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Virus Integration and Genome Influence in Approaches to Stem Cell Based Therapy for Andro-Urology

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Abstract

Despite the potential of stem cells in cell-based therapy, major limitations such as cell retention, ingrowth, and trans-differentiation after implantation remain. One technique for genetic modification of cells for tissue repair is the introduction of specific genes using molecular biology techniques, such as virus integration, to provide a gene that adds new functions to enhance cellular function, and to secrete trophic factors for recruiting resident cells to participate in tissue repair. Stem cells can be labelled to track cell survival, migration, and lineage. Increasing evidence demonstrates that cell therapy and gene therapy in combination remarkably improve myogenic differentiation of implanted mesenchymal stromal cells (MSCs), revascularization, and innervation in genitourinary tissues, especially to treat urinary incontinence, erectile dysfunction, lower urinary tract reconstruction, and renal failure. This review discusses the benefits, safety, side effects, and alternatives for using genetically modified MSCs in tissue regeneration in andro-urology.

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Keywords

Stem cells; cell therapy; gene therapy; urinary incontinence; skeletal muscle genesis; innervation; angiogenesis; erectile dysfunction; endothelial cells

1. Introduction

Autologous stem cells or mesenchymal stromal cells (MSCs)-based therapy for urological diseases caused by congenital defects, trauma, cancer, or chronic inflammation has recently offered a promising alternative for tissue regeneration. MSCs possess certain characters of stem cells, i.e. distinct differentiation potential to promote tissue regeneration via their trans-differentiation and trophic factor secretion although they are lack of self-renew capacity of totipotent stem cells [1]. Major benefits of autologous MSCs include avoiding adverse events related to the rejection of implanted xenogenous or allogeneous tissues, and reducing risk of tumorigenesis, and bypassing ethical concerns about application of induced pluripotent stem cells (iPSCs) or embryonic stem cells. Skeletal muscle-derived progenitor cells[2–5], bone marrow-derived mesenchymal stromal cells (BMSCs) [6–16], adipose-derived stem cells (ASCs)[17–21], hair follicle stem cells[22], endothelial progenitor cells[23], amniotic fluid-derived cells [24–30] and urine-derived stem cells (USCs)[31–36] are regarded as candidates for cell therapy in genito-urinary tract tissue repair. However, a lower retention rate of implanted cells limits the therapeutic effect of donor stem cells in tissue repair processes. In cell-based therapy, stem cells are often induced to become fully differentiated cells *in vitro* and then implanted into the host to participate in tissue repair. Thus, an extra step of *in vitro* pre-conditioning processing is needed. Furthermore, the lifespan of *in vitro* pre-differentiated cells is shorter than that of non-pre-conditioned cells when they are implanted *in vivo*.

Since vascular support is a key for these donor cells to survive in the implanted sites, angiogenic gene manipulation is often used in stem cell therapy to improve donor cell viability, ingrowth, differentiation and functional tissue formation *in vivo*. Several studies have reported that MSCs that express vascular epithelial growth factor (VEGF), fibroblast growth factor (FGF), or insulin-like growth factor-1 (IGF1) can improve cell survival, ingrowth, innervation, and myogenesis of stem cells[37–42] via angiogenesis. Most experimental studies on genetically-modified stem cells have focused on augmenting the inherent capabilities of these cells to facilitate urological tissue repair *in vivo*. As excellent carriers of transgenes, stem cells can be feasibly manipulated *in vitro* for delivery of therapeutic genes to genitourinary tissues [31, 42, 43]. With anti-fibrotic and angiogenic properties, MSCs are an optimal gene carrier cell source for urological tissue regeneration compared to other somatic cells.

Stem cell therapy has been used in tissue defect with minimal scarring tissues; gene therapy is suitable in treatment of inherited disorders or neurodegenerative diseases; stem cell and gene therapy offer an alternative for treating a range of diseases, many of which currently have no cure. In this review, we discuss the advantages and limitations of stem cell therapy combined with gene modification, and describe future directions for cellular therapy in

improving cell retention, engraftment, differentiation, and host cell recruitment in urinary tract tissue repair.

2. Stem cell therapy

Cell-based therapy provides therapeutic potential for treatment of genitourinary diseases, such as stress urinary incontinence (SUI) due to urethral sphincter dysfunction, erectile dysfunction (ED) due to nerve or endothelial dysfunction, bladder or urethral defects, and renal ischemia injuries. MSCs are commonly used cell sources when the native target cells are unhealthy or unavailable. Multiple types of stem cells have been used in preclinical animal models to repair or regenerate tissue, including pluripotent stem cells i.e. embryonic stem cells (ESCs) [44–47], iPSCs [48] or multi-differentiated potent MSCs. As a cell source for tissue repair, MSCs can secrete paracrine factors, recruit resident stem cells, foster trans-differentiation, and appear to be less prone to malignant tumors. In addition, MSCs can give rise to skeletal, smooth muscle cells, and endothelial cells for creating urethral sphincter, blood vessels, or urinary tract muscle wall[49]. They can be implanted into the host via local administration, intravenously, or by intra-peritoneal injection.

In cell therapy for ED, SUI, and renal failure, paracrine factors secreted by stem cells appear to play a dominant role in stimulating host cells to participate in tissue repair. Most studies have demonstrated that numbers of implanted stem cells decrease with time during tissue repair[18, 24, 25, 36]. The most likely reasons include: 1) loss of proliferative function after repeated cellular de-attachment processes during culture; 2) over-expansion of the cell population *in vitro* that shortens cell lifespan; and 3) low retention rate of grafted cells due to a poor blood supply, fibrosis, or inflammation at the implantation site. Improving the microenvironment by adding exogenous angiogenic growth factors is a logical approach to increase the rate of stem cell survival *in vivo*.

3. Gene therapy

Gene therapy promises treatment of inherited disorders or defected tissues by drug (DNA) delivered to the nucleus of the patient's cells in order to function. The most common methods of gene therapy in treatment of inherent disease involve 1) replacing the mutated gene by a plasmid DNA; 2) inactivating, or knocking out a mutated gene [42]; or 3) introducing a functional therapeutic gene (such as IGF1) into the host to promote tissue regeneration[42, 50–52] or help fight a disease. In gene therapy, plasmid DNA encoding a therapeutic protein is inserted into the host cells. Once inside, the DNA starts expressed by cellular machinery, causing the regeneration of functional proteins, which can in turn be healing the host tissue defects.

DNA is delivered into cells by two main methods: recombinant viruses and non-viral methods (i.e. naked DNA) [53]. In the viral vector approach, viruses insert their genetic material into the cells as part of their replication cycle in the host. Certain viruses can be used for human gene therapy via using the virus after removing DNA as a vehicle to insert the specific function DNA (Table 1).

3.1. Virus integration

Two classes of vectors lead to transient (or short-term) and permanent (or long-term) expression of the transgene, based on whether the vector is integrated into the cellular DNA. Because adenoviral vectors usually do not incorporate into the cellular genome and can infect both proliferating and non-proliferating (or quiescent) cells with large amounts of exogenous DNA, these vectors are often used as tools for stem cell gene therapy. However, adenovirus can cause humoral and cellular immune reactions in the hosts (Table 1). The new generation of adenoviral vectors offers promising alternatives, because the constructs only hold minimum amounts of residual adenoviral DNA, causing declined immunogenicity upon rejection. The benefit of utilizing these adenoviral vectors is that new vectors offer higher-level transgene expression during a restricted period of time. Other viral vectors, such as retroviral, lentiviral, and adeno-associated viral vectors, lead to permanent expression, which increases the risk of insertional mutagenesis. Because of safety concerns, these three viral vectors are currently used only in experimental research, not in clinical applications.

3.2. Non-viral methods of integration

Non-viral approaches display certain benefits over viral approaches, such as broad production and little host immunogenicity[54]. Low levels of transfection and gene expression previously limited the application of non-viral methods. Nevertheless, recent advances in vector technology have generated molecules and techniques to make the virus transfection more efficient. Other approaches for non-viral gene therapy can be more effective to transfect stem cells, such as the electroporation, sonoporation, the gene gun, injection of naked DNA, magnetofection, and the use of lipoplexes, oligonucleotides, inorganic nanoparticles and dendrimers.

3.3. Labelling cells

Reporter genes [55] (i.e. GFP, Lac Z, and luciferase (Table 2) are commonly used to monitor cell viability, location, and ingrowth of implanted cells *in vivo*. In addition, both reporter genes and genes of interest (such as VEGF, FGFs, or IGF1) are often combined in the same DNA construct for cell insertion. Thus, expression of a reporter gene is a marker for successful uptake of a gene of interest, allowing easy visualization of genetically-modified stem cells. This tracking and expression technology is often used to monitor survival, localization, migration, self-renewal, and differentiation of visualized reporter gene-expressing stem cells after transplantation *in vivo*. GFP or other fluorescent proteins are generally stable and continue to emit fluorescence long after the host has died. These fluorescent labeling provides better images when tissue blocks are freshly sliced, since stored tissue blocks show a slow decrease in GFP fluorescence intensity with time. In addition to reporter genes, several tracking markers, such as bromodeoxyuridine (BrdU) or magnetic labels to mark nuclei, are often used to track the implanted cells *in vivo* [43]. As a thymidine analog incorporating DNA of dividing cells during the S-phase of the cell cycle, BrdU is a marker of DNA synthesis. For monitoring cell proliferation, BrdU labeling is commonly used technique for studying the implanted cells in tissue repair *in situ*. These nucleic markers can be used in tissue blocks for reliable detection of human cells even after long-term preservation. However, BrdU is a toxic and mutagenic substance to cause cell

death, teratomas formation, the cell cycle extension, alternation in DNA stability, and mitogenic, transcriptional and translational impacts on cells that incorporate it, which causes the limitation of its application.

3.4. Gain- or loss-of-function studies

Stem cells are genetically modified to determine the functions and role of certain genes in tissue regeneration[42]. These could include effects on the cellular phenotype, in which the gene is expressed, or what other genes it relates to. These assessments often engage gain of function and loss of function. To identify a key gene or signaling pathway, genetically manipulated stem cells are often used to overexpress or inhibit the gene of interest with high expression and transfection efficiencies with viral or non-viral approaches [56].

Loss-of-function experiments (i.e. gene knockout experiment *in vitro*) are those in which cells are engineered to make one or more genes inactive. This gene knockout assessment contains the generation and alteration of a DNA construct. In a standard knockout model, this involves a copy of the non-function of desired gene.

Gain-of-function experiments are often achieved in combination with knockout experiments to more finely create the function of the desired gene. In this experiment, the DNA construct is designed to strengthen the function of the gene, commonly by bringing synthesis of the protein or using extra copies of the gene. With gain-of-function strategy, a recent study demonstrated that IGF1 gene delivery has therapeutic potential to treat SUI simulating trauma induced by childbirth in women[42]. To examine the effects of IGF1 on urethral sphincter function in a rat model of vaginal distention, IGF-1 was continuously delivered. IGF-1, IGF1R mRNA and protein levels were significantly increased in urethral and pudendal nerves one week after distention injury. IGF1-treated animals showed that leak point pressure, urethral baseline pressure, and urethral responses were significantly improved following distention. Moreover, IGF1 treatment promoted cell ingrowth, anti-apoptotic effects and increased the expression level of Akt phosphorylation around urethral tissue, suggesting that IGF1 accelerated recovery from SUI by activation of the Akt signal transduction pathway in rat model.

4. Gene and stem cell therapy

As reliable carriers of transgenes, stem cells are easily manipulated *in vitro* for transfer of therapeutic genes to genito-urolological tissues [18, 33, 36, 42, 57–59]. The genetic modification of the cells can dramatically increase the efficiency of stem cell therapy. Gene and stem cell therapy have several advantages: 1) *in vitro* genetic modification of stem cells can be used to optimize the type of gene, levels of transgene delivery and expression efficacies; 2) *in vitro* gene modification is simpler and more efficient than *in vivo* experiments, and 3) it offers the opportunity to regulate the expression levels of the transgene within stem cells for tissue repair. Thus, therapeutic genes can be used in stem cell-based therapy for defective tissue, including ones for various functional growth factors, cytokines, and signaling molecules (Figure 1). Compared to stem cells alone, genetically-modified stem cells are able to achieve better outcome in cell retaining, anti-apoptosis,

extracellular matrix remodeling, resident cell recruitment, angiogenesis, myogenesis, and innervation to promote genitourinary tissue regeneration.

The mechanisms by which MSCs participate in tissue repair include trans-differentiation and paracrine effects. In preclinical studies, MSCs have been administered via three routes: 1). directly into the injured site, as a way to test the trans-differentiation of labeled stem cells and their paracrine effects; 2). Intra-peritoneally, as a way to test paracrine effects, and 3). intravenously, as a way to test homing, paracrine effects, and trans-differentiation of labeled stem cells (Figure 1). Although a therapeutic stem cell-based gene approach has been successful in pre-clinical studies [18, 33, 36], no clinical studies based on this therapy have been done for the treatment of genitourinary diseases.

4.1. Improvement of grafted cell retention

Transplanted stem cells show low survival rates in genitourinary tissues with scarring, chronic inflammation in sites such as the sphincter in SUI, fibrosis in the urethra or bladder, or chronic renal diseases. This is a major obstacle in the clinical application when stem cells are used alone in the tissues related to chronic diseases. Several approaches have been developed based on preconditioning (such as in stem cells cultured in hypoxic conditions, the conditioned medium or growth factors) and reprogramming of stem cells before transplantation. One of the most effective approaches is the genetic engineering stem cells with plasmids encoding for signaling molecules for angiogenesis, anti-apoptosis and improvement of cell survival, such as VEGF[18, 33, 60], FGF2[36], Akt[61, 62], or IGF1[58, 59] to prepare these cells. Such manipulation enhanced the viability and lifespan of the transplanted cells after transplantation and increased engraftment, improving genitourinary tissue regeneration, and functional recovery.

VEGF is one of the most commonly used growth factors acting through the VEGFR-1 and VEGFR-2 receptors. It enhances angiogenesis and promotes vascularity via its specific mitogenic and anti-apoptotic effects on endothelial cells. Except multiple differentiation capacity, MSCs possess angiogenic capacity because of they can secrete various trophic factors, including VEGF in response to ischemic injuries. BMSCs modified with the VEGF gene significantly promoted graft angiogenesis, increased size of the engraftment, and enhanced genitourinary functional recovery (such as erectile function in diabetic ED [63]) compared to non-preconditioned stem cells in an *in vivo* model. In a study of potential treatment for SUI, we showed that USCs can secrete VEGF into the culture medium *in vitro* and induce moderate angiogenesis *in vivo*[57]. This angiogenic gene modification markedly increased neovascularization. In addition, stem cells expressing VEGF and endothelial cell in combination promoted better neovascularization compared to the cells expressing VEGF alone. It appears that VEGF transfection of USCs can significantly enhance cell survival ratio and revascularization in treatment of SUI.

Fibroblast growth factor (FGF) family is termed as a “pluripotent” growth factor due to its varied interactions with multiple cell types [21,22]. FGFs are multifunctional proteins with a wide variety of functions; they are most commonly mitogens, but also have regulatory, morphological, and endocrine effects. FGF1 and FGF2 can both promote endothelial cell proliferation and enhance its tube-like structures *in vitro* [23]. In an *in vivo* model, FGF

induces angiogenesis and enhances the growth of new blood vessels from the pre-existing vasculature [24]. As angiogenic factors, FGF1 and FGF2 are both more important than VEGF or PDGF [25]. FGF1 mainly expressed in the central nervous system, while FGF2 expression is throughout each of tissues [26, 27]. Furthermore, FGF2 is more potent than other angiogenic growth factors such as VEGF, insulin-like growth factor (IGF), or epidermal growth factor (EGF) in inducing adipose stem cells to give rise to endothelial cells [28]. In the absence of VEGF, IGF, or EGF, adipose stem cells can also display endothelial properties when cultured in an FGF2-supplemented medium.

4.2. Promotion of transdifferentiation of stem cells

Gene modification can more effectively induce stem cells to differentiate and improve cell function, compared to the use of a commercial inductive media supplied with bioactive growth factors. A comprehensive analysis of 14 types of bone morphogenic proteins (BMPs) was conducted to compare their abilities to regulate multilineage-specific differentiation of MSCs [64–66]. Most BMPs could regulate the expression of Runx2, Sox9, MyoD, and PPAR γ 2 [67]. After selected, BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9 are showed to effectively promote both adipogenic and osteogenic differentiation [67]. BMPs activated several signaling pathways to regulate lineage commitment and terminal differentiation of MSCs. Our recent study demonstrated that gene transfection with BMP2 and BMP9 guided USCs to differentiate into osteocytes, chondrocytes, and adipocytes *in vivo* in a highly efficient manner[68].

As one of the most potent biological activators of the AKT signaling pathway, IGF1 is a stimulator of cell growth and proliferation, and a potent inhibitor of programmed cell death. IGF1 also possesses growth-promoting properties on almost every cell, which promote systemic body growth, especially skeletal muscle, nerve, and renal cells. Sumino *et al.* reported that IGF1 prompted the myogenic differentiation of human urethral sphincter skeletal muscle progenitor cells, instead of hepatocyte growth factor (HGF) [69]. Cultured progenitor cells extended their lifespan and induced myogenic differentiation after transfected with temperature-sensitive simian virus-40 T antigen (ts-SV40 Tag). The transfected progenitor cells grew slowly, expressed myosin heavy chain protein, and fused to form multinucleated myotubes. Moreover, IGF1 significantly accelerated myogenic protein expression in a dose-dependent fashion via activation of the PI3-kinase pathway.

MSCs modified with a VEGF gene promote angiogenesis and enhance skeletal and smooth myogenic differentiation and innervation [31, 43] (Figure 1). Our recent study showed that USCs expressing VEGF significantly increased myogenic gene and protein marker expression after implanted *in vivo* compared to control USCs that did not express VEGF. More vessels formed and more cells expressing muscle markers were observed when USCs expressing VEGF along with endothelial cells were used. Our data suggest that a direct effect of VEGF angiogenesis and an indirect effect mediated by an autocrine factor from host muscle cells both promote myogenic differentiation of USCs and muscle tissue engineering *in vivo*.

4.3. Enhancement of paracrine effect of stem cells

To improve tissue repair, a strategy of genetically modifying stem cells is to modulate their paracrine behavior for enhanced expression of bioactive growth factors or cytokines. The angiogenic growth factors released from genetically modified stem cells significantly improved survival of the grafted cells, enhanced the engraftment, preserved injured tissue function by multiple mechanisms, including cytoprotection, anti-apoptosis, angiogenesis, and resident stem cell recruitment. We have already shown that USC_s infected with Ad-VEGF secreted VEGF in high levels as a part of their modified paracrine activity, and reached a peak two weeks after infection[43]. Overexpression of VEGF significantly promoted the survival of the grafted USC_s, and promoted revascularization, myogenesis, and innervation *in vivo* compared to USC_s alone. In a recent study, we modulated the paracrine activity of USC_s by transgenic expression of FGF2. USC_s overexpressing FGF2 secreted significantly higher levels of VEGF, compared to USC_s alone *in vitro*. USC_s-FGF2 increased the number of cells expressing the endothelial markers and restored intracavernous pressure, thereby enhancing erectile function in a rat model of diabetic ED [36].

4.4. Preclinical experiments

4.4.1. Stress urinary incontinence—Stress urinary incontinence (SUI) is associated with the loss of various amounts of urine when intra-abdominal pressure increases due to muscle and nerve injury within the urethral sphincter. Three types of tissues around the urethra, including muscle, vessels and peripheral nerve, are associated with urinary continence. The sphincter muscle unit of the urethra contains both internal and external sphincter muscles. The internal sphincter is the extension of the detrusor muscle and is made of smooth muscle; thus, it is under involuntary or autonomic control, and is the primary muscle for stopping the release of urine. The external sphincter is made of skeletal muscle which under the voluntary control of the somatic nervous system. The goal of treating SUI is to recover the defect of both skeletal and smooth muscles, and neurons in the urethral proximal segment

In women, the injury from vaginal delivery during childbirth is an initial cause of SUI, which results in damage to the external urethral sphincter, pelvic floor muscle, and the neural plexus posterolateral to the vagina[70, 71]. Although success rates of cell-based therapy for patients with SUI reach up to 80%[71], preventing SUI recurrence is critical after cell implantation. To achieve a long-term effect of stem cell transplantation, a favorable microenvironment is needed to enhance angiogenesis, myogenesis, and innervation to promote urethral sphincter tissue regeneration (Figure 1). Three critical criteria are important for stem cell therapy in correction of urethral sphincter muscle defects, i.e. enhancing local blood circulation, improving skeletal and smooth muscle tissue regeneration, and restoring peripheral nerve function, particularly with chronic inflammatory, scarred tissues or lack of blood supply in the neighboring tissue of the urethral sphincter.

Our recent studies demonstrated that use of genetically modified stem cells via transfection of the angiogenic factor genes such as VEGF or FGF2 significantly promoted myogenic differentiation of USC_s, and improved angiogenesis and innervation [31, 57]. Implanted

USCs alone improved neo-angiogenesis and myogenic regeneration compared to cell-free controls, suggesting that USCs display endothelial and myogenic differentiation potential *in vivo*. When VEGF-expressing USCs are transplanted *in vivo*, the graft size retained and the number of survived cells significantly increased. Importantly, angiogenesis induced by VEGF-modified USCs significantly improved both skeletal and smooth muscle regeneration[31, 43]. Analyses by real-time PCR and immunocytochemical staining consistently demonstrated myogenic differentiation and angiogenic effects of VEGF-expressing USCs compared to human skeletal muscles or USCs implants. The number of cells expressing skeletal muscle cell markers (Myo-D, desmin, myogenin, and myosin) and endothelial markers (CD31 and vWF) significantly increased with the graft tissue of USCs expressing VEGF. In addition, VEGF-expressing USCs promote innervation assessed with target cell-specific markers (i.e. neurofilament and S-100) at the gene and protein levels [31, 43]. The regenerated nerve fibers most likely initiated from the host peripheral nerve via improved angiogenesis, instead of differentiation from the implanted human USCs because these regenerated nerve cells had little human nuclear staining. Further studies are needed to establish whether nerve regeneration could help recover urethral sphincter function in a SUI animal model.

4.4.2. Diabetic erectile dysfunction—Erectile dysfunction associated with type 2 diabetes is more prevalent and more severe than in non-diabetic patients [72]. The management of diabetic ED is complex and challenging, and underlying endothelial dysfunction and subsequent neuropathy (nerve damage) and decreased smooth muscle content may be pivotal reasons. Therefore, new therapeutic strategies targeted towards repairing endothelial function, particularly in the early stage of this disorder, are needed. Our recent study demonstrated that human USCs genetically modified with FGF2 improved ED in a rat model of type 2 diabetes. The USCs-FGF2 displayed significantly higher intracavernous pressure and a higher ratio of intracavernous pressure/mean artery pressure 28 days after intracavernous injection ($p<0.01$). Although few implanted cells were detected within the grafted tissues, histologic and Western blot analyses demonstrated increased expression of endothelial CD31 and Von Willebrand factor (vWF) within the penile corpus cavernosum tissues. In addition, the number of cells expressing endothelial nitric oxide synthase (eNOS) within corporal tissue in USC-FGF2-treated rats was significantly higher than in control rats. Furthermore, USC-FGF2 increased the amount of cells expressing the smooth muscle markers (i.e. desmin and smoothelin) within the cavernous tissue [62]. In our other study, ASCs expressing VEGF similarly improved erectile function in our rat model of type 2 diabetes by recruiting resident cells and increasing the endothelial expression and contents of smooth muscle[18].

4.4.3. Lower urinary tract reconstruction—Transplanted grafts require rapid establishment of vascular ingrowth to allow cell survival and support bladder wall or urethral regeneration, particularly given the large size of the bladder [73] or when long segments of urethra require replacement [60]. To explore the role of bioactive growth factors on bladder or urethral tissue regeneration, a few studies have been conducted with gene stem cell-based therapy.

Bladder regeneration using tissue engineering techniques is challenging because of the bladder's unique properties and functions. It appears that revascularization rates of grafts are critical for the extent of pre-existing bladder injury. In a porcine model of a partial bladder cystectomy, VEGF/GFP gene-modified autologous endothelial progenitor cells seeded onto bladder acellular matrix grafts enhanced blood supply in tissue-engineered bladders [74]. Significant increases in micro-vessel density and bladder regeneration were noted in the experimental group, compared with the control group. In another study, bone marrow MSCs transfected with the nerve growth factor (NGF) gene survived and continued to express NGF in diabetic rat bladder tissues 4 weeks following implantation [75].

Urethral strictures or urethrocutaneous fistulas are common complications of urethral injuries, due to dense scar formation and inadequate blood supply. Thus, an adequate blood supply is a key factor for successful urethral tissue reconstruction. Guan et al subcutaneously implanted decellularized artery matrix-seeded bladder urothelial cells modified with VEGF/GFP in mice to test the neovascular potential of urethral grafts. VEGF-modified urothelial cells secreted VEGF in a time-dependent manner and significantly enhanced neovascularization and formation of a urethral layer compared to GFP-modified cells [60]. This approach provides a possible alternative method for urethral reconstruction.

4.4.4. Renal insufficiency—Hepatocyte growth factor (HGF) gene therapy reverses glomerulosclerosis in diabetic nephropathy [76, 77]. Stem cells and HGF gene therapy might be opportunities to prompt renal regeneration in diabetic nephropathy. A recent study showed that HGF gene therapy enhances renal expression of stromal-cell-derived factor-1 and increases the number of BMSCs in the injured kidneys. These cells are mainly monocyte-derived macrophages, which may contribute to renal tissue repair and regeneration *in vivo*. In addition, HGF gene therapy increased number of Bowman's capsule parietal epithelial cells, led BMSCs to fuse with the parietal cells and improved podocyte repopulation[78, 79].

Diabetic nephropathy as a major complication of diabetes is a leading cause of end-stage renal diseases. As potent angiogenic factor, pigment epithelium-derived factor (PEDF) expressed at high levels in healthy kidneys and other tissues. PEDF levels are significantly declined in the kidneys of streptozotocin (STZ)-induced diabetic rats. Ma's group demonstrated the therapeutic effects of PEDF on diabetic nephropathy in a STZ-diabetic rat model [56] using an adenovirus expressing PEDF (Ad-PEDF). Increased PEDF expression was associated with significantly improved microalbuminuria at the early stages of diabetic kidney disease. Administration of Ad-PEDF significantly reduced generation of extracellular matrix (ECM) protein via preventing the expression of two major fibrogenic factors, transforming growth factor-beta (TGF- β)1 and connective tissue growth factor (CTGF) within the diabetic kidney. Therefore, PEDF acts as an endogenous anti-collagen deposit and anti-fibrogenic factor in diabetic nephropathy. Thus, the therapeutic potential of PEDF was supported by its down-regulation; its inhibition of the overexpression of TGF- β , CTGF, and ECM proteins; and its improvement of proteinuria in rats with diabetic kidney after Ad-PEDF treatment.

5. Safety concerns and limitations

DNA introduced into target cells for therapeutic reasons should remain functional, and cells containing the therapeutic DNA should be long-lived and stable. However, integrating therapeutic DNA into the genome and the rapidly dividing nature of many cells remains risky, preventing this technique from achieving any long-term benefits in the clinic. Stem cell gene therapy is still under study to make sure that it will be safe and effective.

Some unsolved problems with the technology underlying gene therapy include:

- i. Potential genome changes (i.e. gene mutation; tumor genesis): the safety of angiogenic gene transfection of stem cells is an issue, as this approach can potentially induce tumorigenesis if the DNA is integrated in the wrong site in the genome [80, 81]. Although we noted no tumor formation in any of the 5 implanted graft groups for up to one month [31, 57], a long-term study needs to be performed before this approach is considered safe for clinical use.
- ii. A few animals in other studies [82] and ours have died from hemorrhage in the liver or other sites. In our study, 8 of 45 mice died within one week following injection with VEGF-expressing USCs in the presence or absence of ECs (unpublished data). Extensive hemorrhage was seen only in liver tissue and implanted grafts (Figure 2); the remaining organs were normal. Most likely the mice died from internal organ hemorrhage due to excessive VEGF secretion, not adenovirus infection. VEGF not only promotes angiogenesis, but can also render the vessels hyper-permeable by increasing circulating macromolecules by about 50,000 times through histamine secretion [82, 83]. The onset of VEGF's vascular permeabilization is rapid, becoming evident within several minutes of injection [82]; thus, creating a slower release of VEGF becomes highly critical in angiogenic gene modification of stem cells, and merits further investigation.
- iii. Short-lived nature of gene therapy: Therapeutic gene transferred into target cells must remain functional and the cells containing the functional gene must be long-lived and stable when gene therapy becomes a permanent cure. Problems with integrating a therapeutic gene into the genome and the rapidly dividing nature of the cells prevents gene therapy from achieving long-term benefits. Thus, patients will have to undergo multiple rounds of gene therapy. In addition, tracing cells with gene transfection can adversely affect the function of the gene, causing side effects.
- iv. Immune response: Autologous MSCs might be a foreign object after genetically modified. When the cells are transferred into host tissues, the immune system can be activated and stimulated to attack the invader. The risk of stimulating the immune system is always a probability even when the effectiveness of gene therapy reduces. In addition, the immune system enhanced response to invaders, which makes it difficult for gene therapy to be repeated in hosts.
- v. Drawbacks with viral vectors: Viruses present a variety of potential problems to the host: immune and inflammatory responses, toxicity, and gene control and targeting

issues. There is also always the concern that the viral vector, once inside the host, may recover its ability to result in diseases.

- vi. Optimal amounts of genetically modified MSCs: The amount of stem cells expressing angiogenic growth factors needs to be optimized. Too few cells might have limited effects; too many could lead to bleeding, hematoma, or even death.

Nonviral methods as an alternative gene transfer vehicle have gained considerable attention due to their promising properties, such as low toxicity, lack of immunogenicity, and potential for tissue specificity[53]. To promote current virus or nonvirus gene transfer systems, several key steps are needed to increase efficiency, reduce complications of insertion mutation and cytotoxicity, enhance intracellular delivery and long-term expression, and promote extracellular transfer and targeting. Strategies to develop the new vectors, such as nonviral and viral vectors in combination, might be beneficial to lead to a safe, efficient, long-lasting, and nontoxic gene delivery system.

Other than viral or nonviral gene delivery systems, a few alternative approaches are available. These include controlled release of growth factors entrapped within nanoparticles, alginate or gelatin microsphere beads, and hydrogel and collagen matrix binding with growth factors via heparin or collagen binding domains. To provide safe, site-specific delivery and targeted release of growth factors to implanted stem cells, microbeads of alginate or hydrogel containing growth factors provide an alternative to correct muscular deficiency in SUI [43]. Our recent studies demonstrated that a combination of growth factors released locally from the alginate microbeads significantly enhanced stem cells to differentiate into a myogenic lineage, enhanced angiogenesis and innervation, and stimulated resident cell growth *in vivo* (Figure 3). Three groups of growth factors with low doses, including angiogenic (VEGF, FGF, PDGF, IGF1), myogenic (IGF1, PFGF, TGF- β), and neurogenic (FGF, IGF, NGF) growth factors in combination, achieved more efficient outcomes compared to use of a single group of growth factors in grafted cells retention, engraftment, muscle tissue formation, revascularization, and nerve regeneration *in vivo*. This approach could be used for cell therapy in the treatment of SUI and promote regeneration of other urinary tract tissues.

Conclusions

Viral integration is an excellent tool to monitor the fate of implanted cells and to study the mechanisms of interaction between growth factors and regrowth and differentiation of stem cells through loss-of-function (gene knockdown) and gain-of-function (gene overexpression) techniques in urinary tract tissue repair. In addition, compared to use of stem cells alone, genetically modified stem cells with angiogenic growth factor appreciably increased cell viability, myogenic regeneration, and innervation, indicating that this approach may offer more effective treatment for andro-urolological tissue defects. In particular, stem cell and gene therapy aims to rebuild the contractile function of the striated muscles and rhabdosphincter to correct SUI. However, dual therapy has not been used clinically due to safety concerns regarding gene transfection. Therefore, developing new vectors that are target-specific, efficient and confer fewer complications is needed.

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Figure 1.
Gene therapy and stem cell transplantation for treatment of SUI.

Figure 2. Abundant hemorrhage in implanted grafts and livers in dead athymic mice with USCs/ VEGF-GFP

Histochemical staining displayed hemorrhage in retrieved livers (**a**, arrows) and implanted grafts (**b**, arrows) in the dead animals one week after USC/VEGF-GFP plus EC were subcutaneously implanted *in vivo*, compared to the livers (**c**) and grafts (**d**) in the live animals. H&E staining for (**a**, **c**) and Masson's trichrome staining for (**b**, **d**). Scale bar= 50 μm .

Figure 3.
Experimental stem cell approaches to create controlled release of growth factors for treatment of SUI

Table 1

Commonly used vectors in genetically modified MSCs

	Adenovirus	Retrovirus	Lentivirus	Plasmid
Insert size	~30kb	8kb	4kb	unlimited
Integration	no	yes	yes	rare
Production	$>10^{11}$	$>10^6$ cfu/ml	10^6	unlimited
Administration	<i>ex/in vivo</i>	<i>ex vivo</i>	<i>ex/in vivo</i>	<i>ex/in vivo</i>
Expression style	Transient	Long-term	Long-term	Transient
Express level	high	moderate	moderate	high
Immune	extensive	few	low	None
Safety concerns:	Insertional none	Insertional Inflammatory	Mutagenesis response	Mutagenesis toxic
Cells infected	all cells, including proliferating cells and non-proliferating cells	dividing cells or proliferating cells	all cells	need help

Table 2

Common reporter genes

Gene products	Fluorescent proteins	β -galactosidase	Luciferase
Reporter genes	GFP, RFP, YFP, mCherry	LacZ	Catalyzes a reaction with luciferin to produce light
Assay	Bioluminescence imaging	Enzyme assay or histochemical	Fluorescent, microscopic, spectrophotometry
Advantages	<ul style="list-style-type: none"> Simple to visualize with no reagents required Visualized in living cells Single-cell analysis Cell sorting Broad host applicability >10 different color variants 	Prolonged staining can visualize relatively low levels of expression	<ul style="list-style-type: none"> Short half-life (3–4 hrs) High sensitivity of detection High signal to background ratio Broad dynamic range, minimal DNA input Low expense of a luminometer
Limitations	<ul style="list-style-type: none"> Longer half-life (26 hrs) Significant DNA input Less sensitive Create background and limit fluorescent reporter detection Expensive equipment needed. 	<ul style="list-style-type: none"> Require cumbersome or insensitive assays Purchase of costly and potentially toxic chemicals for the assay and the lysis of cells Visualized only in dead cells 	<ul style="list-style-type: none"> No single cell analysis; Assay reagents required