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Gene therapy of benign gynecological diseases[☆]

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Abstract

Gene therapy is the introduction of genetic material into patient's cells to achieve therapeutic benefit. Advances in molecular biology techniques and better understanding of disease pathogenesis have validated the use of a variety of genes as potential molecular targets for gene therapy based approaches. Gene therapy strategies include: mutation compensation of dysregulated genes; replacement of defective tumor-suppressor genes; inactivation of oncogenes; introduction of suicide genes; immunogenic therapy and antiangiogenesis based approaches. Preclinical studies of gene therapy for various gynecological disorders have not only shown to be feasible, but also showed promising results in diseases such as uterine leiomyomas and endometriosis. In recent years, significant improvement in gene transfer technology has led to the development of targetable vectors, which have fewer side-effects without compromising their efficacy. This review provides an update on developing gene therapy approaches to treat common gynecological diseases such as uterine leiomyoma and endometriosis.

Keywords

Gene therapy; Adenovirus; Uterine leiomyoma; Endometriosis

1. Introduction

1.1. Gene therapy

Gene therapy is defined as the transfer of genetic material (DNA or RNA) into target cells for the purpose of curing or alleviating disease(s) [1]. The first authorized human gene therapy trial was initiated in 1990 for the treatment of an autosomal recessive form of severe

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combined immunodeficiency (ADASCID) [2]. Although limited significant clinical benefit was observed, an avenue of hope was opened especially when the first report of success with gene therapy was reported by Cavazzana-Calvo et al. [3]. This involved the treatment of two infants with the X-linked form of severe combined immunodeficiency (SCID-X1) caused by mutations in the common gamma chain (γ c) gene. This landmark success was subsequently overshadowed by the occurrence of acute leukemia as a complication of gene therapy in two of 14 infants treated. During the past decade, there has been a growing appreciation of the broader scope for gene therapy.

Achieving therapeutic benefit requires the genetic repair of large numbers of cells and, depending on the nature of the target disease, durable long-term expression of the gene(s) of interest. For the vast majority of potentially amenable diseases, existing gene delivery technologies are simply not efficient enough to repair sufficient numbers of cells to confer optimal and permanent therapeutic benefit. Recently, tremendous efforts have been focusing on constructing ideal vectors and selecting the suitable disease that can be targeted by gene therapy approach to overcome these drawbacks [4]. In this regard uterine fibroids represent a very good candidate for gene therapy because of many inherent characters [4].

1.2. Gene delivery methods

Gene delivery technologies can be broadly divided into those that exploit relatively simple non-viral strategies and those that exploit favorable aspects of virus biology. Examples of the former include microinjection, calcium phosphate co-precipitation, electroporation, microparticle bombardment and liposome technology. The basic drawbacks of these approaches are a very low efficiency of gene delivery, and the absence of specific mechanisms to secure a favorable intracellular fate for the introduced DNA.

Although tremendous advances have been made in gene transfer technology, an ideal vector system has not yet been constructed [4]. The ideal vector for gene delivery would fulfill the following criteria: (i) the vector should be easy to produce at a higher titer to allow the delivery of a small volume and should be amenable to commercial production and processing; (ii) the vector should be able to express the transgene over a sustained period and the expression should be precisely regulated; (iii) immunologically inert, and therefore allow for repeated administration; (iv) specificity for the targeted cells; (v) the vector should have no size limit to the genetic materials it can deliver; (vi) the vector should allow for site-specific integration into chromosome of target cells or should reside in the nucleus as an episome and (vii) the vector should have the ability to transfect both dividing and non-dividing cells [4–6]. The fulfillment of these criteria represents a challenge.

1.2.1. Non-viral vector—A naked DNA injection, without any carrier, into local tissues or into the systemic circulation is the simplest and safest physical/mechanical approach for gene therapy [7]. The use of this approach is limited at present, by its comparatively low efficiency. Among the physical methods commonly used for gene delivery are microinjection, particle bombardment (gene gun) and electroporation.

- *Microinjection*: Microinjection is the simplest gene delivery method. Microinjection of naked DNA directly into the nucleus was shown to bypass

cytoplasmic degradation, resulting in a much higher level of gene expression than intracytoplasmic injection [8].

- *Gene gun*: The concept of gene-mediated particle bombardment, gene gun, is to move naked DNA plasmid into target cells on an accelerated particle carrier with a device called the gene gun [7]. Because of their small size, the DNA-loaded particles penetrate the cell membrane and carry the bound DNA into the cell. The DNA dissociates from and is expressed intra-cellularly [9,10]. The major application of this technology is genetic immunization, with the most obvious target being the skin.
- *Electroporation*: Electroporation designates the use of electric pulses to transiently permeabilize the cell membrane and produces an electrophoretic effect on DNA, leading the polyanionic molecule to move towards or across the destabilized membrane [11].
- *Synthetic vectors*: Synthetic vectors provide opportunities for improved safety, greater flexibility and more facile manufacturing [12]. In general, synthetic vectors (cationic lipids and polymers), complex with DNA, condense the genetic material into small particles. Such complexes of plasmid DNA with cationic lipids and polymers are known, respectively, as lioplexes and polyplexes. These small particles mediate their cellular entry and protect the delivered genes by sterically blocking the access of nucleolytic enzymes [13]. Technically, these vectors are relatively straightforward and easily scaled up and produced in large amounts [14].

1.2.2. Viral vectors—The basic concept of viral vectors is to utilize the innate ability of the virus to deliver genetic material into the infected cells [4]. Viral vectors are derived from viruses with either RNA or DNA genomes and are represented as both integrating and non-integrating vectors. The viral vectors include retrovirus, adenovirus, adeno associated virus (AAV), lentivirus and herpes simplex virus (HSV). They are different in their immunogenicity, vector tropism, maximum size of gene that can be packaged and integration into the host genome leading to persistent transgene expression in dividing cells [4].

1.2.2.1. Adenovirus vectors

1.2.2.1.1. Adenovirus vector structure and pathway of infection: Adenoviruses are DNA viruses. They contain about 36 kb of double-stranded DNA. Adenoviral infection is mediated by binding of the knob region, located at the carboxy terminus of the fiber, to its corresponding receptor, which is the Coxsackie-Adenovirus Receptor (CAR) for most serotypes [15]. This binding is followed by interaction between cellular integrins and an arginine–glycine–aspartic acid motif (RGD-motif) located at the adenovirus penton base. This binding leads to the formation of endosomes and viral internalization. Following this receptor-mediated endocytosis, the virus escapes from the endosome and the adenoviral DNA is transported into the nucleus. Then the virus docks to the nuclear core complex receptor and genes from the early region 1 (E1a and E1b) are quickly transcribed (Fig. 1) or in case of non replicating Ads, transgene expression begins [16].

The natural tropism of intravascular Ad results in accumulation mainly in the liver, spleen, heart, lung and kidneys [17–20]. Tissue macrophages, such as Kupffer cells of the liver, have a major role in clearing Ad from blood.

At least three regions of the viral genome can accept insertions or substitutions of DNA to generate a helper independent virus: a region in E1, a region in E3, and a short region between E4 and the end of the genome [21]. In the fifth-generation vectors (Ad5), the E1, E3 regions are removed to make room for the therapeutic transgene and to prohibit viral replication [22] (Fig. 2).

1.2.2.1.2. Adenovirus vectors: merits and demerits: Replication-incompetent adenoviral vectors (Ad) have emerged as very popular and safe vectors for human gene therapy and are the most commonly used vehicle in clinical trials [23]. The use of Ad vectors in gene therapy is advantageous because of many positive attributes including the following: (1) Ad has the ability to provide efficient in vivo gene transfer to both dividing and non-dividing cells [24]; (2) Ad has exhibited higher in vivo stability; (3) Ad stocks can be prepared to ultra-pure high concentrations (10^{13} particles/ml), which allow the delivery of large amounts of viral particles in finite volumes; (4) Ad is nononcogenic and stays episomic, which makes it relatively safe [25]; (5) Ad can accommodate larger transgenes of up to 7.5 kb [22]; (6) Ad supports high levels of gene targeting to the nucleus, which results in significant gene expression [23]; (7) Ad tropism can be manipulated permitting cell-specific transfection; targeting concept which aims at specific transduction of the target cells and hence decreases the tropism of Ad towards the normal cells and enhances the ability of the virus to infect tumor cells [26].

However, the gene delivery efficiency of human serotype 5 recombinant adenoviruses (Ad5) in tumor gene therapy clinical trials to date face some disadvantages, mainly due to the development of Ad-neutralizing antibodies within the human population limiting the gene transfer to target cells [27], short life span of gene expression due to local immune response against Ad5 [28], the inability of Ad5-based vectors to transduce important therapeutic target cell types due to paucity of the primary Coxsackie/Ad receptor (CAR) in many human tumors [29,30] as well as virus dissemination to normal tissues [28,30,31].

1.2.2.1.3. Adenovirus serotype 5 vectors modifications: It is well-established that Ad5-luc is CAR-dependent for cell entry [32]. Conversely, CAR is scarce in tumor tissues [31,33] or, at best, is relatively lacking on target cells in comparison with normal cells [34]. So Ad5 targeting to tumors could be useful to enhance the Ad5 tumor transfection as well as to limit the non target tissue transfection. Three principal means for achieving this goal exist: a) transductional targeting, (b) transcriptional targeting and (c) oncolytic virus principle.

- i. Transductional targeting Transductional targeting aims at deletion of the broad tropism of Ad5 toward normal epithelial cells and/or enhances virus infectivity of CAR deficient tumor cells [35]. One such modification is the insertion of short peptide (21 amino acid) composed of arginine, glycine, and aspartate (RGD) to the H1 loop of the wild Ad5 fiber knob domain to reroute Ad5 binding to the cellular integrin [36,37]]. Another strategy is to employ knob switching by creating

chimeric fibers possessing the knob domains of alternate human Ad serotypes beside the Ad5 native fiber, Ad5/3 and Ad5-sigma, which are created by substituting one Ad5 fiber with adenovirus serotype 3 (Ad3) or reovirus fiber, respectively. Functionally, Ad5-sigma utilizes CAR, sialic acid, and the junctional adhesion molecule 1 (JAM1) for cell entry [38], while Ad5/3 is redirected to bind the putative Ad3 receptors (CD80, CD86, or CD46) [32]. Recently, more radical modifications based on xenotype knob switching with nonhuman adenovirus have been exploited, e.g., Ad5-CAV2 in which the Ad5 fiber knob is switched to that of the canine Ad serotype 2 [39].

- ii. **Transcriptional targeting** In transcriptional targeting, the expression of the gene of interest is placed under the control of a tumor-specific promoter (TSP) that maintains a tumor-on status, a normal tissue-off status, especially liver off status [40,41] in order to achieve a higher level of tumor cell selectivity.

- Tumor-specific promoters (TSP)

A promoter is the component of the genetic transcriptional unit that is involved in binding of the RNA polymerase, required for initiation of mRNA transcription [42,43]. Further, the promoter is activated by transcription factors presented under tissue-specific control [43]. Therefore, in order for a promoter to be activated in a particular tissue type, that tissue must express specific factors that recognize the promoter. Although a number of TSPs have been studied for cancer gene therapy, recent research has focused rigorously on evaluating candidate TSPs with regard to their specificity and selectivity attributes for transcriptional targeting of viruses [43–45].

- iii. **Oncolytic adenovirus principle** Oncolytic or conditionally replicating adenovirus (CRAD) concept is another improvement in Ad based gene and viral therapy approaches. In these vectors, the viral genome and subsequent replication is modified in such a way that it will only be complemented by the tumor cell and lead to virus replication, oncolysis, and subsequent release of the virus progeny [46,47]. Normal tissue is spared due to lack of replication [48]. This replication cycle is important because it allows dramatic local amplification of the input dose, and, in theory, a CRAd could replicate until all tumor cells are lysed [49]. Several adenovirus examples have been developed for diseases such as cervical and prostate cancer [46,47]. The oncolytic virus implies two principles:

- Deletion-based conditionally replicative adenovirus (type I CRADs)

Two approaches are utilized in type I CRADs. In the first approach, two mutations are introduced in the viral gene coding for the E1B protein, a region responsible for binding and inactivation of wild type p53 in infected cells leading to induction of S-phase-required for viral replication [50,51]. In the second approach, a 24 bp deletion was introduced in the constant region 2 (CR2) of the E1A gene [52]. This domain of the E1A protein is responsible for binding the retinoblastoma tumor-suppressor (Rb)/cell cycle regulator protein, thereby allowing the Ad to induce S phase entry [53]. As a result, CRADs with the either 24 E1A or E1B deletions have

reduced ability to overcome G1-S checkpoint in normal cells and therefore cannot replicate. Conversely, both Rb and p53 pathways are defective in most tumor cells, and hence viruses can replicate [54,47].

- Promoter-based conditionally replicative adenovirus (Type II CRAd)

In type II CRAd, a TSP such as mesothelin promoter (MSLN), replaces endogenous viral promoter to control early translated genes (E1A) of the virus [49]. This restricts viral replication to target tissues, actively expressing the transcription factors that stimulate this TSP.

1.3. Gene therapy strategies

These treatment strategies include mutation compensation, molecular chemotherapy, immunopotentialiation and alteration of drug resistance.

1.3.1. Mutation compensation—Mutation compensation involves correction of the genetic lesions that are etiologic for neoplastic transformation [55]. This gene therapy strategy is also known as correctional gene therapy and focuses on functional ablation of expression, dysregulated oncogenes, replacement or augmentation of the expression of tumor-suppressor genes or interferences with signaling pathways of some growth factors or other biochemical processes that contribute to the initiation or the progression of the tumor [56–58]. The purest examples of this gene therapy strategy are the restoration of normal function of tumor-suppressor genes (P53) and interference with over expressed wild type estrogen receptor (ER) using dominant negative ER (DNER) [59].

1.3.2. Molecular chemotherapy—Molecular therapy, which is known also as cytoreductive gene therapy or suicide gene therapy, refers to the delivery of genes that kill cells by direct or indirect effects. These strategies include gene-directed enzyme prodrug therapy, pro-apoptotic, anti-angiogenic and gene-directed radioisotopic therapy [60–63]. Gene-directed enzyme prodrug therapy involves delivering a gene that encodes an enzyme, which then converts an innocuous prodrug to a toxic agent within tumor cells. The two most widely used gene-directed enzyme prodrug systems are Herpes simplex virus thymidine kinase (HSVTK) plus ganciclovir (GCV) and *Escherichia coli* bacterial cytosine deaminase plus 5-fluorocytosine [64].

1.3.3. Immunopotentialiation—Modulation of immune response is particularly attractive as a key focus of tumor gene therapy. It involves the enhancement of the immune system's ability to destroy tumor cells. The immunopotentialiation gene therapy capitalizes on strategies such as the expression of cytokine genes which may enhance the activity of antigen-presenting cells and T cells [65], the expression of co-stimulatory molecules, such as B7.1 and B7.2, which facilitate the recognition and killing of tumor cells [66,67] or the delivery of exogenous immunogens, which generate local inflammatory reaction that increases the ability of antigen-presenting cells to recognize tumor-associated antigens [68].

1.3.4. Alteration of drug resistance—A major hurdle in cancer chemotherapy is anti-cancer drug-related toxicity, mainly the myelosuppression [69]. Multidrug resistance gene

(MDR1) confers multidrug resistance towards several anti-cancer drugs [70]. Gene therapy approaches involving retroviral transfer of MDR1 gene, aldehyde dehydrogenase class 1 gene (*ALDH-1*) and mutated dihydrofolate reductase gene to haematopoietic progenitor cells protects against the myelosuppression induced by these drugs [70–72].

1.3.5. Oncolytic virus concept—Oncolytic viruses such as conditionally replicating adenoviruses (CRAds), take the advantage of tumor-specific changes, which allow preferential replication in and subsequent death of tumor cells [46,47].

1.4. Genes targeted in gene therapy of benign gynecological diseases

1.4.1. Dominant negative estrogen receptor (DNER) concept—Estrogen receptor (ER) consists of six domains (A–F), which include at least two activation functions (present in the A/B and E domains), a DNA binding region (domain C), ligand binding and dimerization regions present in domain E [73,74] (Fig. 3). Previous biochemical studies have identified particular amino acids and regions of the receptor that are critically involved in some of these functions [75–77].

Several dominant negative ER mutants have been generated [78–80], including truncated receptors (ER1-530 and ER1-536, missing the last 65 or 59 amino acid residues), a point mutant (L540Q), and a frameshift mutant (S554fs). These mutants are altered such that the ligand-dependent transcription activation region in domain E of the ER is inactivated (S554fs and L540Q mutants) or missing (ER1-530 mutant) [78]. However, the three mutants bind estradiol with good affinity [75,78]. These dominant negative receptors were shown to effectively suppress the ability of wild type ER to activate transcription when they are either co-expressed with wild type ER in cells or introduced into cells containing endogenous ER [78,79].

Dominant negative forms of the ER have been suggested as a method to inactivate the wild type ER [81] demonstrated that adenovirus-directed expression of the frame-shifted ER (S554fs) suppressed the proliferation of ER-positive breast cancer cells. Lee et al. [82] reported that adenovirus-mediated expression of a truncated receptor (ER1-536) induced apoptosis in rat pituitary prolactinoma cells and inhibited tumor growth in nude mice. This ER mutant exerts its growth-inhibiting effect by making inactive heterodimers with wild type ER α (the dominant form of ER in leiomyoma tissue) as well as ER β . These aberrant heterodimers are either unable to bind to the estrogen-responsive elements (ERE) in different growth-related genes or unable to activate transcription when bound to ERE [78].

1.4.2. Herpes simplex virus 1 thymidine kinase gene/Ganciclovir approach—

The Herpes simplex virus thymidine kinase (HSV1TK) suicide gene therapy system involves the targeted transfection of cells with the HSV1-TK gene, followed by treatment with the prodrug Ganciclovir (GCV) to induce tumor cell death (Fig. 4).

GCV, a nontoxic guanosine analogue, is specifically metabolized into the toxic triphosphorylated form (GCVTP) in presence of herpes simplex thymidine kinase [83]. Incorporation of this toxic metabolite into polymerizing DNA results in nascent chain termination by inhibiting DNA polymerase, and single-strand breaks [83,84], thereby

inhibiting DNA synthesis and blocking the cell cycle, ultimately leading to cell death via apoptosis [84,85].

Fortunately intratumoural delivery of HSV1TK gene followed by GCV administration results in targeted killing of both HSV1TK-positive cells and neighboring negative cells (by stander effect) [61,86,87]. The by stander phenomenon has been observed both in vitro [88] and in vivo [89]. The effectiveness of cytotoxic gene therapy depends greatly on the presence of a “bystander effect” because currently, it is impossible to achieve 100% gene transfer in vivo.

1.5. Benign gynecological diseases

1.5.1. Uterine leiomyoma and gene therapy—Uterine leiomyomas are the most common gynecological tumors affecting more than 25% of reproductive-age women [91]. Symptomatic fibroids are associated with a host of clinical problems including menorrhagia, pelvic pressure, pelvic pain, spontaneous abortions, and infertility [92]. As a consequence of these complications, uterine fibroids rank as the major reason for hysterectomy in the United States, accounting for approximately 67% of all hysterectomies performed among middle-aged women [93,94], or one-third of all total hysterectomies performed annually in the United States [91].

Unfortunately, there is no optimal treatment option for uterine leiomyoma and the gold standard treatment is surgery [95,96]. This surgical option is extremely costly, carries a risk of major complications and constitutes a clinical dilemma for patients desire to preserve their fertility [97,4]. Thus, the development of novel therapeutic strategies would be a welcome addition for uterine leiomyoma; especially, if it is conservative, relatively safe localized method of treatment which is able to ablate the uterine fibroid without interfering with ovulation, local blood supply to uterus and systemic sex hormonal milieus and hence the fertility [4,95,98–100].

Uterine leiomyoma is an attractive target for gene therapy because of several inherent biologic features. The disease is localized and well-circumscribed in the uterus which simplifies targeting the treatment to the tumor by direct ultrasound-guided intratumor injection using existing technology [4]. Another favorable feature is that uterine fibroids are slow-growing tumors and marked clinical improvement of fibroid-related symptoms (e.g., irregular vaginal bleeding, pelvic pain, and infertility) and patient satisfaction does not necessitate complete resolution of the fibroid but rather a modest decrease in their size [101,102].

The first reported attempt to develop a gene therapy approach for the treatment of uterine fibroids was developed by Niu et al. [103] as a non-viral mediated transfer of the suicide gene for thymidine kinase into human leiomyoma cells. They demonstrated significant cell death when only 5% of the leiomyoma cells transfected. Unfortunately, this study was limited to in vitro study using human leiomyoma cell cultures.

Our earlier laboratory studies demonstrated the optimal ability of adenovirus to transfect both human and Eker rat derived uterine leiomyoma cells (ELT3) at 10 PFU/cells and 100

PFU/cell respectively [104] and thus pointed out the potential use of adenovirus (Ad)-mediated gene therapy for uterine leiomyoma (Fig. 5).

Applying mutation compensation gene therapy strategy via delivery of DNER by adenovirus vehicle to perturb the estrogen-signaling pathway was very encouraging [104–106]. In an in vitro animal model, Ad5-DNER induced significant death in ELT3 cells. In addition, Ad-DNER induced an increase in both caspase-3 levels and the Bax/Bcl-2 ratio, with evident apoptosis in the TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assay [104]. In nude mice, rat leiomyoma cells transduced *ex vivo* with Ad-DNER supported significantly smaller tumors compared with Ad-LacZ treated cells 5 weeks after implantation. In mice treated by direct intratumor injection into pre-existing lesions, Ad-DNER caused immediate overall arrest of tumor growth [104] (Fig. 6). Furthermore, our subsequent in vitro work using both primary and immortalized human LM cells has also demonstrated that such treatment intercepted with the wild type estrogen receptor functions (Fig. 7) and modulated the expression of several genes implicated in leiomyoma growth, apoptosis, estrogen metabolism and extracellular matrix formation in cultured human leiomyoma cells [105].

Also we demonstrated that Ad-DNER was able to markedly shrink Eker rat uterine leiomyoma volume. The shrinkage reached a peak of 80% and was sustained for 30 days after a single Ad-DNER inoculation (Fig. 8). Furthermore, Ad-DNER modulated the expression of several estrogen regulated genes controlling apoptosis (Fig. 9), proliferation and extracellular matrix formation [106]. This provided potential mechanisms of action of this approach and explains the rapid and impressive tumor shrinkage. Noteworthy, locally injected Ad in these approaches was minimally disseminated, only into liver and uterus of injected animals. Also this treatment approach was safe and did not induce any macroscopic or microscopic tissue damage nor caused any significant changes in serum liver function tests over the follow up period (one month) (Fig. 10).

In a similar way suicidal gene therapy strategy has been tested in the context of uterine leiomyoma with impressive results [69,103,107]. Ad-HSV1TK/GCV effectively inhibited the growth of both ELT-3 cells and the primary human LM cells with impressive strong bystander effect [69]. This finding confirmed previous report by Niu et al. [103], using plasmid vector with the advantage of high probability of in vivo tumor transfection efficiency.

In Eker rat, an immunocompetent animal model of uterine leiomyoma, we demonstrated that the Ad-HSV1TK/GCV approach was very efficient in decreasing uterine leiomyoma volume whereas Ad-HSV1TK/GCV treatment significantly decreased uterine fibroid volume by 75 ± 16 , 58.7 ± 6.3 , and 67.5 ± 27.5 of pretreatment volume at days 10, 20, and 30, respectively. (Fig. 11). These effects were mediated via increased caspase 3 activity, Bax expression, TUNEL apoptosis marker; and decreased cyclin D1, PCNA, Bcl2, and PARP protein expressions [107]. Additionally, Ad-transfection was tumor-localized and safe to non target tissues which confirmed the safety. Furthermore, Ad-transfection induced local CD4+ and CD8+ infiltration which add back to the success of this approach.

To optimize gene therapy of leiomyoma, the expression of the transgene should be restricted exclusively to leiomyoma. Selective expression of transgenes in leiomyoma can be achieved by transductional and/or transcriptional targeting. Fortunately, the localized nature of leiomyoma helps to identify the target easily for the clinical introduction of gene therapy vectors and hence avoid inadvertent transfection to the surrounding normal uterine myometrium (transductional targeting). For transcriptional targeting, however, the biological uniqueness of leiomyoma (such as leiomyomas-specific transcription factors or proteins) should be identified to design leiomyoma-specific promoter/vectors which would drive therapeutic gene expression only in leiomyoma tumor cells. Generating improved delivery systems and refining the mechanisms that control gene expression will be a key step in the development of safe clinical protocols for gene therapy of uterine leiomyoma. Thus we have presented the utility of several modified Ad vectors in the human leiomyoma cell line. Our goal was to identify the best Ad vector to enable the targeting of therapeutic genes to human leiomyoma cells with minimal effect on normal myometrial cells, as well as normal liver cells [108]. Our results demonstrated that Ad5-RGD-luc and Ad5-CAV2-luc, Ad5-SLPI-luc and Ad5-MSLN-CRAD-luc at 5, 10 and 50 PFU/cell, showed significantly higher expression levels of luciferase activity in both primary and immortalized human leiomyoma cells when compared with Ad5-Luc (Fig. 12). Additionally, these modified viruses demonstrated selectivity toward leiomyoma cells, compared with myometrial cells. In addition, these targeted modified Ad vectors exhibited lower liver cell transduction, compared with Ad5-luc, at the same dose levels [108].

Construction of these candidate modified (improved) adenoviral vectors with promising therapeutic gene such as DNER or HSV1TK then testing these modalities in Eker rat, an immune competent animal model resembling the human situation, for long period of time might lead to a human clinical trial. This approach is not far from becoming a medical reality. By the end of the year 2005, worldwide, over 600 clinical trials using gene therapy have been conducted [109], and by the end of July 2007, worldwide, over 1300 clinical trials were done [110]. Most of these clinical trials were phase I and or phase II, which means their primary goal is determining the safety of the agents (phase I) or their potential efficacy (phase II) [111–114]. The fields of gynecology and gynecologic oncology now exist at the forefront of evolving novel gene therapy approaches to both benign and malignant disease [115,116]. However, gynecologic gene therapy has advanced to human clinical trials for ovarian carcinoma [115,116]. A phase III human clinical trial has begun in patients with stage III epithelial ovarian cancer [117].

1.5.2. Endometriosis and gene therapy—Endometriosis is a chronic and recurrent disease characterized by the presence of endometrial tissue (glands and stroma) outside the uterine cavity [118]. It affects 5% to 10% of reproductive-age women [119]. Endometriosis associated pelvic pain and subfertility impose a substantial burden on the quality of life of affected women. It also represents a significant financial burden in terms of the amount of money that has to be spent on expensive therapies [120]. The annual healthcare costs and costs of productivity loss associated with endometriosis at \$2801 and \$1023 per patient, respectively [121].

Endometriosis is an estrogen dependent disease and current medical therapy aims at suppressing ovulation with reduction in circulating estrogen to a postmenopausal level [122]. Such treatment is limited to short duration because of substantial hypoestrogenic effects including: hot flushes, dry vagina and loss of bone mineral density. Moreover, recurrence of pelvic pain occurs in 30–70% of patients within 6–18 months after discontinuation of treatment [123] and there is a 24% probability of recurrence of pelvic pain within 3 years after conservative surgery [99]. In addition, medical treatment has no beneficial effects on the treatment of endometriosis associated subfertility [124].

Seemingly, we are still far from having an ideal treatment for endometriosis. New therapies that act on endometriosis cells at a molecular level rather than to suppress ovarian estrogen production are still required for the therapeutic armamentarium against this disease [125].

Gene therapy is an emerging form of molecular medicine that has expanded in scope from malignant to benign diseases [104]. Gene therapy involves the introduction of genetic material to the cell, whose subsequent products will address the therapeutic outcome [115]. In the new millennium, endometriosis started to find its way to gene therapy applications.

In 2002, Dabrosin et al. [126] reported on gene therapy of endometriosis in a mouse model. Those investigators transplanted mouse endometrial tissues into the peritoneal cavity of estrogen-supplemented ovariectomized mice to develop endometriosis lesions. One week after transplantation, the first and second groups of mice were treated with intraperitoneal injection of 1 ml of PBS containing 1×10^9 PFU of adenovirus expressing murine angiostatin gene (Adangiostatin), which is a natural angiogenesis inhibitor or a control adenovirus, respectively. A third and fourth groups consisted of normally cycling mice, which were treated with intraperitoneal Adangiostatin or control virus, respectively. Mice were sacrificed on a subsequent period from 2 to 18 days post-treatment. In the first two groups, peritoneum and visceral organs were examined with a dissecting microscope, endometriosis lesions detected and removed for H&E staining and immunohistochemistry. Blood was collected for hormonal assays and peritoneal fluid obtained for angiostatin protein expression. Animals were weighted as were their uteri and ovaries.

The results have shown that endometriosis lesions were eradicated in 100% of the Ad-angiostatin treated mice within two weeks in comparison to only 15% of control virus-treated mice in the same time period ($P < 0.0001$). Immunohistochemistry for Factor VIII demonstrated that on day 5 of Ad-Angiostatin treatment, not only the bulk of the experimental endometriosis tissue had decreased, but the content of the vasculature was markedly decreased both in extent and in numbers of vessels as compared to day 0. Western Blot analysis of peritoneal lavage showed bands corresponding to murine angiostatin protein in Ad-angiostatin treated but not in control virus-treated mice. In normal cycling mice, Ad-angiostatin treatment resulted in significant decrease in ovarian steroid production, reduction in ovarian size and weight with disappearance of follicles and corpora lutea, decrease in uterine weight and increase in body weight similar to that of ovariectomized mice.

Dabrosin et al. [126], concluded that inhibition of endometriosis associated angiogenesis through transient over expression of angiostatin gene delivered to the peritoneal cavity via

adenovirus vector is an effective method for eradication of endometriosis lesions. The authors recommended that local or targeted delivery of the vector be considered to avoid untoward effects on normal ovarian and uterine functions.

Dominant negative mutants of estrogen receptors (DNER) are altered estrogen receptor forms that are unable to activate transcription of estrogen-responsive genes when estradiol binds to them. They have the additional property of being able to suppress the transcriptional activity mediated by the wild type estrogen receptor when both are co-expressed in the same cells [79, 127]. Our group studied the effects of transfer of dominant negative estrogen receptor genes to endometriosis cells via adenovirus vector in vitro [59].

We first tested the ability of adenovirus to transfect endometriosis cells in vitro. For this purpose, we used RT-PCR to measure mRNA expression of Coxsackievirus-Adenovirus Receptor (CAR), which is the main portal of adenovirus entry into cells, in primary endometriosis cells versus normal endometrial cells. Then, both cell types were transfected with adenovirus expressing β -galactosidase (Lac-Z) as a marker gene at different multiplicities of infection (MOIs) to determine transduction efficiency of adenovirus in endometriosis versus endometrial cells.

The results of this part of the study showed that CAR mRNA expression is significantly higher in endometriosis versus normal endometrial cells. Transduction of endometriosis and normal endometrial cells by adenovirus expressing marker gene confirmed these findings where adenovirus achieved a transduction efficiency of 80% in endometriosis cells versus 28% in normal endometrial cells at MOI of 50 PFU/cell ($P<0.05$).

The next step was to test the effects of transfection of endometriosis cells by adenovirus expressing dominant negative estrogen receptors (Ad-DNER). For this purpose, we used three groups of endometriosis cells: the first was treated with Ad-DNER, the second group was treated with a control virus and the third group was treated with medium alone. Cells were examined for cellular proliferation using a fluorometric assay implementing Hoechst 33258, cytokine production using Bioplex protein array system and cellular apoptosis using deoxynucleotidyl transferase (TdT)-mediated (dUTP) nick-end labeling (TUNEL) assay.

Our findings have shown that Ad-DNER-treated endometriotic cells, as compared with control virus-treated cells, showed cell rounding and detachment (cell death), a 72% reduction in the number of viable cells 5 days after transduction, significantly less production of monocyte chemotactic protein-1 (MCP-1), vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) and a significantly higher percentage of apoptotic cells.

The authors concluded that adenovirus can efficiently transfect endometriosis cells in vitro. Dominant negative estrogen receptor genes, when transferred to endometriosis cells, can result in cellular death, reduce cellular proliferation, cytokine production and induce cellular apoptosis [59]. This new molecular based therapy allows functional stripping of estrogenic drive to endometriosis cells without suppression of ovarian steroid production thus preventing hypoestrogenic effects. Also, the ability of Ad-DNER treatment to reduce proinflammatory and angiogenic cytokine production by endometriosis cells conveys dual

ablative effect on endometriosis via apoptosis and reduction in cytokine secretion which is essential for the pathogenesis of endometriosis [128].

Both our study and that of Dabrosin et al. utilized adenovirus serotype-5 (Ad5) as a viral vector for gene transfer. Ad5 as a gene transfer vector is easy to propagate in laboratories, has mild pathogenicity and low mutagenesis potential in humans. Also, Ad5 can transduce dividing and non-dividing cells. However, this wide viral tropism means that both target and non target cells will be transfected. This will lead to sequestration of the virus in non target tissues while limiting the amount of virus available for target tissues. Moreover, uptake of the viral vectors and their transgene by non target tissues increases toxicity to these tissues and increases the immune response against adenoviruses if they are taken by immune cells [25].

Transductional targeting involves selective gene transfer by the vector to particular tissue or cells of interest. This is achieved through inducing structural changes in adenovirus fiber to allow it to recognize receptors selectively expressed on pathological tissues only [44]. Transcriptional targeting depends on non selective transfer of the gene to different tissues; however, gene expression will be possible only in pathological tissues through driving the gene expression under tissue-specific promoters that are “on” in pathological tissues but “off” in normal tissues [129]. In a recent publication by our group, we screened a number of transductionally targeted and transcriptionally targeted adenoviruses to detect the ones that can achieve highest gene transfer and expression in endometriosis cells and lowest expression in normal tissues and cells [130]. The targeted adenoviruses used in this study are shown in Table 1.

Targeted adenoviruses were used to transfect primary human endometriosis cells as target cells or human liver tissue slices taken from seronegative donors before transplantation into recipients (Fig. 13). All adenoviruses used were replication deficient viruses with a luciferase reporter gene in the E₁ region under the control of corresponding promoters. Gene transfer and expression activity of each virus was reflected by its luciferase transactivation level.

The results have shown that among transductionally targeted adenoviruses, Ad5-RGD-luc achieved the highest reporter gene transfer efficiency (Fig. 14). Among transcriptionally targeted adeno-viruses, Ad-SLPI-luc and Ad-heparanase-luc achieved significantly higher reporter gene expression than Ad5-luc or the wild type (Fig. 15). In the liver tissues, Ad-survivin-luc and Ad-heparanase-luc achieved significantly lower reporter gene expression levels as compared to the Ad5-luc (Fig. 16).

We concluded from this study that Ad-heparanase-luc has high gene expression level in endometriosis cells but minimal levels in liver tissues so it achieves an “endometriosis on, liver off” phenotype and can work as an excellent vector for gene therapy of endometriosis.

2. Conclusions and future directions

Current nonsurgical management options for uterine leiomyoma and endometriosis are limited. Gene-based therapeutic approaches may represent future viable alternatives,

especially for women who want to preserve their fertility. Further understanding and uncovering of the genetic and environmental mechanisms responsible for uterine leiomyoma and endometriosis will aid in designing more targeted and specific gene therapy protocols. Additional research is needed to improve our understanding of the genetic and cellular mechanisms of these common gynecologic disorders. Dominant negative ER (delivered via an adenovirus) as well as Ad-HSV-tk/GCV provided two promising approaches to induce apoptosis and eventually shrink leiomyoma, and these approaches could conceivably constitute the basis for future clinical trials. Several other biological pathways in leiomyoma represent potential targets for gene therapy application. For instance, several lines of evidence have suggested that the apoptotic mechanisms are downregulated in leiomyoma cells compared with normal myometrium [136]. Thus, gene therapy of leiomyoma can be executed by switching on the apoptosis mechanism through both extrinsic and intrinsic pathways (by delivering apoptosis-inducing ligands, such as TNF, TRAIL, and FasL; or by delivering pro-apoptotic members of the Bcl-2 family, such as Bax or active caspase molecules). In endometriosis, in addition to estrogen dependency, angiogenesis, which is essential for endometriosis growth, is driven by different growth factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and platelet-derived endothelial growth factor (PDGF)[137–140]. Each of these angiogenic factors represents a potential gene therapy target.

To optimize gene therapy of leiomyoma and endometriosis, the expression of the transgene should be restricted exclusively to target tissues. Selective expression of transgenes in the pathological tissues can be achieved by transductional and/or transcriptional targeting. For transcriptional targeting, however, the biological uniqueness of leiomyoma or endometriosis (such as endometriosis-specific transcription factors or proteins) should be identified. This identification will allow the design of disease-specific promoters in vectors driving therapeutic gene expression only in the abnormal cells.

Generating improved delivery systems and refining the mechanisms that control gene expression will be a key step in the development of safe clinical protocols of gene therapy of benign gynecologic disorders.

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Fig. 1. Schematic diagram depicts a) Adenovirus virion b) Adenovirus infection pathway [16].

Fig. 2.
Structure of Adenovirus DNA [22].

Fig. 3.
Schematic diagram of the wild type and mutant estrogen receptors [117].

Fig. 4. HSV1TK/GCV By standard effect: A; normal cells with low GIIJ protein level B; tumor cells with high GIIJ protein level [90].

Fig. 5. Adenovirus-infected human leiomyoma cells. LM-15 cells were optimally transfected with Ad-LacZ at a high multiplicity of infection (MOI) of 10 PFU/cell. At a low MOI of 5 PFU/cell, about 50% of cells were successfully transfected [104].

Fig. 6. Direct intratumor injection of adenovirus with dominant negative estrogen receptor (ER) inhibited tumor growth. Direct intratumor injection of the corresponding agent was performed on day 16 after cell implantation. (A) Ad-ER-DN treatment caused immediate overall arrest of tumor growth. The difference among treatment and control groups was highly significant ($P=0.007$). Results are mean $\pm$ SD ($n=10$) of two independent experiments. (B) Tumors treated with Ad-ER-DN demonstrated inhibition of tumor progression [104].

Fig. 7.

Effect of Ad-DNER on transcriptional activity of endogenous ER of rat uterine leiomyoma cells (ELT3 cell line). The ER transcriptional activity was assayed using Ad-ERE-luc, and the relative light units were normalized to the cell number. Results are plotted as mean $\pm$ SE of three independent experiments. $P < 0.01$ was considered a significant difference from control (a) or from Ad-DNER 1 PFU/cell (b) [105].

Fig. 8. Ad-DNER injection into Eker rat uterine leiomyoma significantly shrinks total leiomyoma volume (compared to both vehicle control and Ad-LacZ treated animals). Tumor volume was calculated as a percentage of its corresponding day zero (pretreatment) volume and presented as $M \pm SE$ of at least three animals at each time point. a,b indicate significant difference from vehicle control and Ad-LacZ treated animals, respectively, at $P < 0.05$ [106].

Fig. 9.

Ad-DNER treatment of fibroid lesions in Eker rats induces modulation of several estrogen regulated genes controlling both (A) intrinsic (p53, BAX, Bcl2 and PARP) and (B) extrinsic (DAXX and fas L) apoptotic pathways. Data represent uterine leiomyoma tissues collected at 8, 15 and 30 days post Ad-DNER treatment compared to either vehicle control or Ad-lacZ treated group: (A) Protein levels assessed by western blot; (B) DAXX and FAS L mRNA expression; (C) demonstrate increased Caspase 3 activity; (D) increase in the percentage of positive TUNNEL cells in the Ad-DNER treated lesions. The letters a or b indicate significant difference from vehicle control or Ad-lac Z treated lesions respectively at $P < 0.05$ using two tailed student *T* test in (C) caspase 3 experiment and Tukey test as post-ANOVA test in (B) RT-PCR and (D) TUNNEL assay experiments [106].

Fig. 10.

Safety studies of the Ad-DNER gene therapy approach after a single direct intra-fibroid treatment of Eker rat. (A) Assessment of adenovirus particles dissemination into Eker rat body organs by PCR amplification of the specific Ad5 E4 region detected by 714pb DNA band on 1% agarose gel. The band was detected mainly in tumor tissues while faint bands were detected also in uterus (lane 11) and liver (Lane 12) respectively, in 40% and 30% of the treated animals. (B) Although Ad-DNER particles were detected in the liver of 40% of Eker rats injected with adenovirus, Ad-DNER did not produce any significant change in liver function tests (AST, ALT and total bilirubin) evaluated 30 days post adenovirus injection, compared to vehicle control animals [106].

Fig. 11.

Adenovirus-mediated delivery of herpes simplex virus 1 thymidine kinase gene (Ad-HSV1TK) (3×10^{10} PFU/cm³ of tumor) by a single direct injection into Eker rat uterine leiomyoma followed by a subcutaneous injection of GCV 50 mg/kg/day for 10 days significantly shrinks Eker rat uterine leiomyoma volume when compared with both Ad-LacZ/GCV and vehicle-treated animal groups. Uterine leiomyoma tumor volume was measured by both MRI scanning and caliper measurement. Tumor volume was calculated as a percentage from day 0. Each time point was represented by mean $\pm$ SE of 3 animals. ^{a,b}Indicate a significant difference from control and Ad-LacZ/GCV-treated groups, respectively, at $P < 0.05$ using the Tukey test as a post-ANOVA test [107].

Fig. 12.

Evaluation of modified adenovirus vectors in human leiomyoma cells. (A) fiber-modified adenoviruses; Ad5-SLPI-luc, Ad5-heparanase-luc, and Ad5-MSLN-CRAD-luc and (B) transcriptional targeted adenoviruses; Ad5-SLPI-luc and Ad5-MSLN-CRAD-luc showed higher luciferase expression levels in both human primary (PLM) and immortalized leiomyoma (huLM) cells at 10 PFU/cell when compared with Ad5-luc at the same dose level, while survivin showed uterine leiomyoma cells off-profile. Luciferase activities (RLU) were normalized to the protein content and expressed as mean±standard error of the mean (SEM) of 4 separate experiments and plotted as percentage of Ad5-luc activity. a; Indicates significant differences compared with corresponding Ad5-luc at 10 PFU/cell ($P<0.05$) [108].

Fig. 13.

Evaluation of modified adenovirus transduction efficiency in normal human liver cell line (THLE3) at 10 PFU/cell. Luciferase activities (RLU) were normalized to the protein content, and expressed as mean±standard error of the mean (SEM) of 4 separate experiments, and plotted as percentage of corresponding Ad5-luc activity. a,c; indicate significant difference from Ad5 ($P<.001$ and $P<.01$, respectively) [108].

Fig. 14. Luciferase transactivation in endometriosis cells mediated by transductionally targeted adenoviruses at MOI of 10 PFU/cell.

Fig. 15. Luciferase transactivation in endometriosis cells mediated by transcriptionally targeted adenoviruses at MOI of 10 PFU/cell.

Fig. 16.

Luciferase transactivation in liver tissues mediated by transductionally and transcriptionally targeted adenoviruses at MOI of 10 PFU/cell.

Table 1

Names, abbreviations and descriptions of targeted adenoviruses [36,131–135].

Targeting strategy	Virus name	Abbreviation	Description	Reference
Wild type (non targeted)	Adenovirus serotype 5	Ad5-CMV-luc	Encodes adenovirus serotype-5 fiber with cytomegalovirus (CMV) promoter driving luciferase (luc) transgene expression	
Transductional targeting	Adenovirus-RGD	Ad5-RGD-Luc	Integrin binding peptide (arginine–glycine–aspartate) is attached to the adenovirus fiber HI loop to expand tropism to integrin expressing cells	[36]
	Adenovirus sigma	Ad-sigma-luc	Encoding the wild type Ad5 fibers and the sigma-1 protein which is a reovirus attachment protein that can infect cells expressing sialic acid or junction adhesion molecule-1 (JAM-1)	[131]
	Adenovirus 5/3	Ad5/3-luc	Ad-5 fiber knob has been replaced by that of adenovirus serotype-3 to redirect binding to the putative Ad-3 receptors CD80, CD86 or CD46.	[132]
Transcriptional targeting	Adenovirus-survivin	Ad-survivin-luc	Transgene expression is derived by surviving promoter	[133]
	Adenovirus-secretory leukocyte protease inhibitor	Ad-SLPI-luc	Transgene expression is derived by SLPI promoter	[134]
	Adenovirus-heparanase	Ad-heparanase luc	Transgene expression is derived by heparanase promoter	[135]