J Am Soc Nephrol 15: 1113–1125, 2004

Tissue Engineering, Stem Cells, and Cloning: Opportunities for Regenerative Medicine

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Introduction to Tissue Engineering

As one of the major components of regenerative medicine, tissue engineering follows the principles of cell transplantation, materials science, and engineering toward the development of biologic substitutes that can restore and maintain normal function. Tissue engineering strategies generally fall into two categories: acellular matrices, where matrices are used alone and depend on the body's natural ability to regenerate for proper orientation and direction of new tissue growth, and matrices with cells. Acellular tissue matrices are usually prepared by removing cellular components from tissues via mechanical and chemical manipulation to produce collagen-rich matrices (1–4). These matrices tend to slowly degrade on implantation and are generally replaced by the ECM proteins that are secreted by the ingrowing cells.

When cells are used for tissue engineering, a small piece of donor tissue is dissociated into individual cells. These cells are either implanted directly into the host or are expanded in culture, attached to a support matrix, and then reimplanted into the host after expansion. The source of donor tissue can be heterologous (such as bovine), allogeneic (same species, different individual), or autologous. Ideally, both structural and functional tissue replacements will occur with minimal complications. The most 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 (4–21). The use of autologous cells avoids rejection, and thus the deleterious side effects of immunosuppressive medications can be avoided.

One of the limitations of applying cell-based regenerative medicine techniques toward 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 could lead to 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 last two decades that identified the undifferentiated cells, and kept them undifferentiated during their growth phase (12,22–24). By use of these methods of cell culture, it is now possible to expand a urothelial strain from a single specimen that initially covers a surface area of 1 cm^2 to one covering a surface area of 4202 m^2 (the equivalent area of one football field) within 8 wk (12). 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 human donor in sufficient quantities for reconstructive purposes (12,13,23–32). Major advances have been achieved within the last decade on the possible expansion of a variety of primary human cells, with specific techniques that make the use of autologous cells possible for clinical application.

For cell-based tissue engineering, the expanded cells are seeded onto a scaffold synthesized with the appropriate biomaterial. In tissue engineering, biomaterials replicate the biologic and mechanical function of the native ECM found in tissues in the body by serving as an artificial ECM. As a result, biomaterials provide a three-dimensional space for the cells to form into new tissues with appropriate structure and function, and also can allow for the delivery of cells and appropriate bioactive factors (*e.g.*, cell adhesion peptides, growth factors), to desired sites in the body (33). Because the majority of mammalian cell types are anchorage dependent and will die if no cell-adhesion substrate is available, biomaterials provide a cell-adhesion substrate that can deliver cells to specific sites in the body with high loading efficiency. Biomaterials can also provide mechanical support against *in vivo* forces such that the predefined three-dimensional structure is maintained during tissue development. Furthermore, bioactive signals, such as cell-adhesion peptides and growth factors, can be loaded along with cells to help regulate cellular function.

The ideal biomaterial should be biocompatible in that it is biodegradable and bioresorbable to support the replacement of normal tissue without inflammation. Incompatible materials are destined for an inflammatory or foreign-body response that eventually leads to rejection and/or necrosis. In addition, the degradation products, if produced, should be removed from the body via metabolic pathways at an adequate rate that keeps the concentration of these degradation products in the tissues at a

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1046-6673/1505-1113
Journal of the American Society of Nephrology

DOI: 10.1097/01.ASN.0000119683.59068.F0

tolerable level (34). Furthermore, the biomaterial should provide an environment in which appropriate regulation of cell behavior (*e.g.*, adhesion, proliferation, migration, and differentiation) can occur such that functional tissue can form. Cell behavior in the newly formed tissue has been shown to be regulated by multiple interactions of the cells with their microenvironment, including interactions with cell-adhesion ligands (35) and with soluble growth factors (36). In addition, biomaterials provide temporary mechanical support that allows the tissue to grow in three dimensions while the cells undergo spatial tissue reorganization. The properly chosen biomaterial should allow the engineered tissue to maintain sufficient mechanical integrity to support itself in early development, whereas in late development, the properly chosen biomaterial should have begun degradation such that it does not hinder further tissue growth (33).

Generally, three classes of biomaterials have been used for engineering tissues: naturally derived materials (*e.g.*, collagen and alginate), acellular tissue matrices (*e.g.*, bladder submucosa and small intestinal submucosa), and synthetic polymers (*e.g.*, polyglycolic acid (PGA), polylactic acid (PLA), and poly(lactic-co-glycolic acid) (PLGA). These classes of biomaterials have been tested in respect to their biocompatibility (37,38). Naturally derived materials and acellular tissue matrices have the potential advantage of biologic recognition. However, synthetic polymers can be produced reproducibly on a large scale with controlled properties of their strength, degradation rate, and microstructure.

Collagen is the most abundant and ubiquitous structural protein in the body, and may be readily purified from both animal and human tissues with an enzyme treatment and salt/acid extraction (39). Collagen implants degrade through a sequential attack by lysosomal enzymes. The *in vivo* resorption rate can be regulated by controlling the density of the implant and the extent of intermolecular cross-linking. The lower the density, the greater the interstitial space and generally the larger the pores for cell infiltration, leading to a higher rate of implant degradation. Collagen contains cell-adhesion domain sequences (*e.g.*, RGD) that exhibit specific cellular interactions. This may assist to retain the phenotype and activity of many types of cells, including fibroblasts (40) and chondrocytes (41).

Alginate, a polysaccharide isolated from seaweed, has been used as an injectable cell delivery vehicle (42) and a cell immobilization matrix (43) because of its gentle gelling properties in the presence of divalent ions such as calcium. Alginate is relatively biocompatible and approved by the US Food and Drug Administration (FDA) for human use as wound dressing material. Alginate is a family of copolymers of D-mannuronate and L-guluronate. The physical and mechanical properties of alginate gel are strongly correlated with the proportion and length of polyguluronate block in the alginate chains (42).

Acellular tissue matrices are collagen-rich matrices prepared by removing cellular components from tissues. The matrices are often prepared by mechanical and chemical manipulation of a segment of tissue (1–4). The matrices slowly degrade upon implantation, and are replaced and remodeled by ECM proteins synthesized and secreted by transplanted or ingrowing cells.

Polyesters of naturally occurring α-hydroxy acids, including PGA, PLA, and PLGA, are widely used in tissue engineering. These polymers have gained FDA approval for human use in a variety of applications, including sutures (44). The ester bonds in these polymers are hydrolytically labile, and these polymers degrade by nonenzymatic hydrolysis. The degradation products of PGA, PLA, and PLGA are nontoxic, natural metabolites and are eventually eliminated from the body in the form of carbon dioxide and water (44). The degradation rate of these polymers can be tailored from several weeks to several years by altering crystallinity, initial molecular weight, and the copolymer ratio of lactic to glycolic acid. Because these polymers are thermoplastics, they can be easily formed into a three-dimensional scaffold with a desired microstructure, gross shape, and dimension by various techniques, including molding, extrusion (45), solvent casting (46), phase separation techniques, and gas foaming techniques (47). Many applications in tissue engineering often require a scaffold with high porosity and ratio of surface area to volume. Other biodegradable synthetic polymers, including poly(anhydrides) and poly(orthoesters), can also be used to fabricate scaffolds for tissue engineering with controlled properties (48).

Stem Cells

Most current strategies for tissue engineering depend upon 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 can not be expanded from a particular organ, such as the pancreas. In these situations, pluripotent human embryonic stem cells are envisioned as a viable source of cells because they can serve as an alternative source of cells from which the desired tissue can be derived. Combining the techniques learned in tissue engineering over the past few decades with this potentially endless source of versatile cells could lead to novel sources of replacement organs.

Embryonic stem cells exhibit two remarkable properties: the ability to proliferate in an undifferentiated but pluripotent state (self-renew), and the ability to differentiate into many specialized cell types (49). They can be isolated by immunosurgery from the inner cell mass of the embryo during the blastocyst stage (5 d after fertilization), and are usually grown on feeder layers consisting of mouse embryonic fibroblasts or human feeder cells (50). More recent reports have shown that these cells can be grown without the use of a feeder layer (51), 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 using current published protocols (52,53).

Human embryonic stem cells have been shown to differentiate into cells from all three embryonic germ layers *in vitro*. Skin and neurons have been formed, indicating ectodermal differentiation (54–57). Blood, cardiac cells, cartilage, endo-

thelial cells, and muscle have been formed, indicating mesodermal differentiation (58–60). And pancreatic cells have been formed, indicating endodermal differentiation (61). 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* (62).

Therapeutic Cloning

Nuclear cloning, which has also been called nuclear transplantation and nuclear transfer, involves the introduction of a nucleus from a donor cell into an enucleated oocyte to generate an embryo with a genetic makeup identical to that of the donor.

Although there has been tremendous interest in the field of nuclear cloning since the birth of Dolly in 1997, the first successful nuclear transfer was reported over 50 yr ago by Briggs and King (63). Cloned frogs, which were the first vertebrates derived from nuclear transfer, were subsequently reported by Gurdon in 1962 (64), but the nuclei were derived from nonadult sources. In the past 6 yr, tremendous advances in nuclear cloning technology have been reported, indicating the relative immaturity of the field. Dolly was not the first cloned mammal to be produced from adult cells; in fact, live lambs were produced in 1996 using nuclear transfer and differentiated epithelial cells derived from embryonic discs (65). The significance of Dolly was that she was the first mammal to be derived from an adult somatic cell using nuclear transfer (66). Since then, animals from several species have been grown using nuclear transfer technology, including cattle (67), goats (68,69), mice (70), and pigs (71–74).

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 revolutionary technologies (75,76). 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 (77). Some useful applications would be in the treatment of diseases, such as end-stage kidney disease, neurodegenerative diseases, and diabetes, for which there is limited availability of immunocompatible tissue transplants.

Therefore, therapeutic cloning, which has also been called somatic cell nuclear transfer, provides an alternative source of transplantable cells that theoretically may be limitless. Figure 1 shows the strategy of combining therapeutic cloning with tissue engineering to develop tissues and organs. According to data from the Centers for Disease Control and Prevention, it has been estimated that approximately 3000 Americans die every day of diseases that could have been treated with embryonic stem cell–derived tissues (78). With current allogeneic tissue transplantation protocols, rejection is a frequent complication because of immunologic incompatibility, and immuno-

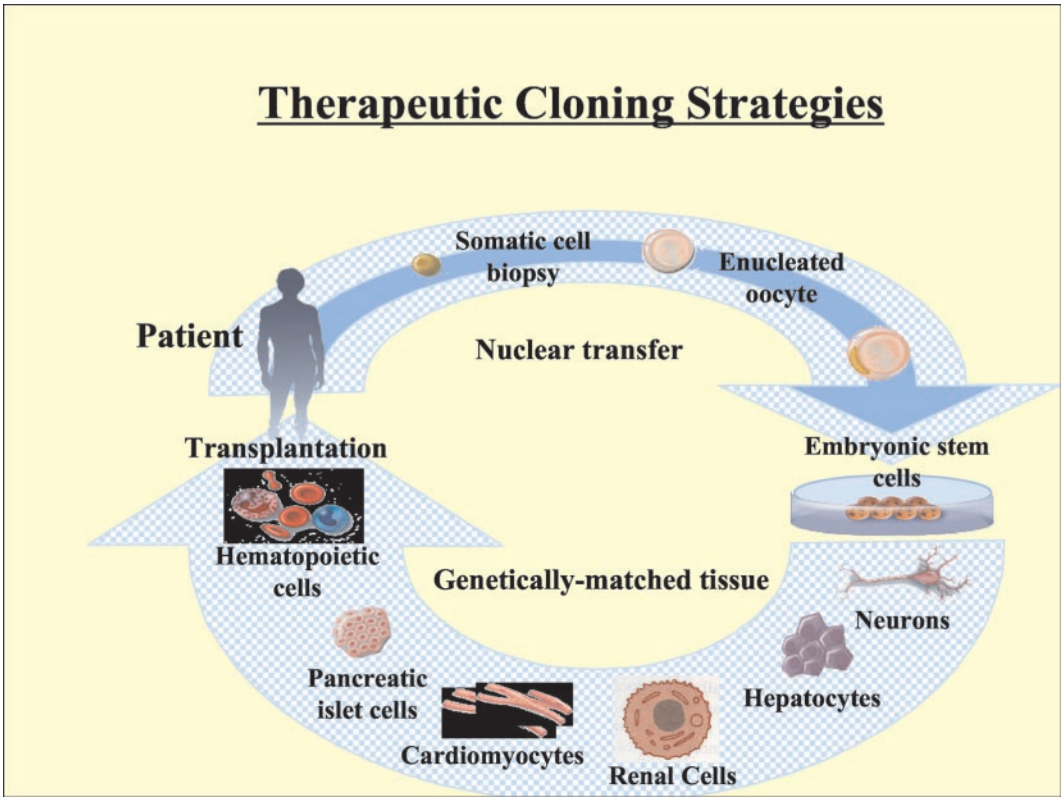

Figure 1. Strategy for therapeutic cloning and tissue engineering.

suppressive drugs are usually administered to treat and hopefully prevent graft-*versus*-host disease (77). The use of transplantable tissue and organs derived from therapeutic cloning may lead to the avoidance of immune responses that typically are associated with transplantation of nonautologous tissues. As a result, with therapeutic cloning, the variety of serious and potentially life-threatening complications associated with immunosuppressive treatments may be avoided (79).

Current Limitations of Cloning Technology

Although promising, somatic cell nuclear transfer technology has certain limitations that require further improvements before therapeutic cloning can be applied widely in replacement therapy.

Currently, the efficiency of the overall cloning process is low. The majority of embryos derived from animal cloning do not survive after implantation (80–82). In practical terms, multiple nuclear transfers must be performed to produce one live offspring for animal cloning applications. The potential for cloned embryos to grow into live offspring is between 0.5% to 18% for sheep, cattle, pigs, and mice (83). However, greater success (80%) has been reported in cattle (84), which may be in part due to the availability of advanced bovine supporting technologies, such as *in vitro* embryo production and embryo transfer, which have been developed for this species for agricultural purposes. To improve cloning efficiencies, further improvements are required in the multiple complex steps of nuclear transfer, such as enucleation and reconstruction, activation of oocytes, and cell cycle synchronization between donor cells and recipient oocytes (85), that will more readily produce viable sources of cells.

Furthermore, common abnormalities have been found in newborn clones if they survive to birth, including enlarged size with an enlarged placenta (large offspring syndrome) (86), respiratory distress and defects of the kidney, liver, heart, and brain (87), obesity (88), and premature death (89). These may be related to the epigenetics of the cloned cells, which involve the reversible modifications of the DNA or chromatin, while the original DNA (genetic) sequences remain intact. Faulty epigenetic reprogramming in clones, where the DNA methylation patterns, histone modifications, and the overall chromatin structure of the somatic nuclei are not being reprogrammed to an embryonic pattern of expression, may explain the above abnormalities (77). Reactivation of key embryonic genes at the blastocyst stage is usually not present in embryos cloned from somatic cells, but embryos cloned from embryos consistently express early embryonic genes (90,91). Proper epigenetic reprogramming to an embryonic state may help to improve the cloning efficiency and reduce the incidence of abnormal cloned cells.

Tissue Engineering of Specific Structures

Investigators around the world, including our laboratory, have been working toward the development of several cell types and tissues and organs for clinical application.

Urethra

Various biomaterials without cells, such as PGA and acellular collagen-based matrices from small intestine and bladder, have been used experimentally (in animal models) for the regeneration of urethral tissue (1,92–96). Some of these biomaterials, like acellular collagen matrices derived from bladder submucosa, have also been seeded with autologous cells for urethral reconstruction. Our laboratory has been able to replace tubularized urethral segments with cell-seeded collagen matrices.

Acellular collagen matrices derived from bladder submucosa by our laboratory 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. Histologic examination showed complete epithelialization and progressive vessel and muscle infiltration, and the animals were able to void through the neourethras (1). These results were confirmed in a clinical study of patients with hypospadias and urethral stricture disease (6,97). Decellularized cadaveric bladder submucosa was used as an onlay matrix for urethral repair in patients with stricture disease and hypospadias (Figure 2). Patent, functional neourethras were noted in these patients with up to a 7-yr 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 (98,99). Therefore, tubularized collagen matrices seeded with autologous cells can be used successfully for total penile urethra replacement.

Bladder

Currently, gastrointestinal segments are commonly used as tissues for bladder replacement or repair. However, gastrointestinal tissues are designed to absorb specific solutes, whereas bladder tissue is designed for the excretion of solutes. Because of the problems encountered with the use of gastrointestinal segments, numerous investigators have attempted alternative materials and tissues for bladder replacement or repair.

The success of the use of cell transplantation strategies for bladder reconstruction depends on the ability to use donor tissue efficiently and to provide the right conditions for long term survival, differentiation and growth. Urothelial and muscle cells can be expanded *in vitro*, seeded onto polymer scaffolds, and allowed to attach and form sheets of cells (7). These principles were applied toward the creation of tissue engineered bladders in an animal model that required a subtotal cystectomy with subsequent replacement with a tissue engineered organ in beagle dogs (18). Urothelial and muscle cells were separately expanded from an autologous bladder biopsy sample and seeded onto a bladder-shaped biodegradable poly-

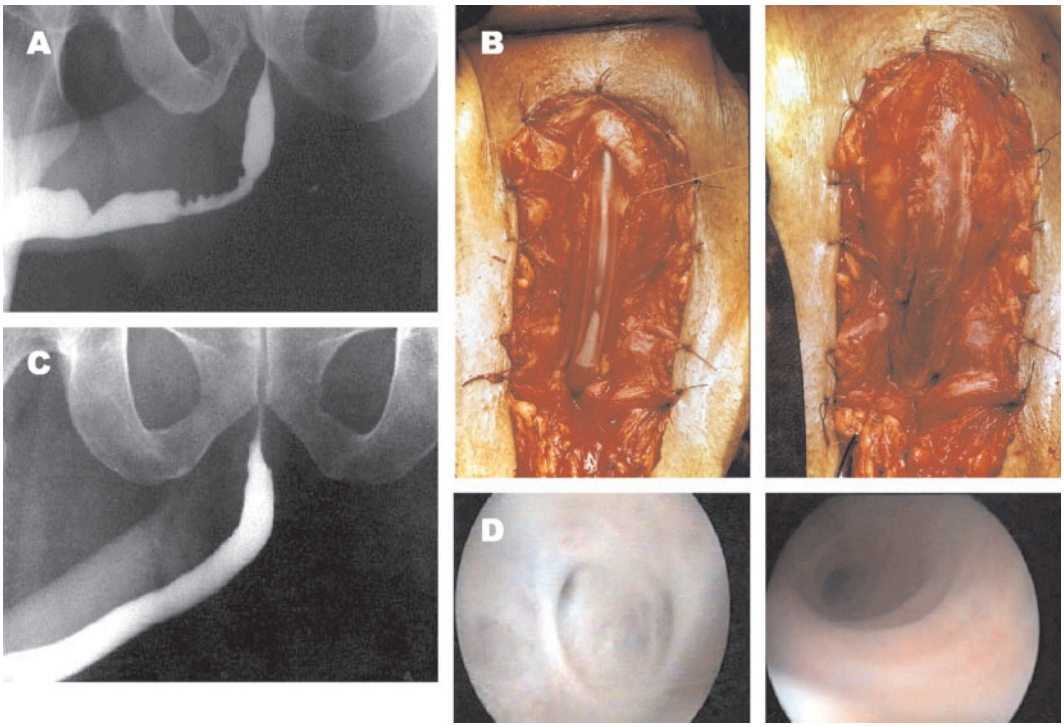

Figure 2. Tissue engineering of the urethra using a collagen matrix. (A) Representative case of a patient with a bulbar stricture. (B) Urethral repair. Strictured tissue is excised, preserving the urethral plate on the left side, and matrix is anastomosed to the urethral plate in an onlay fashion on the right. (C) Urethrogram 6 mo after repair. (D) Cystoscopic view of urethra before surgery on the left side and 4 mo after repair on the right side.

mer scaffold. The results from this study showed that it is possible to tissue-engineer bladders that are anatomically and functionally normal.

Male Genital Tissues

Reconstructive surgery is required for a wide variety of pathologic penile conditions, such as penile carcinoma, trauma, severe erectile dysfunction, and congenital conditions such as ambiguous genitalia, hypospadias, and epispadias. One of the major limitations of phallic reconstructive surgery is the scarcity of sufficient autologous tissue. Phallic reconstruction using autologous tissue, derived from the patient's own cells, may be preferable in selected cases.

The major components of the phallus are corporal smooth muscle and endothelial cells. The creation of autologous functional and structural corporal tissue *de novo* would be beneficial. Autologous cavernosal smooth muscle and endothelial cells were harvested, expanded and seeded on acellular collagen matrices and implanted in a rabbit model (100,101). Histologic examination confirmed the appropriate organization of penile tissue phenotypes, and structural and functional studies, including cavernosonography, cavernosometry, and mating studies, demonstrated that it is possible to engineer autologous functional penile tissue. Our laboratory is currently working on increasing the size of the engineered constructs.

Female Genital Tissues

Congenital malformations of the uterus may have profound implications clinically. Patients with cloacal exstrophy and intersex disorders may not have sufficient uterine tissue present for future reproduction. We investigated the possibility of engineering functional uterine tissue using autologous cells (102). Autologous rabbit uterine smooth muscle and epithelial cells were harvested, then grown and expanded in culture. These cells were seeded onto preconfigured uterine-shaped biodegradable polymer scaffolds, which were then used for subtotal uterine tissue replacement in the corresponding autologous animals. Upon retrieval 6 mo after implantation, histologic, immunocytochemical, and Western blot analyses confirmed the presence of normal uterine tissue components. Biomechanical analyses and organ bath studies showed that the functional characteristics of these tissues were similar to those of normal uterine tissue. Breeding studies that use these engineered uteri are currently being performed.

Similarly, several pathologic conditions, including congenital malformations and malignancy, can adversely affect normal vaginal development or anatomy. Vaginal reconstruction has traditionally been challenging because of the paucity of available native tissue. The feasibility of engineering vaginal tissue *in vivo* was investigated (103). Vaginal epithelial and smooth muscle cells of female rabbits were harvested, grown, and expanded in culture. These cells were seeded onto biodegradable polymer scaffolds, and the cell-seeded constructs were then implanted into nude mice for up to 6 wk. Immunocytochemical, histologic, and Western blot analyses confirmed the presence of vaginal tissue phenotypes. Electrical field stimulation studies in the tissue-engineered constructs showed similar functional properties to those of normal vaginal tissue. When these constructs were used for autologous total vaginal replacement, patent vaginal structures were noted in the tissue-engineered specimens, whereas the non–cell-seeded structures were noted to be stenotic (104).

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, cattle (*Bos taurus*) (105). 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 progastrin-synchronized recipients to allow for further *in vivo* growth. After 12 wk, cloned renal cells were harvested, expanded *in vitro*, and seeded onto biodegradable scaffolds. The constructs, which consisted of the cells and the scaffolds, were then implanted into the subcutaneous space of the same steer from which the cells were cloned to allow for tissue growth.

The kidney is a complex organ with multiple cell types and a complex functional anatomy that renders it one of the most difficult to reconstruct (5,106). Previous efforts in tissue engineering of the kidney have been directed toward the development of extracorporeal renal support systems made of biologic and synthetic components (107–113), and *ex vivo* renal replacement devices are known to be life-sustaining. However, there would be obvious benefits for patients with end-stage kidney disease if these devices could be implanted long term without the need for an extracorporeal perfusion circuit or immunosuppressive drugs.

Cloned renal cells were seeded on scaffolds consisting of three collagen-coated cylindrical polycarbonate membranes (Figure 3A). 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 (Figure 3B). These scaffolds with the collecting devices were transplanted subcutaneously into the same steer from which the genetic material originated and retrieved 12 wk after implantation.

Chemical analysis of the collected urinelike 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.

Histologic examination of the retrieved implants revealed extensive vascularization and self-organization of the cells into glomeruli- and tubulelike structures. A clear continuity between the glomeruli, the tubules, and the polycarbonate mem-

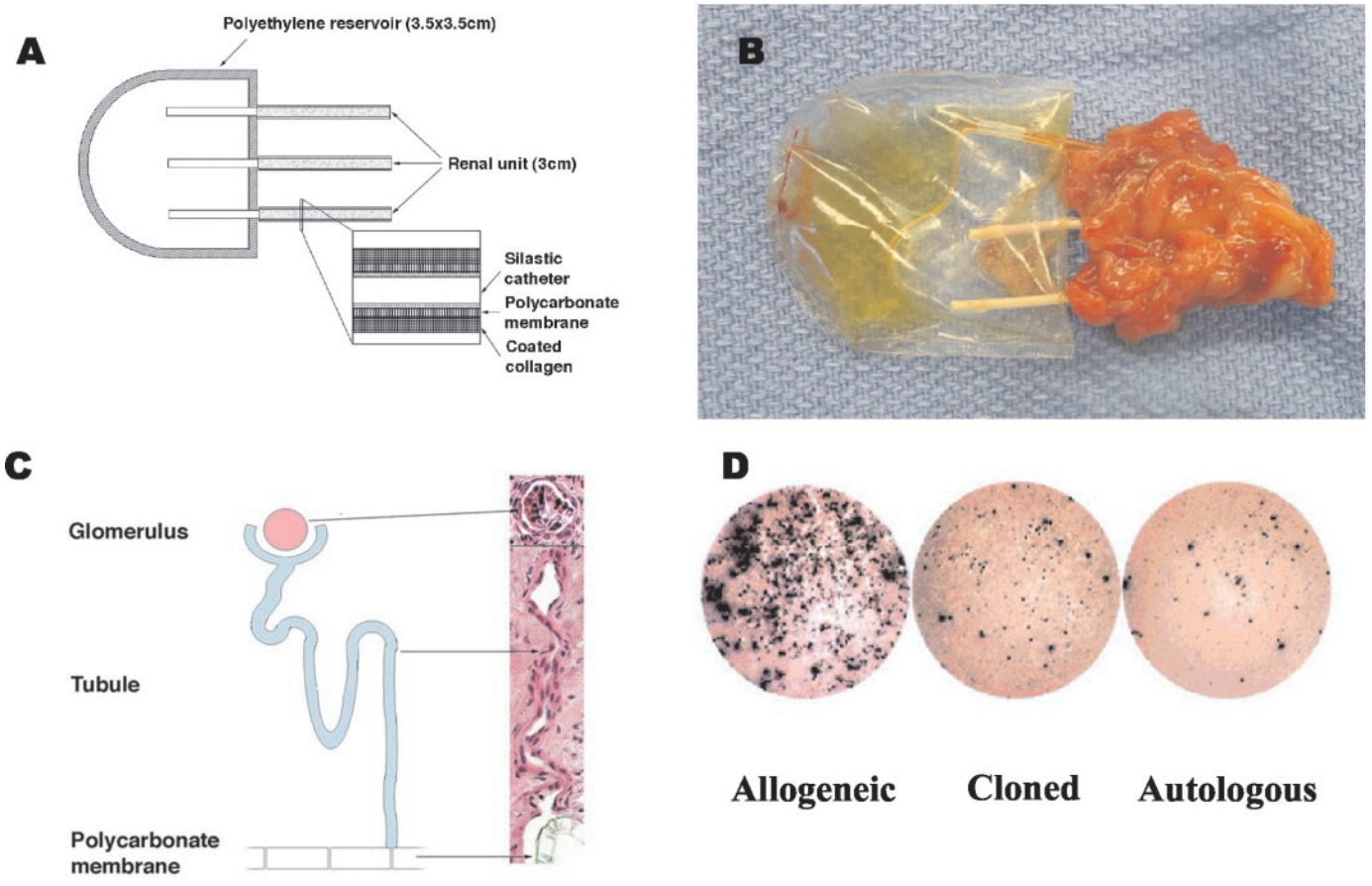

Figure 3. Combining therapeutic cloning and tissue engineering to produce kidney tissue. (A) Illustration of the tissue-engineered renal unit. (B) Renal unit seeded with cloned cells, 3 mo after implantation, showing the accumulation of urinelike fluid. (C) There was a clear unidirectional continuity between the mature glomeruli, their tubules, and the polycarbonate membrane. (D) ELISPOT analyses of the frequencies of T cells that secrete IFN-γ after primary and secondary stimulation with allogeneic renal cells, cloned renal cells, or nuclear donor fibroblasts.

brane was noted that allowed the passage of urine into the collecting reservoir (Figure 3C). Immunohistochemical analysis with renal-specific antibodies revealed the presence of renal proteins, RT-PCR analysis confirmed the transcription of renal specific RNA in the cloned specimens, and Western blot analysis confirmed the presence of elevated renal-specific protein levels.

Because previous studies have shown that bovine clones harbor the oocyte mtDNA (114–116), the donor egg's mtDNA was thought to be a potential source of immunologic incompatibility. Differences in mtDNA-encoded proteins expressed by cloned cells could stimulate a T cell response specific for mtDNA-encoded minor histocompatibility antigens when the cloned cells are implanted back into the original nuclear donor (117). We used nucleotide sequencing of the mtDNA genomes of the clone and fibroblast nuclear donor to identify potential antigens in the muscle constructs. Only two amino acid substitutions were noted to distinguish the clone and the nuclear donor, and as a result, a maximum of two minor histocompatibility antigens could be defined. Given the lack of knowledge regarding peptide-binding motifs for bovine MHC class I molecules, there is no reliable method to predict the effect of these amino acid substitutions on bovine histocompatibility.

Oocyte-derived mtDNA was also thought to be a potential source of immunologic incompatibility in the cloned renal cells. Maternally transmitted minor histocompatibility antigens in mice have been shown to stimulate both skin allograft rejection *in vivo* and cytotoxic T lymphocytes expansion *in vitro* (117), which could prevent the use of these cloned constructs in patients with chronic rejection of major histocompatibility matched human renal transplants (118,119). We tested for a possible T cell response to the cloned renal devices using delayed-type hypersensitivity testing *in vivo* and ELISPOT analysis of IFN-γ–secreting T cells *in vitro*. Both analyses revealed that the cloned renal cells showed no evidence of a T cell response, suggesting that rejection will not necessarily occur in the presence of oocyte-derived mitochondrial DNA (Figure 3D). This finding may represent a step forward in overcoming the histocompatibility problem of stem cell therapy (106).

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 that of the host. These studies were the first demonstration of the use of therapeutic cloning for regeneration of tissues *in vivo*.

Blood Vessels

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 (120).

Tissue-engineered vascular grafts have been constructed using autologous cells and biodegradable scaffolds and have been applied in dog and lamb models (121–125). The key advantage from the use these autografts is that they degrade *in vivo* and thus allow the new tissue to form without the long-term presence of foreign material (120).

Application of these techniques from the laboratory to the clinical setting have begun, where autologous vascular cells were harvested, expanded, and seeded onto a biodegradable scaffold (126). 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 were noted in the recipient.

Cartilage—Articular Cartilage and Trachea

Full-thickness articular cartilage lesions have limited healing capacity and thus represent a difficult management issue for the clinicians who treat adult patients with damaged articular cartilage (127,128). Large defects can be associated with mechanical instability and may lead to degenerative joint disease if left untreated (129,130). Chondrocytes were expanded and cultured onto biodegradable scaffolds to create engineered cartilage for use in large osteochondral defects in rabbits (131). When sutured to a subchondral support, the engineered cartilage was able to withstand physiologic loading and underwent orderly remodeling of the large osteochondral defects in adult rabbits. Thus, the engineered cartilage was able to provide a biomechanically functional template that was able to undergo orderly remodeling when subjected to quantitative structural and functional analyses.

Few treatment options are currently available for patients who have severe congenital tracheal pathology, such as stenosis, atresia, and agenesis, because of 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 congential 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*, then dynamically seeded onto biodegradable scaffolds (132). 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. Furthermore, if native tracheal tissue is unavailable, engineered cartilage may be derived from bone marrow–derived mesenchymal progenitor cells as well (133).

Cellular Therapies

Bulking Agents. Injectable bulking agents can be endoscopically used in the treatment of both urinary incontinence and vesicoureteral reflux. The advantages in treating urinary incontinence and vesicoureteral reflux with this minimally invasive approach include the simplicity of this quick outpatient procedure and the low morbidity associated with it. Several investigators are seeking alternative implant materials that would be safe for human use (16).

The ideal substance for the endoscopic treatment of reflux and incontinence should be injectable, nonantigenic, nonmi-

gratory, volume stable, and safe for human use. Toward this goal, long-term studies were conducted to determine the effect of injectable chondrocytes *in vivo* (134). It was initially determined that alginate, a liquid solution of gluronic and mannuronic acid, embedded with chondrocytes, could serve as a synthetic substrate for the injectable delivery and maintenance of cartilage architecture *in vivo*. Alginate undergoes hydrolytic biodegradation and its degradation time can be varied depending on the concentration of each of the polysaccharides. The use of autologous cartilage for the treatment of vesicoureteral reflux in humans would satisfy all of the requirements for an ideal injectable substance.

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 these experiments, the cartilage matrix replaced the alginate as the polysaccharide polymer underwent biodegradation. This system was adapted for the treatment of vesicoureteral reflux in a porcine model (9). These studies showed that chondrocytes can be easily harvested and combined with alginate *in vitro*, the suspension can be easily injected cystoscopically, and the elastic cartilage tissue formed is able to correct vesicoureteral reflux without any evidence of obstruction.

Two multicenter clinical trials were conducted by using the above engineered chondrocyte technology. Patients with vesicoureteral reflux 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 (Figure 4). Chondrocyte formation was not noted in patients who experienced treatment failure. The patients who were cured would supposedly have a biocompatible region of engineered autologous tissue present, rather than a foreign material (135). Patients with urinary incontinence were also treated endoscopically with injected chondrocytes at three different medical centers. Phase 1 trials showed an approximate success rate of 80% at both 3 and 12 mo postoperatively (136).

Injectable Muscle Cells. The potential use of injectable, cultured myoblasts for the treatment of stress urinary incontinence has been investigated (137,138). Labeled myoblasts were directly injected into the proximal urethra and lateral bladder walls of nude mice with a microsyringe in an open surgical procedure. Tissue harvested up to 35 d after injection contained the labeled myoblasts, as well as evidence of differentiation of the labeled myoblasts into regenerative myofibers. The authors reported that a significant portion of the injected myoblast population persisted *in vivo*. Similar techniques of sphincteric derived muscle cells have been used for the treatment of urinary incontinence in a pig model (139). The fact that myoblasts can be labeled and survive after injection and begin the process of myogenic differentiation further supports the feasibility of the use of cultured cells of muscular origin as an injectable bioimplant.

The use of injectable muscle precursor cells has also been investigated for use in the treatment of urinary incontinence due to irreversible urethral sphincter injury or maldevelopment. Muscle precursor cells are the quiescent satellite cells found in each myofiber that proliferate to form myoblasts and eventually myotubes and new muscle tissue. Intrinsic muscle precursor cells have previously been shown to play an active role in the regeneration of injured striated urethral sphincter (140). In a subsequent study, autologous muscle precursor cells were injected into a rat model of urethral sphincter injury, and both replacement of mature myotubes as well as restoration of functional motor units were noted in the regenerating sphincteric muscle tissue (141). This is the first demonstration of the replacement of both sphincter muscle tissue and its innervation by the injection of muscle precursor cells. As a result, muscle

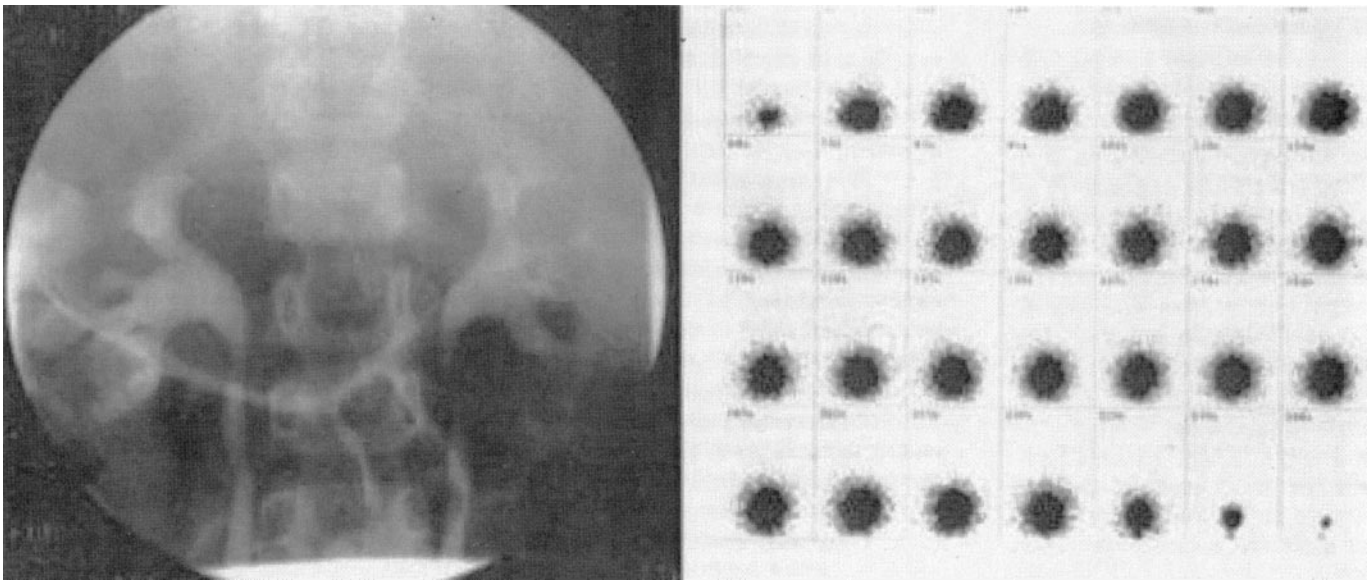

Figure 4. Autologous chondrocytes for the treatment of vesicoureteral reflux. (Left) Preoperative voiding cystourethrogram of a patient with bilateral reflux. (Right) Postoperative radionuclide cystogram of the same patient 6 mo after injection of autologous chondrocytes.

precursor cells may be a minimally invasive solution for urinary incontinence in patients with irreversible urinary sphincter muscle insufficiency.

Endocrine Replacement:. Patients with testicular dysfunction and hypogonadal disorders are dependent on androgen replacement therapy to restore and maintain physiologic levels of serum testosterone and its metabolites, dihydrotestosterone and estradiol (142). Currently available androgen replacement modalities, such as testosterone tablets and capsules, depot injections, and skin patches, may be associated with fluctuating serum levels and complications such as fluid and nitrogen retention, erythropoiesis, hypertension, and bone density changes (143). Because Leydig cells of the testes are the major source of testosterone in men, implantation of heterologous Leydig cells or gonadal tissue fragments has previously been proposed as a method for chronic testosterone replacement (144,145). But these approaches were limited by the failure of the tissues and cells to produce testosterone.

Encapsulation of cells in biocompatible and semipermeable polymeric membranes has been an effective method to protect against a host immune response as well as to maintain viability of the cells while allowing the secretion of desired therapeutic agents (146,147). Alginate-poly-L-lysine–encapsulated Leydig cell microspheres were used as a novel method for testosterone delivery *in vivo* (142). Elevated stable serum testosterone levels were noted in castrated adult rats over the course of the study, suggesting that microencapsulated Leydig cells may be a potential therapeutic modality for testosterone supplementation.

Angiogenic Agents. The engineering of large organs will require a vascular network of arteries, veins, and capillaries to deliver nutrients to each cell. One possible method of vascularization is through the use of gene delivery of angiogenic agents such as vascular endothelial growth factor (VEGF) with the implantation of vascular endothelial cells to enhance neovascularization of engineered tissues. Skeletal myoblasts from adult mice were cultured and transfected with an adenovirus encoding VEGF and combined with human vascular endothelial cells (148). The mixtures of cells were injected subcutaneously in nude mice, and the engineered tissues were retrieved up to 8 wk after implantation. The transfected cells were noted to form muscle with neovascularization by histology and immunohistochemical probing with maintenance of their muscle volume, whereas engineered muscle of nontransfected cells had a significantly smaller mass of cells with loss of muscle volume over time, less neovascularization, and no surviving endothelial cells. These results indicate that a combination of VEGF and endothelial cells may be useful for inducing neovascularization and volume preservation in engineered tissue.

Antiangiogenic Agents. The delivery of antiangiogenic agents may help to slow tumor growth for a variety of neoplasms. Encapsulated hamster kidney cells transfected with the angiogenesis inhibitor endostatin were used for local delivery on human glioma cell line xenografts (111). The release of biologically active endostatin led to significant inhibition of endothelial cell proliferation and substantial reduction in tumor weight. Continuous local delivery of endostatin via encapsulated endostatin-secreting cells may be effective therapeutic option for a variety of tumor types.

Conclusion:

Tissue engineering efforts are currently underway for virtually every type of tissue and organ within the human body. Because tissue engineering incorporates the fields of cell transplantation, materials science, and engineering, personnel who have mastered the techniques of cell harvest, culture, expansion, transplantation, and polymer design are essential for the successful application of this technology. Various engineered 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 because they represent a viable therapeutic option for those who require tissue replacement. More recently, major advances in the areas of stem cell biology, tissue engineering, and nuclear transfer techniques have made it possible to combine these technologies to create the comprehensive scientific field of regenerative medicine.

References

1. Chen F, Yoo JJ, Atala A: Acellular collagen matrix as a possible "off the shelf" biomaterial for urethral repair. *Urology* 54: 407–410, 1999
2. 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* 82: 411–419, 1998
3. 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* 159: 1717–1724, 1998
4. Yoo JJ, Meng J, Oberpenning F, Atala A: Bladder augmentation using allogenic bladder submucosa seeded with cells. *Urology* 51: 221–225, 1998
5. Amiel GE, Atala A: Current and future modalities for functional renal replacement. *Urol Clin North Am* 26: 235–246, 1999
6. Yoo JJ, Park HJ, Lee I, Atala A: Autologous engineered cartilage rods for penile reconstruction. *J Urol* 162: 1119–1121, 1999
7. 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* 150: 608–612, 1993
8. Atala A, Guzman L, Retik AB: A novel inert collagen matrix for hypospadias repair. *J Urol* 162: 1148–1151, 1999
9. Atala A, Kim W, Paige KT, Vacanti CA, Retik AB: Endoscopic treatment of vesicoureteral reflux with a chondrocyte-alginate suspension. *J Urol* 152: 641–644, 1994
10. Atala A, Lanza RP: Preface. In: *Methods of Tissue Engineering*, edited by Atala A, Lanza RP, San Diego, Academic Press, 2001, pp xli
11. Atala A, Mooney D: Preface. In: *Tissue Engineering*, edited by Atala A, Boston, Birkhauser Press, 1997, pp xi
12. Cilento BG, Freeman MR, Schneck FX, Retik AB, Atala A: Phenotypic and cytogenetic characterization of human bladder urothelia expanded in vitro. *J Urol* 152: 665–670, 1994

13. Fauza DO, Fishman SJ, Mehegan K, Atala A: Videofetoscopically assisted fetal tissue engineering: Bladder augmentation. *J Pediatr Surg* 33: 7–12, 1998
14. Fauza DO, Fishman SJ, Mehegan K, Atala A: Videofetoscopically assisted fetal tissue engineering: Skin replacement. *J Pediatr Surg* 33: 357–361, 1998
15. Godbey WT, Atala A: In vitro systems for tissue engineering. *Ann N Y Acad Sci* 961: 10–26, 2002
16. Kershen RT, Atala A: New advances in injectable therapies for the treatment of incontinence and vesicoureteral reflux. *Urol Clin North Am* 26: 81–94, 1999
17. Machluf M, Atala A: Emerging concepts for tissue and organ transplantation. *Graft* 1: 31, 1998
18. Oberpenning F, Meng J, Yoo JJ, Atala A: De novo reconstitution of a functional mammalian urinary bladder by tissue engineering. *Nat Biotechnol* 17: 149–155, 1999
19. Park HJ, Yoo JJ, Kershen RT, Moreland R, Atala A: Reconstitution of human corporal smooth muscle and endothelial cells in vivo. *J Urol* 162: 1106–1109, 1999
20. Yoo JJ, Atala A: A novel gene delivery system using urothelial tissue engineered neo-organs. *J Urol* 158: 1066–1070, 1997
21. Yoo JJ, Lee I, Atala A: Cartilage rods as a potential material for penile reconstruction. *J Urol* 160: 1164–1168, 1998
22. Scriven SD, Booth C, Thomas DF, Trejdosiewicz LK, Southgate J: Reconstitution of human urothelium from monolayer cultures. *J Urol* 158: 1147–1152, 1997
23. Liebert M, Hubbel A, Chung M, Wedemeyer G, Lomax MI, Hegeman A, Yuan TY, Brozovich M, Wheelock MJ, Grossman HB: Expression of mal is associated with urothelial differentiation in vitro: Identification by differential display reverse-transcriptase polymerase chain reaction. *Differentiation* 61: 177–185, 1997
24. Puthenveettil JA, Burger MS, Reznikoff CA: Replicative senescence in human uroepithelial cells. *Adv Exp Med Biol* 462: 83–91, 1999
25. Freeman MR, Yoo JJ, Raab G, Soker S, Adam RM, Schneck FX, Renshaw AA, Klagsbrun M, Atala A: 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* 99: 1028–1036, 1997
26. Nguyen HT, Park JM, Peters CA, Adam RM, Orsola A, Atala A, Freeman MR: Cell-specific activation of the HB-EGF and ErbB1 genes by stretch in primary human bladder cells. *In Vitro Cell Dev Biol Anim* 35: 371–375, 1999
27. Liebert M, Wedemeyer G, Abruzzo LV, Kunkel SL, Hammerberg C, Cooper KD, Grossman HB: Stimulated urothelial cells produce cytokines and express an activated cell surface antigenic phenotype. *Semin Urol* 9: 124–130, 1991
28. Tobin MS, Freeman MR, Atala A: Maturational response of normal human urothelial cells in culture is dependent on extracellular matrix and serum additives. *Surg Forum* 45: 786, 1994
29. Harriss DR: Smooth muscle cell culture: A new approach to the study of human detrusor physiology and pathophysiology. *Br J Urol* 75[Suppl 1]: 18–26, 1995
30. Solomon LZ, Jennings AM, Sharpe P, Cooper AJ, Malone PS: Effects of short-chain fatty acids on primary urothelial cells in culture: Implications for intravesical use in enterocystoplasties. *J Lab Clin Med* 132: 279–83, 1998
31. Lobban ED, Smith BA, Hall GD, Harnden P, Roberts P, Selby PJ, Trejdosiewicz LK, Southgate J: Uroplakin gene expression by normal and neoplastic human urothelium. *Am J Pathol* 153: 1957–1967, 1998
32. Rackley RR, Bandyopadhyay SK, Fazeli-Matin S, Shin MS, Appell R: Immunoregulatory potential of urothelium: Characterization of NF-kappaB signal transduction. *J Urol* 162: 1812–1816, 1999
33. Kim BS, Mooney DJ: Development of biocompatible synthetic extracellular matrices for tissue engineering. *Trends Biotechnol* 16: 224–230, 1998
34. 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* 16: 267–274, 1995
35. Hynes RO: Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* 69: 11–25, 1992
36. Deuel TF: Growth factors. In: *Principles of Tissue Engineering*, edited by Lanza RP, Langer R, Chick WL, New York, Academic Press, 1997, pp 133–149
37. 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* 55: 33–39, 2001
38. 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* 167: 1867–1871, 2002
39. Li ST: Biologic biomaterials: Tissue-derived biomaterials (collagen). In: *The Biomedical Engineering Handbook*, edited by Brozino JD, Boca Raton, FL, CRC Press, 1995, pp 627–647
40. Silver FH, Pins G: Cell growth on collagen: A review of tissue engineering using scaffolds containing extracellular matrix. *J Long Term Eff Med Implants* 2: 67–80, 1992
41. Sams AE, Nixon AJ: Chondrocyte-laden collagen scaffolds for resurfacing extensive articular cartilage defects. *Osteoarthritis Cartilage* 3: 47–59, 1995
42. Smidsrod O, Skjak-Braek G: Alginate as immobilization matrix for cells. *Trends Biotechnol* 8: 71–78, 1990
43. Lim F, Sun AM: Microencapsulated islets as bioartificial endocrine pancreas. *Science* 210: 908–910, 1980
44. Gilding DK: Biodegradable polymers. In: *Biocompatibility of Clinical Implant Materials*, edited by Williams DF, Boca Raton, FL, CRC Press, 1981, pp 209–232
45. Freed LE, Vunjak-Novakovic G, Biron RJ, Eagles DB, Lesnoy DC, Barlow SK, Langer R: Biodegradable polymer scaffolds for tissue engineering. *Biotechnology* 12: 689–693, 1994
46. Mikos AG, Thorsen AJ, Czerwonka LA, *et al.*: Preparation and characterization of poly(L-lactic acid) foams. *Polymer* 35: 1068–1077, 1994
47. Harris LD, Kim BS, Mooney DJ: Open pore biodegradable matrices formed with gas foaming. *J Biomed Mater Res* 42: 396–402, 1998
48. Peppas NA, Langer R: New challenges in biomaterials. *Science* 263: 1715–1720, 1994
49. Brivanlou AH, Gage FH, Jaenisch R, Jessell T, Melton D, Rossant J: Stem cells: Setting standards for human embryonic stem cells. *Science* 300: 913–916, 2003
50. 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* 20: 933–936, 2002
51. Amit M, Margulets V, Segev H, Shariki K, Laevsky I, Coleman R, Itskovitz-Eldor J: Human feeder layers for human embryonic stem cells. *Biol Reprod* 68: 2150–2156, 2003
52. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A: Embryonic stem cell lines from human blastocysts: Somatic

differentiation in vitro. *Nat Biotechnol* 18: 399–404, 2000 [Erratum in: *Nat Biotechnol* 18: 559, 2000]
53. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM: Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145–1147, 1998 [Erratum in: *Science* 282: 1827, 1998]
54. Reubinoff BE, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, Ben-Hur T: Neural progenitors from human embryonic stem cells. *Nat Biotechnol* 19: 1134–1140, 2001
55. Schuldiner M, Eiges R, Eden A, Yanuka O, Itskovitz-Eldor J, Goldstein RS, Benvenisty N: Induced neuronal differentiation of human embryonic stem cells. *Brain Res* 913: 201–205, 2001
56. 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* 97: 11307–11312, 2000
57. Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA: In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol* 19: 1129–1133, 2001
58. 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* 98: 10716–10721, 2001
59. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L: Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* 108: 407–414, 2001
60. 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* 99: 4391–4396, 2002
61. Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M: Insulin production by human embryonic stem cells. *Diabetes* 50: 1691–1697, 2001
62. Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N: Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med* 6: 88–95, 2000
63. Briggs R, King TJ: Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. *Proc Natl Acad Sci U S A* 38: 455–463, 1952
64. Gurdon JB: Adult frogs derived from the nuclei of single somatic cells. *Dev Biol* 4: 256–273, 1962
65. Campbell KH, McWhir J, Ritchie WA, Wilmut I: Sheep cloned by nuclear transfer from a cultured cell line. *Nature* 380: 64–66, 1996
66. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH: Viable offspring derived from fetal and adult mammalian cells. *Nature* 385: 810–813, 1997 [Erratum in: *Nature* 386: 200, 1997]
67. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM: Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* 280: 1256–1258, 1998
68. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW, Echelard Y: Production of goats by somatic cell nuclear transfer. *Nat Biotechnol* 17: 456–461, 1999
69. Keefer CL, Keyston R, Lazaris A, Bhatia B, Begin I, Bilodeau AS, Zhou FJ, Kafidi N, Wang B, Baldassarre H, Karatzas CN: Production of cloned goats after nuclear transfer using adult somatic cells. *Biol Reprod* 66: 199–203, 2002
70. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R: Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 394: 369–374, 1998
71. Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Jurgella G, Koppang R, Lesmeister T, Mallon K, Mell G, Misica P, Pace M, Pfister-Genskow M, Strelchenko N, Voelker G, Watt S, Thompson S, Bishop M: Production of cloned pigs from in vitro systems. *Nat Biotechnol* 18: 1055–1059, 2000
72. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH: Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* 407: 86–90, 2000
73. Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H, Perry AC: Pig cloning by microinjection of fetal fibroblast nuclei. *Science* 289: 1188–1190, 2000
74. De Sousa PA, Dobrinsky JR, Zhu J, Archibald AL, Ainslie A, Bosma W, Bowering J, Bracken J, Ferrier PM, Fletcher J, Gasparrini B, Harkness L, Johnston P, Ritchie M, Ritchie WA, Travers A, Albertini D, Dinnyes A, King TJ, Wilmut I: Somatic cell nuclear transfer in the pig: Control of pronuclear formation and integration with improved methods for activation and maintenance of pregnancy. *Biol Reprod* 66: 642–650, 2002
75. Colman A, Kind A: Therapeutic cloning: Concepts and practicalities. *Trends Biotechnol* 18: 192–196, 2000
76. Vogelstein B, Alberts B, Shine K: Genetics: Please don't call it cloning! *Science* 295: 1237, 2002
77. Hochedlinger K, Jaenisch R: Nuclear transplantation, embryonic stem cells, and the potential for cell therapy. *N Engl J Med* 349: 275–286, 2003
78. Lanza RP, Cibelli JB, West MD, Dorff E, Tauer C, Green RM: The ethical reasons for stem cell research. *Science* 292: 1299, 2001
79. Lanza RP, Cibelli JB, West MD: Prospects for the use of nuclear transfer in human transplantation. *Nat Biotechnol* 17: 1171–1174, 1999
80. Solter D: Mammalian cloning: Advances and limitations. *Nat Rev Genet* 1: 199–207, 2000
81. Rideout WM 3rd, Eggan K, Jaenisch R: Nuclear cloning and epigenetic reprogramming of the genome. *Science* 293: 1093–1098, 2001
82. Hochedlinger K, Jaenisch R: Nuclear transplantation: Lessons from frogs and mice. *Curr Opin Cell Biol* 14: 741–748, 2002
83. Tsunoda Y, Kato Y: Recent progress and problems in animal cloning. *Differentiation* 69: 158–161, 2002
84. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y: Eight calves cloned from somatic cells of a single adult. *Science* 282: 2095–2098, 1998
85. Dinnyes A, De Sousa P, King T, Wilmut I: Somatic cell nuclear transfer: Recent progress and challenges. *Cloning Stem Cells* 4: 81–90, 2002
86. Young LE, Sinclair KD, Wilmut I: Large offspring syndrome in cattle and sheep. *Rev Reprod* 3: 155–163, 1998
87. Cibelli JB, Campbell KH, Seidel GE, West MD, Lanza RP: The health profile of cloned animals. *Nat Biotechnol* 20: 13–14, 2002
88. Tamashiro KL, Wakayama T, Akutsu H, Yamazaki Y, Lachey JL, Wortman MD, Seeley RJ, D'Alessio DA, Woods SC, Yanagimachi R, Sakai RR: Cloned mice have an obese phenotype not transmitted to their offspring. *Nat Med* 8: 262–270, 2002

89. Ogonuki N, Inoue K, Yamamoto Y, Noguchi Y, Tanemura K, Suzuki O, Nakayama H, Doi K, Ohtomo Y, Satoh M, Nishida A, Ogura A: Early death of mice cloned from somatic cells. *Nat Genet* 30: 253–254, 2002
90. Bortvin A, Eggan K, Skaletsky H, Akutsu H, Berry DL, Yanagimachi R, Page DC, Jaenisch R: Incomplete reactivation of Oct4-related genes in mouse embryos cloned from somatic nuclei. *Development* 130: 1673–1680, 2003
91. Boiani M, Eckardt S, Scholer HR, McLaughlin KJ: Oct4 distribution and level in mouse clones: Consequences for pluripotency. *Genes Dev* 16: 1209–1219, 2002
92. Bazeed MA, Thuroff JW, Schmidt RA, Tanagho EA: New treatment for urethral strictures. *Urology* 21: 53–57, 1983
93. Atala A, Vacanti JP, Peters CA, Mandell J, Retik AB, Freeman MR: Formation of urothelial structures in vivo from dissociated cells attached to biodegradable polymer scaffolds in vitro. *J Urol* 148: 658–662, 1992
94. Olsen L, Bowald S, Busch C, Carlsten J, Eriksson I: Urethral reconstruction with a new synthetic absorbable device: An experimental study. *Scand J Urol Nephrol* 26: 323–326, 1992
95. Kropp BP, Ludlow JK, Spicer D, Rippy MK, Badylak SF, Adams MC, Keating MA, Rink RC, Birhle R, Thor KB: Rabbit urethral regeneration using small intestinal submucosa onlay grafts. *Urology* 52: 138–142, 1998
96. 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* 163: 1958–1965, 2000
97. El-Kassaby AW, Retik AB, Yoo JJ, Atala A: Urethral stricture repair with an off-the-shelf collagen matrix. *J Urol* 169: 170–173, 2003
98. De Filippo RE, Pohl HG, Yoo JJ, *et al.*: Total penile urethral replacement with autologous cell-seeded collagen matrices [Abstract]. *J Urol* 167[Suppl]: 152, 2002
99. De Filippo RE, Yoo JJ, Atala A: Urethral replacement using cell seeded tubularized collagen matrices. *J Urol* 168: 1789–1792, 2002
100. Kershen RT, Yoo JJ, Moreland RB, Krane RJ, Atala A: Reconstitution of human corpus cavernosum smooth muscle in vitro and in vivo. *Tissue Eng* 8: 515–524, 2002
101. Kwon TG, Yoo JJ, Atala A: Autologous penile corpora cavernosa replacement using tissue engineering techniques. *J Urol* 168: 1754–1758, 2002
102. Wang T, Koh CJ, Yoo JJ, *et al.*: Creation of an engineered uterus for surgical reconstruction [Abstract]. Presented at the Proceedings of the American Academy of Pediatrics Section on Urology, New Orleans, LA, 2003
103. De Filippo RE, Yoo JJ, Atala A: Engineering of vaginal tissue in vivo. *Tissue Eng* 9: 301–306, 2003
104. De Filippo RE, Yoo JJ, Atala A: Engineering of vaginal tissue for total reconstruction. *J Urol* 169[Supplement]: A1057, 2003
105. Lanza RP, Chung HY, Yoo JJ, Wettstein PJ, Blackwell C, Borson N, Hofmeister E, Schuch G, Soker S, Moraes CT, West MD, Atala A: Generation of histocompatible tissues using nuclear transplantation. *Nat Biotechnol* 20: 689–696, 2002
106. Auchincloss H, Bonventre JV: Transplanting cloned cells into therapeutic promise. *Nat Biotechnol* 20: 665–666, 2002
107. Aebischer P, Ip TK, Panol G, Galletti PM: The bioartificial kidney: Progress towards an ultrafiltration device with renal epithelial cells processing. *Life Support Syst* 5: 159–168, 1987
108. Amiel GE, Yoo JJ, Atala A: Renal therapy using tissue-engineered constructs and gene delivery. *World J Urol* 18: 71–79, 2000
109. Humes HD, Buffington DA, MacKay SM, Funke AJ, Weitzel WF: Replacement of renal function in uremic animals with a tissue-engineered kidney. *Nat Biotechnol* 17: 451–455, 1999
110. Ip TK, Aebischer P, Galletti PM: Cellular control of membrane permeability: Implications for a bioartificial renal tubule. *ASAIO Trans* 34: 351–355, 1988
111. Joki T, Machluf M, Atala A, Zhu J, Seyfried NT, Dunn IF, Abe T, Carroll RS, Black PM: Continuous release of endostatin from microencapsulated engineered cells for tumor therapy. *Nat Biotechnol* 19: 35–39, 2001
112. Lanza RP, Hayes JL, Chick WL: Encapsulated cell technology. *Nat Biotechnol* 14: 1107–1111, 1996
113. MacKay SM, Funke AJ, Buffington DA, Humes HD: Tissue engineering of a bioartificial renal tubule. *ASAIO J* 44: 179–183, 1998
114. Evans MJ, Gurer C, Loike JD, Wilmut I, Schnieke AE, Schon EA: Mitochondrial DNA genotypes in nuclear transfer-derived cloned sheep. *Nat Genet* 23: 90–93, 1999
115. Hiendleder S, Schmutz SM, Erhardt G, Green RD, Plante Y: Transmitochondrial differences and varying levels of heteroplasmy in nuclear transfer cloned cattle. *Mol Reprod Dev* 54: 24–31, 1999
116. Steinborn R, Schinogl P, Zakhartchenko V, Achmann R, Schernthaner W, Stojkovic M, Wolf E, Muller M, Brem G: Mitochondrial DNA heteroplasmy in cloned cattle produced by fetal and adult cell cloning. *Nat Genet* 25: 255–257, 2000
117. Fischer Lindahl K, Hermel E, Loveland BE, Wang CR: Maternally transmitted antigen of mice: A model transplantation antigen. *Annu Rev Immunol* 9: 351–372, 1991
118. Hadley GA, Linders B, Mohanakumar T: Immunogenicity of MHC class I alloantigens expressed on parenchymal cells in the human kidney. *Transplantation* 54: 537–542, 1992
119. Yard BA, Kooymans-Couthino M, Reterink T, van den Elsen P, Paape ME, Bruyn JA, van Es LA, Daha MR, van der Woude FJ: Analysis of T cell lines from rejecting renal allografts. *Kid Intl Suppl* 39: S133–138, 1993
120. 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* 108: 1729–1734, 2003
121. Watanabe M, Shin'oka T, Tohyama S, Hibino N, Konuma T, Matsumura G, Kosaka Y, Ishida T, Imai Y, Yamakawa M, Ikada Y, Morita S: Tissue-engineered vascular autograft: Inferior vena cava replacement in a dog model. *Tissue Eng* 7: 429–439, 2001
122. Shinoka T, Breuer CK, Tanel RE, Zund G, Miura T, Ma PX, Langer R, Vacanti JP, Mayer JE Jr: Tissue engineering heart valves: Valve leaflet replacement study in a lamb model. *Ann Thorac Surg* 60[6 Suppl]: S513–516, 1995
123. Shinoka T, Ma PX, Shum-Tim D, Breuer CK, Cusick RA, Zund G, Langer R, Vacanti JP, Mayer JE Jr: Tissue-engineered heart valves: Autologous valve leaflet replacement study in a lamb model. *Circulation* 94[9 Suppl]: II164–II168, 1996
124. Shinoka T, Shum-Tim D, Ma PX, Tanel RE, Langer R, Vacanti JP, Mayer JE Jr: Tissue-engineered heart valve leaflets: Does cell origin affect outcome? *Circulation* 96[9 Suppl]: II-102–II-107, 1997
125. Shinoka T, Shum-Tim D, Ma PX, Tanel RE, Isogai N, Langer R, Vacanti JP, Mayer JE Jr: Creation of viable pulmonary artery

autografts through tissue engineering. *J Thorac Cardiovasc Surg* 115: 536–545, 1998
126. Shin'oka T, Imai Y, Ikada Y: Transplantation of a tissue-engineered pulmonary artery. *N Engl J Med* 344: 532–533, 2001
127. Hunter W: Of the structure and disease of articulating cartilages. 1743. *Clin Orthop* Aug (317): 3–6, 1995
128. O'Driscoll SW: The healing and regeneration of articular cartilage. *J Bone Joint Surg Am* 80: 1795–1812, 1998
129. Buckwalter JA, Lohmander S: Operative treatment of osteoarthrosis. Current practice and future development. *J Bone Joint Surg Am* 76: 1405–1418, 1994
130. Buckwalter JA, Mankin HJ: Articular cartilage repair and transplantation. *Arthritis Rheum* 41: 1331–1342, 1998
131. Schaefer D, Martin I, Jundt G, Seidel J, Heberer M, Grodzinsky A, Bergin I, Vunjak-Novakovic G, Freed LE: Tissue-engineered composites for the repair of large osteochondral defects. *Arthritis Rheum* 46: 2524–2534, 2002
132. Fuchs JR, Terada S, Ochoa ER, Vacanti JP, Fauza DO: Fetal tissue engineering: In utero tracheal augmentation in an ovine model. *J Pediatr Surg* 37: 1000–1006, 2002
133. Fuchs JR, Hannouche D, Terada S, Vacanti JP, Fauza DO: Fetal tracheal augmentation with cartilage engineered from bone marrow–derived mesenchymal progenitor cells. *J Pediatr Surg* 38: 984–987, 2003
134. Atala A, Cima LG, Kim W, Paige KT, Vacanti JP, Retik AB, Vacanti CA: Injectable alginate seeded with chondrocytes as a potential treatment for vesicoureteral reflux. *J Urol* 150: 745–747, 1993
135. Diamond DA, Caldamone AA: Endoscopic correction of vesicoureteral reflux in children using autologous chondrocytes: Preliminary results. *J Urol* 162: 1185–1188, 1999
136. 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* 20: 157–165, 2001
137. Yokoyama T, Chancellor MB, Watanabe T, *et al.*: Primary myoblasts injection into the urethra and bladder as a potential treatment of stress urinary incontinence and impaired detrusor contractility: Long-term survival without significant cytotoxicity. *J Urol* 161: 307, 1999
138. Chancellor MB, Yokoyama T, Tirney S, Mattes CE, Ozawa H, Yoshimura N, de Groat WC, Huard J: Preliminary results of myoblast injection into the urethra and bladder wall: A possible method for the treatment of stress urinary incontinence and impaired detrusor contractility. *Neurourol Urodyn* 19: 279–287, 2000
139. Strasser H, Marksteiner R, Eva M, *et al.*.: Transurethral ultrasound guided injection of clonally cultured autologous myoblasts and fibroblasts: Experimental results [Abstract]. Presented at the Proceedings of the 2003 International Bladder Symposium, Arlington, VA, 2003
140. Yiou R, LaFleuchuer J, Atala A: The regeneration process of the striated urethral sphincter involves the activation of intrinsic satellite cells. *Anat Embryol* 206: 429–435, 2003
141. Yiou R, Yoo JJ, Atala A: Restoration of functional motor units in a rat model of sphincter injury by muscle precursor cell autografts. *Transplantation* 76: 1053–1060, 2003
142. Machluf M, Orsola A, Boorjian S, Kershen R, Atala A: Microencapsulation of Leydig cells: A system for testosterone supplementation. *Endrocrinology* 144: 4975–4979, 2003
143. Santen RJ, Swerdloff RS: Clinical aspects of androgen therapy [Abstract]. Presented at the Workshop Conference on Androgen Therapy: Biologic and Clinical Consequences, Penn State Workshop Proceedings, Philadelphia, PA, 1990
144. Tai J, Johnson HW, Tze WJ: Successful transplantation of Leydig cells in castrated inbred rats. *Transplantation* 47: 1087–1089, 1989
145. van Dam JH, Teerds KJ, Rommerts FF: Transplantation and subsequent recovery of small amounts of isolated Leydig cells. *Arch Androl* 22: 123–129, 1989
146. De Vos P, De Haan B, Van Schilfgaarde R: Effect of the alginate composition on the biocompatibility of alginate-polylysine microcapsules. *Biomaterials* 18: 273–278, 1997
147. Tai IT, Sun AM: Microencapsulation of recombinant cells: A new delivery system for gene therapy. *FASEB J* 7: 1061–1069, 1993
148. Nomi M, Atala A, Coppi PD, Soker S: Principals of neovascularization for tissue engineering. *Mol Aspects Med* 23: 463–483, 2002