same species, different individual), or autologous. The preferred cells to use are autologous cells, where a biopsy of tissue

is obtained from the host, the cells are dissociated and expanded in culture, and the expanded cells are implanted into the same host.[5,21-28] The use of autologous cells, although it may cause an inflammatory response, avoids rejection, and thus the deleterious side effects of immunosuppressive medications can be avoided.

Ideally, both structural and functional tissue replacement will occur with minimal complications when autologous native cells are used. However, one of the limitations of applying cell-based regenerative medicine techniques to organ replacement has been the inherent difficulty of growing specific cell types in large quantities. Even when some organs, such as the liver, have a high regenerative capacity *in vivo*, cell growth and expansion *in vitro* may be difficult. By studying the privileged sites for committed precursor cells in specific organs, as well as exploring the conditions that promote differentiation, one may be able to overcome the obstacles that limit cell expansion *in vitro*. For example, urothelial cells could be grown in the laboratory setting in the past, but only with limited expansion. Several protocols were developed over the past two decades that identified the undifferentiated cells and kept them undifferentiated during their growth phase.[27,29,30-32] With the use of these methods of cell culture, it is now possible to expand a urothelial strain from a single specimen that initially covered a surface area of 1 cm^2 to one covering a surface area of 4,202 m^2 (the equivalent of one football field) within 8 weeks.[27] These studies indicated that it should be possible to collect autologous bladder cells from human patients, expand them in culture, and return them to the donor in sufficient quantities for reconstructive purposes.[27,30] Major advances have been achieved within the past decade on the possible expansion of a variety of primary human cells, with specific techniques that make the use of autologous cells for clinical application possible.

Most current strategies for tissue engineering depend on a sample of autologous cells from the diseased organ of the host. However, for many patients with extensive end-stage organ failure, a tissue biopsy may not yield enough normal cells for expansion and transplantation. In other instances, primary autologous human cells cannot be expanded from a particular organ, such as the pancreas. In these situations, stem cells are envisioned as being an alternative source of cells from which the desired tissue can be derived. Stem cells can be derived from discarded human embryos (human embryonic stem cells), from fetal tissue, or from adult sources (bone marrow, fat, skin).

3. Stem cells for use in tissue engineering

1) Embryonic stem cells: Human embryonic stem (hES) cells exhibit two remarkable properties: the ability to proliferate in an undifferentiated but pluripotent state (self-renewal), and the ability to differentiate into many specialized cell types.[36] They can be isolated by aspirating the inner cell mass from the embryo during the blastocyst stage (5 days post-fertilization) and are usually grown on feeder layers consisting of mouse embryonic fibroblasts or human feeder cells.[37] More recent reports have shown that these cells can be grown without the use of a feeder layer[38] and thus avoid the exposure of these human cells to mouse viruses and proteins. These cells have demonstrated longevity in culture by maintaining their undifferentiated state for at least 80 passages when grown by use of current published protocols.[2,39] In addition, hES cells are able to differentiate into cells from all three embryonic germ layers *in vitro*. Skin and neurons have been formed, indicating ectodermal differentiation.[40-43] Blood, cardiac cells, cartilage, endothelial cells, and muscle have been formed, indicating mesodermal differentiation.[44-46] Pancreatic cells have been formed, indicating endodermal differentiation.[47] In addition, as further evidence of their pluripotency, embryonic stem cells can form embryoid bodies, which are cell aggregations that contain all three embryonic germ layers while in culture and can form teratomas *in vivo*.[48] However, there are many ethical and religious concerns associated with hES cells because embryos are destroyed in order to obtain them. Thus, the use of these cells is currently banned in many countries.

2) Stem cells from somatic cell nuclear transfer: Stem cells for tissue engineering could also be generated through cloning procedures. There has been tremendous interest in the field of nuclear cloning since the birth of the cloned sheep Dolly in 1997, but actually, Dolly was not the first animal produced by using nuclear transfer. In fact, frogs were the first successfully cloned vertebrates derived from nuclear transfer.[49] However, in the frog experiment, the nuclei used for cloning were derived from non-adult sources. In fact, live lambs were produced in 1996 by using nuclear transfer as well, but they were produced from differentiated epithelial cells derived from embryonic discs.[50] The significance of Dolly was that she was the first mammal to be derived from an adult somatic cell by use of nuclear transfer.[51] Since then, animals from several species have been grown by using nuclear transfer technology, including cattle,[52] goats,[53] mice,[54] and pigs.[55-56]

Two types of nuclear cloning, reproductive cloning and therapeutic cloning, have been described, and a better understanding of the differences between the two types may help to alleviate some of the controversy that surrounds these technologies.[57-58] Banned in most countries for human applications, reproductive cloning is used to generate an embryo that has the identical genetic material as its cell source. This embryo can then be implanted into the uterus of a female to give rise to an infant that is a clone of the donor. On the other hand, therapeutic cloning is used to generate early stage embryos that are explanted in culture to produce embryonic stem cell lines whose genetic material is identical to that of its source. These autologous stem cells have the potential to become almost any type of cell in the adult body, and thus would be useful in tissue and organ replacement applications.[59] Therefore, therapeutic cloning, which has also been called somatic cell nuclear transfer, may provide an alternative source of transplantable cells. According to data from the Centers for Disease

Control and Prevention, an estimated 3,000 Americans die every day of diseases that could have been treated with stem cell–derived tissues.[60-61] With current allogeneic tissue transplantation protocols, rejection is a frequent complication because of immunologic incompatibility, and immunosuppressive drugs are usually required.[59] The use of transplantable tissue and organs derived from therapeutic cloning could lead to the avoidance of immune responses that typically are associated with transplantation of nonautologous tissues.[60]

While promising, somatic cell nuclear transfer technology has certain limitations that require further study before this technique can be applied widely in tissue or organ replacement therapy. First, the efficiency of the cloning process is very low, as evidenced by the fact that most embryos derived from the cloning process do not survive.[62-64] To improve cloning efficiency, further improvements are required in many of the complex steps of nuclear transfer, such as the enucleation process for oocytes, the actual transfer of a nucleus to this enucleated oocyte, and the activation process that instructs the cloned oocytes to begin dividing. In addition, cell cycle synchronization between donor cells and recipient oocytes must be accomplished.[65]

3) Reprogramming and generation of iPS cells: Within the past few years, exciting reports of the successful transformation of adult somatic cells into pluripotent stem cells through genetic "reprogramming" have been published. Reprogramming is a technique that involves de-differentiation of adult somatic cells (such as fibroblasts) to produce patient-specific pluripotent stem cells. This process is especially exciting because it allows pluripotent stem cells to be obtained without the use of embryos. Also, cells generated by reprogramming are genetically identical to the somatic cells used (and thus to the patient who donated these cells) and should not be rejected. Yamanaka was the first to discover that mouse embryonic fibroblasts (MEFs) and adult mouse fibroblasts could be reprogrammed into an "induced pluripotent state (iPS)."[66] They examined 24 genes that were thought to be important for embryonic stem cells and identified 4 key genes that, when introduced into the reporter fibroblasts via retroviral vectors, resulted in drug-resistant cells. These were *Oct3/4, Sox2, c-Myc,* and *Klf4*. The resultant iPS cells possessed the immortal growth characteristics of self-renewing embryonic stem cells, expressed genes specific for embryonic stem cells, and generated embryoid bodies *in vitro* and teratomas *in vivo*. When iPS cells were injected into mouse blastocysts, they contributed to a variety of cell types. However, although iPS cells selected in this way were pluripotent, they were not identical to embryonic stem cells. Unlike embryonic stem cells, chimeras made from iPS cells did not result in full-term pregnancies. Gene expression profiles of the iPS cells showed that they possessed a distinct gene expression signature that was different from that of embryonic stem cells. In addition, the epigenetic state of the iPS cells was somewhere between that found in somatic cells and that found in embryonic stem cells, suggesting that the reprogramming was incomplete.

These results were improved significantly by Wernig and Jaenisch in July 2007.[67] Fibroblasts were infected with retroviral vectors and selected for the activation of endogenous *Oct4* or *Nanog* genes. Results from this study showed that DNA methylation, gene expression profiles, and the chromatin state of the reprogrammed cells were similar to those of embryonic stem cells. Teratomas induced by these cells contained differentiated cell types representing all three embryonic germ layers. Most importantly, the reprogrammed cells from this experiment could form viable chimeras and contribute to the germline-like embryonic stem cells, suggesting that these iPS cells were completely reprogrammed. Wernig et al observed that the number of reprogrammed colonies increased when drug selection was initiated later (day 20 rather than day 3 post-transduction). This suggests that reprogramming is a slow and gradual process and may explain why previous attempts resulted in incomplete reprogramming.

It has recently been shown that reprogramming of human cells is possible.[68-69] Yamanaka generated human iPS cells that are similar to hES cells in terms of morphology, proliferation, gene expression, surface markers, and teratoma formation. Thompson's group showed that retroviral transduction of the stem cell markers *OCT4, SOX2, NANOG,* and *LIN28* could generate pluripotent stem cells. However, in both studies, the human iPS cells were similar but not identical to hES cells. Although reprogramming is an exciting phenomenon, our limited understanding of the mechanism underlying it currently limits the clinical applicability of the technique, but the future potential of reprogramming is quite exciting.

4) Amniotic fluid and placental stem cells: An alternate source of stem cells is the amniotic fluid and placenta. Amniotic fluid and the placenta are known to contain multiple partially differentiated cell types derived from the developing fetus. We isolated stem cell populations from these sources, called amniotic fluid and placental stem cells (AFPSC), that express embryonic and adult stem cell markers.[70] The undifferentiated stem cells expand extensively without feeders and double every 36 hours. Unlike hES cells, the AFPSC do not form tumors *in vivo*. Lines maintained for over 250 population doublings retained long telomeres and a normal karyotype. AFS cells are broadly multipotent. Clonal human lines verified by retroviral marking can be induced to differentiate into cell types representing each embryonic germ layer, including cells of adipogenic, osteogenic, myogenic, endothelial, neuronal, and hepatic lineages. In this respect, they meet a commonly accepted criterion for pluripotent stem cells, without implying that they can generate every adult tissue. Examples of differentiated cells derived from AFS cells and displaying specialized functions include neuronal lineage cells secreting the neurotransmitter L-glutamate or expressing G-protein-gated inwardly rectifying potassium (GIRK) channels, hepatic lineage cells producing urea, and osteogenic lineage cells forming tissue engineered bone. The cells could

be obtained either from amniocentesis or chorionic villous sampling in the developing fetus, or from the placenta at the time of birth. The cells could be preserved for self-use and used without rejection, or they could be banked. A bank of 100,000 specimens could potentially supply 99% of the US population with a perfect genetic match for transplantation. Such a bank may be easier to create than with other cell sources, because there are approximately 4.5 million births per year in the USA.[70]

5) Adult stem cells: Adult stem cells, especially hematopoietic stem cells, are the best understood cell type in stem cell biology.[71] The presence of stem cells in the adult was first discerned by Till and McCulloch, who were investigating the mechanisms by which the bone marrow could regenerate after exposure to radiation.[72] However, adult stem cell research remains an area of intense study, because their potential for therapy may be applicable to a myriad of degenerative disorders. Within the past decade, adult stem cell populations have been found in many adult tissues other than the bone marrow and the gastrointestinal tract, including the brain,[73-74] skin,[75] and muscle.[76] Many other types of adult stem cells have been identified in organs all over the body and are thought to serve as the primary repair entities for their corresponding organs.[77] The discovery of such tissue-specific progenitors has opened up new avenues for research.

A notable exception to the tissue-specificity of adult stem cells is the mesenchymal stem cell (MSC), also known as the multipotent adult progenitor cell. This cell type is derived from bone marrow stroma.[78-79] Such cells can differentiate *in vitro* into numerous tissue types[80-81] and can also differentiate developmentally if injected into a blastocyst. Multipotent adult progenitor cells can develop into a variety of tissues including neuronal,[82] adipose,[76] muscle,[76,83] liver,[84-85] lungs,[86] spleen,[87] and gut tissue,[79] but notably not bone marrow or gonads.

In addition, stem cells derived from adipose tissue may also be an autologous and self-renewing cell source. Adipose-derived stem cells (ADSCs) have been shown to differentiate into a variety of cell phenotypes, and since they are easily obtained, they show great promise for future types of reconstructive surgery based on tissue engineering and there have been several clinical trials using these cells. Wilson and Mizuno have both provided excellent, detailed reviews of these.[88-89]

Research into more differentiated types of adult stem cells has, however, progressed slowly, mainly because investigators have had great difficulty in maintaining adult non-mesenchymal stem cells in culture. Some cells, such as those of the liver, pancreas, and nerve, have very low proliferative capacity *in vitro*, and the functionality of some cell types is reduced after the cells are cultivated. Isolation of cells has also been problematic, because stem cells are present in extremely low numbers in adult tissue.[84,90] While the clinical utility of adult stem cells is currently limited, great potential exists for future use of such cells in tissue-specific regenerative therapies. The advantage of adult stem cells is that they can be used in autologous therapies, thus avoiding any complications associated with immune rejection.

CELLULAR THERAPIES

The simplest regenerative medicine strategies are those that are based on the actions of cells, which can be implanted either alone or within a type of carrier material, such as a hydrogel. These cell therapies are designed to inject or implant healthy cells to replace populations of cells that are no longer functioning properly owing to disease or injury. The cells used in these therapies can be autologous cells derived from a tissue biopsy and expanded in culture, or they can be stem cells from various sources that can be guided to differentiate into appropriate cell types by using both endogenous and exogenous biochemical cues.

For example, one area of intense study in regenerative medicine is the pancreas, because the ability to replace or regenerate the insulin-producing cells of this organ could lead to novel treatments or a cure for diabetes. In a series of exciting experiments, Zhou et al demonstrated that regeneration of the insulin-producing cells of the pancreas, the β-cells, may be possible by using cellular reprogramming techniques[91] Using a mouse model, they showed that *in vivo* activation of a specific combination of three transcription factors (Ngn3, Pdx1, and Mafa) by use of adenoviral vectors led to the reprogramming of adult differentiated pancreatic exocrine cells into cells that closely resembled β-cells. These cells were similar to native β-cells in size, shape, and ultrastructure, and they expressed genes that are specific to β-cells as well. Interestingly, these cells secreted insulin and expressed vascular endothelial growth factor (VEGF), which allowed them to remodel the local vasculature in a manner similar to native β-cells. In fact, these reprogrammed cells were able to partially ameliorate hyperglycemia in diabetic mice, suggesting that reprogramming techniques for treating disease may one day become a reality.

Degenerative muscle diseases such as Duchenne's muscular dystrophy have devastating effects on quality of life. To date, these genetic disorders have no suitable treatment. Early enthusiasm for gene therapy interventions has been tempered by issues of vector toxicity and inadequate gene transfer to target muscle cells *in vivo*. However, natural mechanisms of muscle repair have suggested that cell-based therapy could take advantage of natural homing mechanisms to direct cells to the proper location.[92] Experiments using the mdx mouse model, in which the dystrophin gene is mutated, indicate that injection of normal muscle precursors and dermal fibroblasts into skeletal muscle can lead to increased expression of dystrophin and improved functional outcomes. However, this treatment option requires further studies before it can be widely applied in the clinic.

Although many of these cell therapies are still in the experimental stage, some are being translated to the clinic

and clinical trials are being performed. Vesicoureteral reflux (VUR; a condition in which urine flows backwards from the bladder into the ureter and kidney) and stress urinary incontinence are two urologic conditions that can result from dysfunction of a specific sphincter muscle. When severe, these conditions are repaired surgically. However, cell-based therapies for both VUR and incontinence would be an important alternative to surgical repair of these conditions. Ideally, such a therapy would be easily administered by injection and well tolerated by the patient. The injectable therapy should be non-antigenic, non-migratory, volume stable, and safe for human use, and in addition, it should be able to carry cells and serve as a matrix *in vivo*.

Toward this goal, long-term studies were conducted to determine the effects of injectable chondrocytes for the treatment of VUR *in vivo*.[93] Chondrocytes were chosen because the use of autologous cartilage for the treatment of VUR in humans would satisfy all of the requirements for an ideal injectable cell-based therapy. Chondrocytes derived from an ear biopsy can be readily grown and expanded in culture. Neocartilage formation can be achieved *in vitro* and *in vivo* by using chondrocytes cultured on synthetic biodegradable polymers. In the VUR experiments, chondrocytes were suspended in an alginate matrix and injected around the vesicoureteral sphincter. In time, normal cartilage replaced the alginate as the alginate slowly degraded. This system was then adapted for the treatment of VUR in a porcine model.[94] These studies show that chondrocytes can be easily harvested and combined with alginate *in vitro*, that the suspension can be easily injected cystoscopically, and that the elastic cartilage tissue formed can correct the VUR without any evidence of obstruction.

Two multicenter clinical trials were conducted by use of this engineered chondrocyte technology. First, patients with VUR were treated at 10 centers throughout the United States. The patients had a similar success rate as with other injectable substances in terms of cure. Cartilage formation was not noted in patients with treatment failure. Patients who were cured probably had a biocompatible region of engineered autologous tissue present.[95] Secondly, patients with urinary incontinence were treated endoscopically with injected chondrocytes at three different medical centers. Phase 1 trials showed an approximate success rate of 80% at 3 and 12 months postoperatively.[96]

TISSUE THERAPIES

Tissue engineering strategies are often referred to as "growing organs in the laboratory." In these strategies, differentiated cells or stem cells are seeded onto a biomaterial scaffold and this construct is allowed to mature *in vitro* in a bioreactor for a short time before implantation *in vivo*. These constructs are designed to replace a malfunctioning organ in its entirety. In recent years, it has been shown that hollow organs, such as the urinary bladder, urethra, and blood vessels, can be successfully engineered in the laboratory, and these successes are described below.

The urethra can be repaired by using tissue-engineered grafts in several ways. It has been shown that various biomaterials without cells, such as PGA and acellular collagen-based matrices from small intestine and bladder, can be used experimentally (in animal models) for the regeneration of urethral tissue.[7,97-99] Acellular collagen matrices derived from bladder submucosa have been used experimentally and clinically. In animal studies, segments of the urethra were resected and replaced with acellular matrix grafts in an onlay fashion. Histological examination showed complete epithelialization and progressive vessel and muscle infiltration, and the animals were able to void through the neo-urethras.[7] These results were confirmed in a clinical study of patients with hypospadias and urethral stricture disease.[100] Decellularized cadaveric bladder submucosa was used as an onlay matrix for urethral repair in patients with stricture disease and hypospadias. Patent, functional neo-urethras were noted in these patients with up to a 7-year follow-up. The use of an off-the-shelf matrix appears to be beneficial for patients with abnormal urethral conditions and obviates the need for obtaining autologous grafts, thus decreasing operative time and eliminating donor site morbidity.

Unfortunately, the above techniques are not applicable for tubularized urethral repairs. The collagen matrices are able to replace urethral segments only when used in an onlay fashion. However, if a tubularized repair is needed, the collagen matrices should be seeded with autologous cells to avoid the risk of stricture formation and poor tissue development.[101] In addition, cell-seeded matrices must be used if the segment of urethra to be replaced is longer than about 1 cm.[102] Recently, Raya-Rivera and colleagues used tissue-engineered urethras that had been created from patients' own cells for tubularized urethral reconstruction. In this preliminary study, five boys who had urethral defects were treated. A tissue biopsy was taken from each patient, and the muscle and epithelial cells derived from the biopsy sample were expanded and seeded onto tubularized polyglycolic acid:poly(lactide-co-glycolide acid) scaffolds to create neo-urethras for implantation. The patients then underwent urethral reconstruction with the engineered urethras. After surgery, these patients were followed for up to 6 years. All of the patients experienced an increase in urinary flow rate, and serial radiographic and endoscopic studies showed that they maintained wide urethral calibers throughout the follow-up period and did not develop strictures. In addition, urethral biopsies were performed in these patients, and these revealed that by 3 months postsurgery, the engineered grafts had developed a normal appearing tissue architecture consisting of a urothelial layer surrounded by a muscular layer.[103]

In addition, similar techniques have been used to create tissue-engineered bladder constructs. Urothelial and muscle cells can be expanded *in vitro*, seeded onto polymer scaffolds, and allowed to attach and form sheets of cells.[104] These principles were applied in several studies in which

tissue-engineered bladders were used to repair subtotal cystectomies in beagle dogs.[28,105-106] The first clinical experience in which engineered bladder tissue for cystoplasty reconstruction in patients was conducted starting in 1999. A small pilot study of seven patients was reported, using a collagen scaffold seeded with cells either with or without omentum coverage, or a combined PGA-collagen scaffold seeded with cells and omental coverage. The patients reconstructed with the engineered bladder tissue created with the PGA-collagen cell-seeded scaffolds showed increased compliance, decreased end-filling pressures, increased capacities, and longer dry periods.[107] Although the experience is promising in terms of showing that engineered tissues can be implanted safely, it is just a start in terms of accomplishing the goal of engineering fully functional bladders. Further experimental and clinical work is being conducted.

Xenogenic or synthetic materials have been used as replacement blood vessels for complex cardiovascular lesions. However, these materials typically lack growth potential, and may place the recipient at risk for complications such as stenosis, thromboembolization, or infection.[108] Tissue-engineered vascular grafts have been constructed by using autologous cells and biodegradable scaffolds and have been applied in dog and lamb models.[109-112] The key advantage of using these autografts is that they degrade *in vivo* and thus allow the new tissue to form without the long-term presence of foreign material.[108] Translation of these techniques from the laboratory to the clinical setting has begun, with autologous vascular cells harvested, expanded, and seeded onto a biodegradable scaffold.[113] The resultant autologous construct was used to replace a stenosed pulmonary artery that had been previously repaired. Seven months after implantation, no evidence of graft occlusion or aneurysmal changes was noted in the recipient. In addition, another group created tissue-engineered blood vessels by using the cell-sheet multilayer method and then used these constructs to successfully create vascular access points for hemodialysis in 10 patients.[114] More recently, the same group completed a larger study on these engineered vessels for hemodialysis, which indicated that the 1-month and 6-month patency of the grafts was 78% and 60%, respectively, which meets the approved criteria for a high-risk patient cohort.[115]

Finally, few treatment options are currently available for patients who suffer from severe congenital tracheal pathology, such as stenosis, atresia, and agenesis, due to the limited availability of autologous transplantable tissue in the neonatal period. Tissue engineering in the fetal period may be a viable alternative for the surgical treatment of these prenatally diagnosed congenital anomalies, because cells could be harvested and grown into transplantable tissue in parallel with the remainder of gestation. Chondrocytes from both elastic and hyaline cartilage specimens have been harvested from fetal lambs, expanded *in vitro*, and then dynamically seeded onto biodegradable scaffolds.[116] The constructs were then implanted as replacement tracheal tissue in fetal lambs. The resultant tissue-engineered cartilage was noted to undergo engraftment and epithelialization, while maintaining its structural support and patency.

Recently, Martin Birchall's group moved this technology into a human patient with end-stage airway disease.[117] This group was able to remove the cellular material and MHC antigens from a human donor trachea and, using a specialized bioreactor, seed this acellular matrix with chondrocytes and epithelial cells derived from the patient to receive the graft. This construct was then used to replace the patient's left main bronchus. There were no perioperative complications, and the left lung ventilated normally as soon as the graft was placed. At 3 months after surgery, the patient's lung function was in the normal range for her age and sex, and she was able to function normally. Although longer follow-up and larger study populations are needed, this report indicates that tissue engineering may be a new option for patients with airway disease.

However, whereas there has been exciting progress with tissue engineering techniques for hollow organs, the development of methods to generate larger, solid organs with more complex histological structure has been much more difficult. A number of issues must be addressed before fully functional, engineered organs such as liver and kidney can be prepared in the laboratory. First, these organs contain extremely complex internal structures made up of numerous cell types arranged in very specific ways, and simple cell-seeding techniques may not be sufficient for reconstructing these structures. In addition, the large size of these organs dictates that the delivery of oxygen and nutrients to each part of the organ will be a challenge, unless a method for engineering a functional vascular network within the organ can be found. However, despite the challenges, there have been some encouraging results from several studies. For example, the kidney contains multiple cell types and a complex functional anatomy that renders it one of the most difficult to reconstruct,[21,118] yet we were able to create a rudimentary form of this organ that appeared to have at least the filtration properties of the native kidney.

We applied the principles of both tissue engineering and therapeutic cloning in an effort to produce genetically identical renal tissue in a large animal model, the cow (*Bos taurus*).[119] Bovine skin fibroblasts from adult Holstein steers were obtained by ear notch, and single donor cells were isolated and microinjected into the perivitelline space of donor enucleated oocytes (nuclear transfer). The resulting blastocysts were implanted into progestin-synchronized recipients to allow for further *in vivo* growth. After 12 weeks, cloned renal cells were harvested and expanded *in vitro*. Next, the cloned renal cells were seeded on scaffolds consisting of three collagen-coated cylindrical silastic catheters. The ends of the three membranes of each scaffold were connected to catheters that terminated into a collecting reservoir. This created a renal neo-organ with a mechanism for collecting the excreted urinary fluid. These scaffolds with the collecting devices were transplanted sub-

cutaneously into the same steer from which the genetic material originated and then retrieved 12 weeks after implantation.

At this time, a yellow urine-like fluid was observed collecting within the reservoir of the device. Chemical analysis of this fluid, including urea nitrogen and creatinine levels, electrolyte levels, specific gravity, and glucose concentration, revealed that the implanted renal cells possessed filtration, reabsorption, and secretory capabilities. Histological examination of the retrieved implants revealed extensive vascularization and self-organization of the cells into glomeruli and tubule-like structures. A clear continuity between the glomeruli, the tubules, and the silastic catheter was noted that allowed the passage of urine into the collecting reservoir. These studies demonstrated that cells derived from nuclear transfer can be successfully harvested, expanded in culture, and transplanted *in vivo* with the use of biodegradable scaffolds on which the single suspended cells can organize into tissue structures that are genetically identical to those of the host. These studies were the first demonstration of the use of therapeutic cloning for regeneration of tissues *in vivo*. However, the size of this device was small, and the challenge will be to create a larger device with functioning vasculature and innervations, so that it can replace all of the myriad metabolic functions of the kidney.

CHALLENGES AND FUTURE DIRECTIONS IN REGENERATIVE MEDICINE: TRANSLATING REGENERATIVE THERAPIES TO THE CLINIC

The experiences with urethral, bladder, blood vessel, and tracheal replacement using tissue engineering provide encouragement for future efforts to engineer other organs in the laboratory. These experiences also cast light on unsolved problems. For example, innervation of tissues and organs is important for achieving full functionality. In the canine engineered bladder experiments, the observation of positive S-100 staining was consistent with growth of neural structures into the neo-bladders, and bladder function was restored soon after implantation.[28] Innervation of tissue-engineered constructs has been observed in other systems such as the small intestine.[120] Not only is successful connection with the nervous system important for the functionality of neo-organs, but evidence suggests that it can enhance tissue regeneration.[121-122] The controlled release of neurotrophic factors is one potential approach to promote peripheral nerve regeneration and synapse formation with engineered tissue.[123] Direct electrical stimulation has proven useful in muscle regeneration[124] and may have broader applicability.

An even more fundamental issue for the ultimate success of laboratory-grown organs, particularly those with complex three-dimensional structure, is the provision of adequate oxygen and the generation of new vasculature. It has been appreciated for some years that in metabolically active tissues, the distance over which oxygen typically must diffuse from a capillary bed to reach a cell is about 0.1 mm, but that in clinical grafts, the distance from the edge to the center of the graft is likely to exceed that by at least 50-fold.[125-126] Therefore, with few exceptions (e.g., cartilage), oxygen is rate-limiting for the viability of grafted cells, and thus for organ engineering. Neovascularization, an intricate morphogenetic process that allows the formation of extensively branched vessels, even in an adult, must occur rapidly and efficiently for a grafted neo-organ to thrive after implantation.[127] Moreover, special measures may be necessary to ensure survival of grafted tissue during the initial period after implantation, until a functional vascular bed is in place. Currently, three types of strategies have been devised to solve the oxygen supply problem.

The first strategy involves the use of mechanical or chemical sources of oxygen that can support the construct before and immediately after implantation, until the neovascularization process is completed and can provide the neo-organ with sufficient blood circulation. An intra-tissue perfusion system utilizing an array of micro-needles to deliver oxygen and nutrients and eliminate waste enhances the viability and functionality of thick (1 mm) slices of liver tissue *in vitro* and might facilitate *in vivo* grafting.[128] In addition, the use of oxygen-carrying molecules such as perfluorocarbons could promote the function of cells in culture and of encapsulated cells and organ constructs implanted into animals.[129-130] Our laboratory recently showed that a PLGA film incorporating an oxygen-generating system (sodium percarbonate) could prevent the necrosis of ischemic tissue over several days *in vivo*.[131] We hope to develop such novel scaffold materials further to support the survival of large, complex organ constructs in the initial period after implantation.

Second, "prevascularization" strategies aim to generate neo-organs engineered with a preexisting channel structure to facilitate the generation of a competent vascular network.[130,132] To accomplish this, endothelial lineage cells can be pre-seeded into the channels or may be recruited *in vivo* by using biochemical signals that are embedded in or released by the scaffold. However, there is still the question of how to create channels in a way that will be interpreted as a natural vascular network by the body. One solution would be to employ decellularized tissue as the scaffold. A recent study demonstrated that perfusion of an entire heart with detergents yields an acellular structure in which the native vascular channels remain intact.[133] We independently devised perfusion-decellularization technology using liver tissue and have found that the vascular tree of the whole organ scaffold remains patent and can be repopulated with large numbers of endothelial cells.[134] Alternatively, several technologies can be used to manufacture scaffolds with preformed channels, potentially with cells incorporated, designed to promote neo-vascularization. For example, laser guided "writing" was used to pattern endothelial cells and promote their aggregation into tubular vessels.[135] Similarly, ink-jet-based bioprinting of cells and biomaterials by thermal ink jet technology can

provide remarkable control of the fine structure of engineered tissues, including the generation of intricate vessel networks.[136] We have used layer-by-layer ink jet printing to produce three-dimensional constructs containing endothelial cells and showed that these develop functional microvascularization when implanted *in vivo*, as assessed by magnetic resonance imaging.[137] Electrospinning of living cells with biomaterials offers similar potential to fabricate organ structures with pre-patterned vessels.[138] Mathematical modeling of scaffolds designed to contain a preexisting arteriovenous loop shows how the provision of an oxygen source within the scaffold can dynamically support further neo-vascularization and tissue development.[139]

Third, it is well established that growth factors such as VEGF and FGF can promote vascularization in engineered tissues.[140] Recent efforts have extended this approach by incorporating additional pro-angiogenic molecules into scaffolds, such as organ-specific ECM from liver to support sinusoidal endothelial cells.[141] Synthetic biomaterials designed to provide signals normally presented by the ECM will complement, and may eventually supersede, the use of the native molecules.[142]

Finally, several of the clinical trials involving bioengineered products have been placed on hold because of the costs involved with the specific technology. With a bioengineered product, costs are usually high because of the biological nature of the therapies involved, and as with any therapy, the cost that the medical health care system can allow for a specific technology is limited. Therefore, the costs of bioengineered products have to be lowered before they can have an impact clinically. This is currently being addressed for multiple tissue-engineered technologies. As the technologies advance over time, and the volume of the application is considered, costs will naturally decrease.

SUMMARY AND CONCLUSION

Regenerative medicine efforts are currently underway experimentally for virtually every type of tissue and organ within the human body. As regenerative medicine incorporates the fields of tissue engineering, cell biology, nuclear transfer, and materials science, personnel who have mastered the techniques of cell harvest, culture, expansion, transplantation, and polymer design are essential for the successful application of these technologies to extend human life. Various tissues are at different stages of development, with some already being used clinically, a few in preclinical trials, and some in the discovery stage. Recent progress suggests that engineered tissues may have an expanded clinical applicability in the future and may represent a viable therapeutic option for those who would benefit from the life-extending benefits of tissue replacement or repair.

REFERENCES

1. Atala A. Lanza RP. Preface. In: Atala A. Lanza RP, eds. Methods of tissue engineering. San Diego: Academic Press, 2001.
2. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145-7.
3. Hasetine W. A brave new medicine. A conversation with William Haseltine. Interview by Joe Flower. Health Forum J 1999;42:28-30.
4. Dahms SE, Piechota HJ, Dahiya R, Lue TF, Tanagho EA. Composition and biomechanical properties of the bladder acellular matrix graft: comparative analysis in rat, pig and human. Br J Urol 1998;82:411-9.
5. Yoo JJ, Meng J, Oberpenning F, Atala A. Bladder augmentation using allogenic bladder submucosa seeded with cells. Urology 1998;51:221-5.
6. Piechota HJ, Dahms SE, Nunes LS, Dahiya R, Lue TF, Tanagho EA. In vitro functional properties of the rat bladder regenerated by the bladder acellular matrix graft. J Urol 1998;159:1717-24.
7. Chen F, Yoo JJ, Atala A. Acellular collagen matrix as a possible "off the shelf" biomaterial for urethral repair. Urology 1999;54: 407-10.
8. Kim BS, Mooney DJ. Development of biocompatible synthetic extracellular matrices for tissue engineering. Trends Biotechnol 1998;16:224-30.
9. Bergsma JE, Rozema FR, Bos RR, Boering G, de Bruijn WC, Pennings AJ. In vivo degradation and biocompatibility study of in vitro pre-degraded as-polymerized polyactide particles. Biomaterials 1995;16:267-74.
10. Li ST. Biologic biomaterials: tissue-derived biomateri-als (collagen). In: Brozino JD, ed. The Biomedical Engineering Handbook. Boca Raton, FL: CRS Press, 1995:627-47.
11. Silver FH, Pins G. Cell growth on collagen: a review of tissue engineering using scaffolds containing extracellular matrix. J Long Term Eff Med Implants 1992;2:67-80.
12. Sams AE, Nixon AJ. Chondrocyte-laden collagen scaffolds for resurfacing extensive articular cartilage defects. Osteoarthritis Cartilage 1995;3:47-59.
13. Smidsrød O, Skjåk-Braek G. Alginate as immobilization matrix for cells. Trends Biotechnol 1990;8:71-8.
14. Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 1980;210:908-10.
15. Gilding DK. Biodegradable polymers. In: Williams DF, ed. Biocompatibility of Clinical Implant Materials. Boca Raton, FL: CRC Press, 1981:209-32.
16. Mikos AG, Lyman MD, Freed LE, Langer R. Wetting of poly (L-lactic acid) and poly (DL-lactic-co-glycolic acid) foams for tissue culture. Biomaterials 1994;15:55-8.
17. Choi JS, Lee SJ, Christ GJ, Atala A, Yoo JJ. The influence of electrospun aligned poly (epsilon-caprolactone)/collagen nanofiber meshes on the formation of self-aligned skeletal muscle myotubes. Biomaterials 2008;29:2899-906.
18. Lee SJ, Liu J, Oh SH, Soker S, Atala A, Yoo JJ. Development of a composite vascular scaffolding system that withstands physiological vascular conditions. Biomaterials 2008;29:2891-8.
19. Pariente JL, Kim BS, Atala A. In vitro biocompatibility assessment of naturally derived and synthetic biomaterials using normal human urothelial cells. J Biomed Mater Res 2001;55:33-9.
20. Pariente JL, Kim BS, Atala A. In vitro biocompatibility evaluation of naturally derived and synthetic biomaterials using normal human bladder smooth muscle cells. J Urol 2002;167:1867-71.

21. Amiel GE, Atala A. Current and future modalities for functional renal replacement. Urol Clin North Am 1999;26:235-46.
22. Amiel GE, Komura M, Shapira O, Yoo JJ, Yazdani S, Berry J, et al. Engineering of blood vessels from acellular collagen matrices coated with human endothelial cells. Tissue Eng 2006;12:2355-65.
23. Yoo JJ, Park HJ, Lee I, Atala A. Autologous engineered cartilage rods for penile reconstruction. J Urol 1999;162:1119-21.
24. Atala A. Autologous cell transplantation for urologic reconstruction. J Urol 1998;159:2-3.
25. Atala A. Bladder regeneration by tissue engineering. BJU Int 2001;88:765-70.
26. Atala A. Creation of bladder tissue in vitro and in vivo. A system for organ replacement. Adv Exp Med Biol 1999;462:31-42.
27. Cilento BG, Freeman MR, Schneck FX, Retik AB, Atala A. Phenotypic and cytogenetic characterization of human bladder urothelia expanded in vitro. J Urol 1994;152:665-70.
28. Oberpenning F, Meng J, Yoo JJ, Atala A. De novo reconstitution of a functional mammalian urinary bladder by tissue engineering. Nat Biotechnol 1999;17:149-55.
29. Scriven SD, Booth C, Thomas DF, Trejdosiewicz LK, Southgate J. Reconstitution of human urothelium from monolayer cultures. J Urol 1997;158:1147-52.
30. Liebert M, Hubbel A, Chung M, Wedemeyer G, Lomax MI, Hegeman A, et al. Expression of mal is associated with urothelial differentiation in vitro: identification by differential display reverse-transcriptase polymerase chain reaction. Differentiation 1997;61:177-85.
31. Liebert M, Wedemeyer G, Abruzzo LV, Kunkel SL, Hammerberg C, Cooper KD, et al. Stimulated urothelial cells produce cytokines and express an activated cell surface antigenic phenotype. Semin Urol 1991;9:124-30.
32. Puthenveettil JA, Burger MS, Reznikoff CA. Replicative senescence in human uroepithelial cells. Adv Exp Med Biol 1999;462: 83-91.
33. Freeman MR, Yoo JJ, Raab G, Soker S, Adam RM, Schneck FX, et al. Heparin-binding EGF-like growth factor is an autocrine growth factor for human urothelial cells and is synthesized by epithelial and smooth muscle cells in the human bladder. J Clin Invest 1997;99:1028-36.
34. Nguyen HT, Park JM, Peters CA, Adam RM, Orsola A, Atala A, et al. Cell-specific activation of the HB-EGF and ErbB1 genes by stretch in primary human bladder cells. In Vitro Cell Dev Biol Anim 1999;35:371-5.
35. Harriss DR. Smooth muscle cell culture: a new approach to the study of human detrusor physiology and pathophysiology. Br J Urol 1995;75 Suppl 1:18-26.
36. Brivanlou AH, Gage FH, Jaenisch R, Jessell T, Melton D, Rossant J. Stem cells. Setting standards for human embryonic stem cells. Science 2003;300:913-6.
37. Richards M, Fong CY, Chan WK, Wong PC, Bongso A. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol 2002;20: 933-6.
38. Amit M, Shariki C, Margulets V, Itskovitz-Eldor J. Feeder layer- and serum-free culture of human embryonic stem cells. Biol Reprod 2004;70:837-45.
39. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 2000;18:399-404.
40. Reubinoff BE, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, et al. Neural progenitors from human embryonic stem cells. Nat Biotechnol 2001;19:1134-40.
41. Schuldiner M, Eiges R, Eden A, Yanuka O, Itskovitz-Eldor J, Goldstein RS, et al. Induced neuronal differentiation of human embryonic stem cells. Brain Res 2001;913:201-5.
42. Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2000;97:11307-12.
43. Zhang SC, Wernig M, Duncan ID, Brüstle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 2001;19:1129-33.
44. Kaufman DS, Hanson ET, Lewis RL, Auerbach R, Thomson JA. Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2001;98:10716-21.
45. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 2001;108:407-14.
46. Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R. Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2002;99:4391-6.
47. Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M. Insulin production by human embryonic stem cells. Diabetes 2001;50:1691-7.
48. Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, et al. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 2000;6:88-95.
49. Gurdon JB, Elsdale TR, Fischberg M. Sexually mature individuals of Xenopus laevis from the transplantation of single somatic nuclei. Nature 1958;182:64-5.
50. Campbell KH, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. Nature 1996;380:64-6.
51. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature 1997;385:810-3.
52. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, et al. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 1998;280:1256-8.
53. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, et al. Production of goats by somatic cell nuclear transfer. Nat Biotechnol 1999;17:456-61.
54. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998;394:369-74.
55. Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, et al. Production of cloned pigs from in vitro systems. Nat Biotechnol 2000;18:1055-9.
56. De Sousa PA, Dobrinsky JR, Zhu J, Archibald AL, Ainslie A, Bosma W, et al. Somatic cell nuclear transfer in the pig: control of pronuclear formation and integration with improved methods for activation and maintenance of pregnancy. Biol Reprod 2002; 66:642-50.

57. Colman A, Kind A. Therapeutic cloning: concepts and practicalities. Trends Biotechnol 2000;18:192-6.
58. Vogelstein B, Alberts B, Shine K. Genetics. Please don't call it cloning! Science 2002;295:1237.
59. Hochedlinger K, Rideout WM, Kyba M, Daley GQ, Blelloch R, Jaenisch R. Nuclear transplantation, embryonic stem cells and the potential for cell therapy. Hematol J 2004;5 Suppl 3:S114-7.
60. Lanza RP, Cibelli JB, West MD. Prospects for the use of nuclear transfer in human transplantation. Nat Biotechnol 1999;17: 1171-4.
61. Lanza RP, Cibelli JB, West MD, Dorff E, Tauer C, Green RM. The ethical reasons for stem cell research. Science 2001;292:1299.
62. Rideout WM 3rd, Eggan K, Jaenisch R. Nuclear cloning and epigenetic reprogramming of the genome. Science 2001;293:1093-8.
63. Solter D. Mammalian cloning: advances and limitations. Nat Rev Genet 2000;1:199-207.
64. Hochedlinger K, Jaenisch R. Nuclear transplantation: lessons from frogs and mice. Curr Opin Cell Biol 2002;14:741-8.
65. Dinnyés A, De Sousa P, King T, Wilmut I. Somatic cell nuclear transfer: recent progress and challenges. Cloning Stem Cells 2002;4:81-90.
66. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663-76.
67. Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, et al. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 2007;448:318-24.
68. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861-72.
69. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318:1917-20.
70. De Coppi P, Bartsch G Jr, Siddiqui MM, Xu T, Santos CC, Perin L, et al. Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol 2007;25:100-6.
71. Ballas CB, Zielske SP, Gerson SL. Adult bone marrow stem cells for cell and gene therapies: implications for greater use. J Cell Biochem Suppl 2002;38:20-8.
72. McCulloch EA, Till JE. Proliferation of hemopoietic colony-forming cells transplanted into irradiated mice. Radiat Res 1964;22: 383-97.
73. Jiao J, Chen DF. Induction of neurogenesis in nonconventional neurogenic regions of the adult central nervous system by niche astrocyte-produced signals. Stem Cells 2008;26:1221-30.
74. Taupin P. Therapeutic potential of adult neural stem cells. Recent Pat CNS Drug Discov 2006;1:299-303.
75. Jensen UB, Yan X, Triel C, Woo SH, Christensen R, Owens DM. A distinct population of clonogenic and multipotent murine follicular keratinocytes residing in the upper isthmus. J Cell Sci 2008;121:609-17.
76. Crisan M, Casteilla L, Lehr L, Carmona M, Paoloni-Giacobino A, Yap S, et al. A reservoir of brown adipocyte progenitors in human skeletal muscle. Stem Cells 2008;26:2425-33.
77. Weiner LP. Definitions and criteria for stem cells. Methods Mol Biol 2008;438:3-8.
78. Devine SM. Mesenchymal stem cells: will they have a role in the clinic? J Cell Biochem Suppl 2002;38:73-9.
79. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002;418:41-9.
80. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol 2007;213:341-7.
81. da Silva Meirelles L, Caplan AI, Nardi NB. In search of the in vivo identity of mesenchymal stem cells. Stem Cells 2008;26:2287-99.
82. Duan X, Chang JH, Ge S, Faulkner RL, Kim JY, Kitabatake Y, et al. Disrupted-In-Schizophrenia 1 regulates integration of newly generated neurons in the adult brain. Cell 2007;130:1146-58.
83. Luttun A, Ross JJ, Verfaillie C, Aranguren X, Prosper F. Unit 22F.9: Differentiation of multipotent adult progenitor cells into functional endothelial and smooth muscle cells. In: Current Protocols in Immunology. Hoboken, NJ: John Wiley and Sons, Inc.; 2006.
84. Mimeault M, Batra SK. Recent progress on tissue-resident adult stem cell biology and their therapeutic implications. Stem Cell Rev 2008;4:27-49.
85. Ikeda E, Yagi K, Kojima M, Yagyuu T, Ohshima A, Sobajima S, et al. Multipotent cells from the human third molar: feasibility of cell-based therapy for liver disease. Differentiation 2008;76: 495-505.
86. Nolen-Walston RD, Kim CF, Mazan MR, Ingenito EP, Gruntman AM, Tsai L, et al. Cellular kinetics and modeling of bronchioalveolar stem cell response during lung regeneration. Am J Physiol Lung Cell Mol Physiol 2008;294:L1158-65.
87. in 't Anker PS, Noort WA, Scherjon SA, Kleijburg-van der Keur C, Kruisselbrink AB, van Bezooijen RL, et al. Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. Haematologica 2003;88: 845-52.
88. Wilson A, Butler PE, Seifalian AM. Adipose-derived stem cells for clinical applications: a review. Cell Prolif 2011;44:86-98.
89. Mizuno H. Adipose-derived stem and stromal cells for cell-based therapy: current status of preclinical studies and clinical trials. Curr Opin Mol Ther 2010;12:442-9.
90. Hristov M, Zernecke A, Schober A, Weber C. Adult progenitor cells in vascular remodeling during atherosclerosis. Biol Chem 2008; 389:837-44.
91. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 2008;455:627-32.
92. Torrente Y, Tremblay JP, Pisati F, Belicchi M, Rossi B, Sironi M, et al. Intraarterial injection of muscle-derived CD34(+)Sca-1(+) stem cells restores dystrophin in mdx mice. J Cell Biol 2001;152: 335-48.
93. Atala A, Cima LG, Kim W, Paige KT, Vacanti JP, Retik AB, et al. Injectable alginate seeded with chondrocytes as a potential treatment for vesicoureteral reflux. J Urol 1993;150:745-7.
94. Atala A, Kim W, Paige KT, Vacanti CA, Retik AB. Endoscopic treatment of vesicoureteral reflux with a chondrocyte-alginate suspension. J Urol 1994;152:641-3.
95. Diamond DA, Caldamone AA. Endoscopic correction of vesicoureteral reflux in children using autologous chondrocytes: preliminary results. J Urol 1999;162:1185-8.

96. Bent AE, Tutrone RT, McLennan MT, Lloyd LK, Kennelly MJ, Badlani G. Treatment of intrinsic sphincter deficiency using autologous ear chondrocytes as a bulking agent. Neurourol Urodyn 2001;20:157-65.
97. Chen F, Yoo JJ, Atala A. Experimental and clinical experience using tissue regeneration for urethral reconstruction. World J Urol 2000;18:67-70.
98. Kropp BP, Ludlow JK, Spicer D, Rippy MK, Badylak SF, Adams MC, et al. Rabbit urethral regeneration using small intestinal submucosa onlay grafts. Urology 1998;52:138-42.
99. Sievert KD, Bakircioglu ME, Nunes L, Tu R, Dahiya R, Tanagho EA. Homologous acellular matrix graft for urethral reconstruction in the rabbit: histological and functional evaluation. J Urol 2000;163:1958-65.
100. El-Kassaby AW, Retik AB, Yoo JJ, Atala A. Urethral stricture repair with an off-the-shelf collagen matrix. J Urol 2003;169: 170-3.
101. De Filippo RE, Yoo JJ, Atala A. Urethral replacement using cell seeded tubularized collagen matrices. J Urol 2002;168:1789-92.
102. Dorin RP, Pohl HG, De Filippo RE, Yoo JJ, Atala A. Tubularized urethral replacement with unseeded matrices: what is the maximum distance for normal tissue regeneration? World J Urol 2008;26:323-6.
103. Raya-Rivera A, Esquiliano DR, Yoo JJ, Lopez-Bayghen E, Soker S, Atala A. Tissue-engineered autologous urethras for patients who need reconstruction: an observational study. Lancet 2011; 377:1175-82.
104. Atala A, Freeman MR, Vacanti JP, Shepard J, Retik AB. Implantation in vivo and retrieval of artificial structures consisting of rabbit and human urothelium and human bladder muscle. J Urol 1993;150:608-12.
105. Jayo MJ, Jain D, Ludlow JW, Payne R, Wagner BJ, McLorie G, et al. Long-term durability, tissue regeneration and neo-organ growth during skeletal maturation with a neo-bladder augmentation construct. Regen Med 2008;3:671-82.
106. Jayo MJ, Jain D, Wagner BJ, Bertram TA. Early cellular and stromal responses in regeneration versus repair of a mammalian bladder using autologous cell and biodegradable scaffold technologies. J Urol 2008;180:392-7.
107. Atala A, Bauer SB, Soker S, Yoo JJ, Retik AB. Tissue-engineered autologous bladders for patients needing cystoplasty. Lancet 2006;367:1241-6.
108. Matsumura G, Miyagawa-Tomita S, Shin'oka T, Ikada Y, Kurosawa H. First evidence that bone marrow cells contribute to the construction of tissue-engineered vascular autografts in vivo. Circulation 2003;108:1729-34.
109. Watanabe M, Shin'oka T, Tohyama S, Hibino N, Konuma T, Matsumura G, et al. Tissue-engineered vascular autograft: inferior vena cava replacement in a dog model. Tissue Eng 2001;7: 429-39.
110. Shinoka T, Breuer CK, Tanel RE, Zund G, Miura T, Ma PX, et al. Tissue engineering heart valves: valve leaflet replacement study in a lamb model. Ann Thorac Surg 1995;60(6 Suppl):S513-6.
111. Shinoka T, Shum-Tim D, Ma PX, Tanel RE, Isogai N, Langer R, et al. Creation of viable pulmonary artery autografts through tissue engineering. J Thorac Cardiovasc Surg 1998;115:536-45.
112. Shinoka T, Shum-Tim D, Ma PX, Tanel RE, Langer R, Vacanti JP, et al. Tissue-engineered heart valve leaflets: does cell origin affect outcome? Circulation 1997;96(9 Suppl):II-102-7.
113. Shin'oka T, Imai Y, Ikada Y. Transplantation of a tissue-engineered pulmonary artery. N Engl J Med 2001;344:532-3.
114. L'Heureux N, McAllister TN, de la Fuente LM. Tissue-engineered blood vessel for adult arterial revascularization. N Engl J Med 2007;357:1451-3.
115. McAllister TN, Maruszewski M, Garrido SA, Wystrychowski W, Dusserre N, Marini A, et al. Effectiveness of haemodialysis access with an autologous tissue-engineered vascular graft: a multicentre cohort study. Lancet 2009;373:1440-6.
116. Fuchs JR, Terada S, Ochoa ER, Vacanti JP, Fauza DO. Fetal tissue engineering: in utero tracheal augmentation in an ovine model. J Pediatr Surg 2002;37:1000-6.
117. Macchiarini P, Jungebluth P, Go T, Asnaghi MA, Rees LE, Cogan TA, et al. Clinical transplantation of a tissue-engineered airway. Lancet 2008;372:2023-30.
118. Auchincloss H, Bonventre JV. Transplanting cloned cells into therapeutic promise. Nat Biotechnol 2002;20:665-6.
119. Lanza RP, Chung HY, Yoo JJ, Wettstein PJ, Blackwell C, Borson N, et al. Generation of histocompatible tissues using nuclear transplantation. Nat Biotechnol 2002;20:689-96.
120. Nakase Y, Nakamura T, Kin S, Nakashima S, Yoshikawa T, Kuriu Y, et al. Endocrine cell and nerve regeneration in autologous in situ tissue-engineered small intestine. J Surg Res 2007;137:61-8.
121. Auger FA, Berthod F, Moulin V, Pouliot R, Germain L. Tissue-engineered skin substitutes: from in vitro constructs to in vivo applications. Biotechnol Appl Biochem 2004;39:263-75.
122. Suuronen EJ, McLaughlin CR, Stys PK, Nakamura M, Munger R, Griffith M. Functional innervation in tissue engineered models for in vitro study and testing purposes. Toxicol Sci 2004;82: 525-33.
123. Burdick JA, Ward M, Liang E, Young MJ, Langer R. Stimulation of neurite outgrowth by neurotrophins delivered from degradable hydrogels. Biomaterials 2006;27:452-9.
124. Dennis RG, Dow DE. Excitability of skeletal muscle during development, denervation, and tissue culture. Tissue Eng 2007;13: 2395-404.
125. Popel AS. Theory of oxygen transport to tissue. Crit Rev Biomed Eng 1989;17:257-321.
126. Muschler GF, Nakamoto C, Griffith LG. Engineering principles of clinical cell-based tissue engineering. J Bone Joint Surg Am 2004;86:1541-58.
127. Risau W. Mechanisms of angiogenesis. Nature 1997;386:671-4.
128. Khong YM, Zhang J, Zhou S, Cheung C, Doberstein K, Samper V, et al. Novel intra-tissue perfusion system for culturing thick liver tissue. Tissue Eng 2007;13:2345-56.
129. Iyer RK, Radisic M, Cannizzaro C, Vunjak-Novakovic G. Synthetic oxygen carriers in cardiac tissue engineering. Artif Cells Blood Substit Immobil Biotechnol 2007;35:135-48.
130. Radisic M, Park H, Chen F, Salazar-Lazzaro JE, Wang Y, Dennis R, et al. Biomimetic approach to cardiac tissue engineering: oxygen carriers and channeled scaffolds. Tissue Eng 2006;12: 2077-91.
131. Harrison BS, Eberli D, Lee SJ, Atala A, Yoo JJ. Oxygen producing biomaterials for tissue regeneration. Biomaterials 2007;28:

4628-34.
132. Tsang VL, Bhatia SN. Fabrication of three-dimensional tissues. Adv Biochem Eng Biotechnol 2007;103:189-205.
133. Ott HC, Matthiesen TS, Goh SK, Black LD, Kren SM, Netoff TI, et al. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. Nat Med 2008;14:213-21.
134. Baptista PM, Siddiqui MM, Lozier G, Rodriguez SR, Atala A, Soker S. The use of whole organ decellularization for the generation of a vascularized liver organoid. Hepatology 2010.
135. Nahmias Y, Schwartz RE, Verfaillie CM, Odde DJ. Laser-guided direct writing for three-dimensional tissue engineering. Biotechnol Bioeng 2005;92:129-36.
136. Xu T, Jin J, Gregory C, Hickman JJ, Boland T. Inkjet printing of viable mammalian cells. Biomaterials 2005;26:93-9.
137. Xu T, Olson J, Zhao W, Atala A, Zhu JM, Yoo JJ. Characterization of cell constructs generated with inkjet printing technology using in vivo magnetic resonance imaging. J Manuf Sci Eng 2008;130: 021013.
138. Stankus JJ, Soletti L, Fujimoto K, Hong Y, Vorp DA, Wagner WR. Fabrication of cell microintegrated blood vessel constructs through electrohydrodynamic atomization. Biomaterials 2007; 28:2738-46.
139. Landman KA, Cai AQ. Cell proliferation and oxygen diffusion in a vascularising scaffold. Bull Math Biol 2007;69:2405-28.
140. Nomi M, Atala A, Coppi PD, Soker S. Principals of neovascularization for tissue engineering. Mol Aspects Med 2002;23: 463-83.
141. Sellaro TL, Ravindra AK, Stolz DB, Badylak SF. Maintenance of hepatic sinusoidal endothelial cell phenotype in vitro using organ-specific extracellular matrix scaffolds. Tissue Eng 2007; 13:2301-10.
142. Lutolf MP, Gilbert PM, Blau HM. Designing materials to direct stem-cell fate. Nature 2009;462:433-41.