Chapter 1

Ovarian cancer gene therapy strategies


Submitted to Expert Opinion on Drug Delivery
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Abstract

Similar to most human malignancies, ovarian cancer is a result of multiple genetic alterations. Advances in molecular biology, tumor biology, immunology, virology and other branches of science have helped in defining specific genes and molecular pathways altered in ovarian cancer. Therefore correcting these genetic alterations by gene therapy is a rational approach. Ovarian cancer is particularly ideal for a gene therapy approach because of its containment of disease mostly to abdominal cavity and easy access to the peritoneal cavity for the delivery of genes. On this basis, many gene therapy strategies have been endeavored to target these altered genes and pathways for the therapy of ovarian carcinoma. This review provides an overview of existing gene therapy strategies for ovarian carcinoma and attempts to develop strategies for their targeted delivery to ovarian carcinoma to increase the therapeutic index of gene therapy.

1. Introduction

Ovarian cancer is the most lethal cause of death among the gynecological cancers. It is estimated that 22,220 new cases will be diagnosed and 16,210 deaths will occur in the United States in 2005. Despite advances in surgery and chemotherapy, the mortality rates for this disease remains unchanged for the past decade. Furthermore, 75-80% of the patients present with advanced stage disease for which 5-year survival rate is 15% to 30%. Given these dismal survival statistics, there is need for development of novel therapeutic approaches for effective management of this disease.

Similar to most human malignancies, ovarian cancer is a result of multiple genetic alterations. Advances in molecular biology, tumor biology, immunology, virology and other branches of science have helped in defining specific genes and molecular pathways altered in ovarian cancer. Therefore, correcting these genetic alterations by gene therapy is a rational approach. Ovarian cancer is particularly ideal for gene therapy approach because of its containment of disease mostly to abdominal cavity and easy access to the peritoneal cavity for the delivery of genes. On this basis, many gene therapy strategies have been endeavored to target these altered genes and pathways for therapy of ovarian carcinoma. Based on the genes that are targeted, gene therapy strategies can be classified into 1) mutation compensation, 2) molecular chemotherapy, 3) immunopotentiation, 4) anti-angiogenesis, and 5) virotherapy. (Table 1)

2. Mutation Compensation

Genetic lesions involved in the pathogenesis of malignant transformation may be thought of as critical compilation of two general types. Aberrant expression of dominant oncogenes or loss of expression or function of tumor suppressor genes. Mutation compensation strategies are designed to replace the altered tumor suppressor genes or to ablate dominant oncogenes, or to interfere with proper function of a growth factor or its receptor and to manipulate genes controlling cellular apoptosis responsible for conferring malignant phenotype in ovarian cancer cells.

2.1. Tumor suppressor genes

Studies understanding the tumor biology of ovarian cancer have identified various tumor suppressor genes such as p53, Rb, PTEN, p16 and BRCA1. These tumor suppressor genes are responsible for preventing cells from proliferating abnormally. Loss of function of these tumor suppressor genes occurs as a result of numerous mechanisms, such as deletions, partial deletions, mutations, failure of transcription, and inactivation of the gene product.
### Table 1. Genes Targeted/Employed For Ovarian Cancer Gene Therapy

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<th>Mutation compensation</th>
<th>Molecular chemotherapy</th>
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<td><strong>Tumor suppressor genes</strong></td>
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<td>K-ras</td>
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<td><strong>Antiangiogenesis</strong></td>
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<td>Transcriptional repressors</td>
<td>Angiostatin</td>
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<td>E1A</td>
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<td>SV large T antigen</td>
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<td><strong>Virotherapy</strong></td>
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<td>Oncolytic HSV</td>
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#### 2.1.1. p53
Wild-type p53 present in normal cells is critically involved in cell cycle control, DNA repair, and apoptosis (1-5). Loss of its normal function constitutes an important step in the malignant transformation process (reviewed in) (6) and is the most common genetic alteration found in ovarian carcinoma. It is estimated that 50-75% of the advanced stage ovarian carcinoma and 16-44% of early carcinoma harbor
p53 alterations. Therefore, p53 appears to be an appealing target for gene therapy. On this basis, replacement of p53 gene is a logical approach.

Delivery of wild-type p53 gene to p53 deficient ovarian carcinoma cells in vitro abrogated the malignant phenotype (7). Initially it was believed that p53 induced apoptosis only in tumor cells which lack wild type p53 or have mutated p53. Subsequently it was also shown to induce apoptosis and inhibit tumor growth in wild-type p53 ovarian cancer cells as well (8). However, cells with mutated p53 were more sensitive to apoptosis and growth suppression than wild type p53 cells. Possible reasons for this resistance could have been the abrogation of induction of the p53-targets or blockade of p53-dependent apoptosis upstream or at the caspase-9 level (9). Another reason could have been that wild type p53 cells express high levels of MDM2 protein which degrade p53. A recombinant adenovirus expressing a p53 variant, rAd-p53 (d 13-19), that is deleted for the amino acid sequence necessary for MDM2 binding (amino acids 13-19) (10) induced higher levels of apoptosis in p53 wild-type tumor lines compared to wild-type p53 treatment. In addition, this variant p53 protein displayed synergy with chemotherapeutic agents to inhibit proliferation of ovarian cancer cell lines.

2.1.1.1. p53 in vivo pre-clinical studies
Based on positive results of in vitro studies, p53 gene therapy was evaluated in vivo. One study showed that adenoviral-mediated ex vivo gene delivery of the wild type p53 gene in ovarian cancer SK-OV-3 cells, which lack p53, and their subsequent implantation in immunodeficient mice prolonged their survival compared to controls (11). A subsequent study demonstrated that direct intraperitoneal delivery of p53 gene via adenoviral vector in a pre-established intraperitoneal human ovarian cancer xenograft mouse model significantly prolonged the survival of treated mice especially when the tumor burden was low (12). In another study, adenoviral-mediated in vivo p53 gene delivery in mice was well tolerated at doses of 1 x 10^8 pfu and showed significant increase in survival time compared to untreated groups. However, no statistical significant survival advantage was observed between p53 and beta-gal treatment at the dose and administration regimen used (13). Increasing the dose of virus increased the therapeutic efficacy; however, it was associated with hepatotoxicity (14).

An alternate way of increasing therapeutic efficacy without increasing toxicity is efficient delivery of therapeutic genes into tumor cells. Towards this end, p53 gene was genetically fused with VP22 (important for intercellular transport) and delivered to cells by means of adenovirus. This strategy showed efficient translocation of VP22-p53 into tumor cells, resulting in inhibition of tumor cell proliferation and induction of apoptosis (15). This strategy was further enhanced when lentivirus expressing VP22-TK was targeted to MUC1 over expressed in ovarian cancer cells using a single chain antibody (16).

2.11.2. p53 gene therapy strategy for chemoresistant ovarian cancer
p53 mutations are frequently associated with chemoresistance. According to a recent study, non-responders to chemotherapy had mutations of p53 gene more frequently (83% for non responders vs. 16% for responders) in epithelial ovarian carcinoma undergoing platinum based therapy. In this regard, p53 gene delivery markedly enhanced the sensitivity of ovarian cancer cells to cisplatin induced apoptosis (17, 18) and significantly reduced tumor growth and prolonged the survival in a mouse model. Additional therapy with cisplatin further reduced tumor growth and increased survival (30-40%) compared to either chemotherapy or p53 therapy alone (19). Similary, combination with paclitaxel showed synergistic therapeutic effect. Although the mechanism of synergy was not clearly understood, paclitaxel treatment was shown to enhance the transduction of Adp53 in tumor cells in vivo in mouse model (20).

In addition to cisplatin and paclitaxel, a combination of p53 and other chemotherapeutic agents such as doxorubicin, 5-floururacil, methotrexate or etoposide significantly inhibited the cell proliferation more effectively than chemotherapy alone (21). Of particular significance, enhanced efficacy was observed when the three drug combination of Adp53, Cisplatin, and paclitaxel was administered (21). A more recent study reported that Ad p53 gene therapy enhanced chemosensitivity irrespective of endogenous p53 status of tumor cells and showed synergism when combined with cisplatin and paclitaxel (22).
study, p53 gene transfer even at 50% transduction level significantly reduced IC50 of carboplatin chemotherapy up to 49-fold and paclitaxel chemotherapy up to 6 fold and paclitaxel/carboplatin chemotherapy up to 19 fold. These results strongly support the studies evaluating combination of Adp53 gene therapy with standard chemotherapy in human clinical trials.

2.1.1.3. p53 gene therapy strategy for TRAIL resistant ovarian cancer
In addition to sensitizing tumor cells to chemotherapy agents, p53 was also shown to sensitize ovarian cancer cells, which are resistant to the cytotoxic ligand TRAIL therapy. These studies shows that p53 has a sensitizing ability to a variety of tumor cell killing agents (23).

2.1.1.4. Targeting p53 at RNA level
Although most studies showed restoration of p53 activity at DNA level by p53 cDNA gene transfer, a recent study showed the feasibility of restoring p53 activity at RNA level by using intron-based ribozyme that can replace endogenous mutant p53 RNA with a wild-type RNA sequence (24).

2.1.1.5. p53 gene therapy clinical trials
To determine the safety, gene transfer efficiency, host immune response, and pharmacokinetics of a replication-deficient adenovirus encoding human, recombinant, wild-type p53 (SCH 58500) delivered into the peritoneal cavity (i.p.), in phase I/II clinical trial, 43 recurrent ovarian cancer patients were administered either Adp53 (intraperitoneally) alone or in combination with intravenous chemotherapy. This therapy was shown to be well tolerated even at a dosage up to 2.5 x 10^{13} particles/dose only showing known chemotherapy side effects and constitutional side effects due to virus. The ability of Ad to transfer p53 gene into tumor cells, was confirmed by RTPCR of tumor biopsies (25). Another study not only confirmed clinical efficiency of gene p53 transfer by adenoviral vector by RTPCR and in situ PCR in tumor biopsies but also confirmed biological activity of transferred p53 gene by observed up regulation of p21/WAF1, bax, and mdm2 and down regulation of survivin (26). These results suggest the feasibility of this gene therapy approach for inhibition of tumor growth in recurrent ovarian cancer patients.

Another phase I study was designed to determine maximum tolerated dose of intraperitoneally delivered Adp53 (27). Patients with platinum and paclitaxel-resistant metastatic epithelial ovarian cancer were administered Adp53 daily for 5 days every three weeks at one of four dosing levels: 3 x 10(10), 3 x 10(11), 1 x 10(12), or 3 x 10(12) viral particle. 15 out of 17 patients who were evaluated for toxicity showed no dose limiting toxicity, and the most common grade 3 toxic effects have been fatigue (6 patients) and abdominal pain (3 patients). Two of 11 (18%) patients evaluated showed partial therapeutic response and 4 patients (36%) showed stable disease for up to 4 courses (27). All patients showed anti-adenovirus immune response. These results, suggest that multiple dosing of intraperitoneal Adp53 is feasible and is well tolerated in chemoresistant patients.

Based on the promising data of pre-clinical studies and clinical Phase I/II safety trials with Adp53, a large international Phase II/III trial was initiated for first line treatment of patients with stage III advanced ovarian cancer who have undergone cytoreduction surgery. Replication deficient Adp53 was given intraperitoneally in combination with standard chemotherapy to patients with ovarian cancers harboring p53 mutations. First interim analysis showed that patients who received standard therapy of six systemic cycles of carboplatin and paclitaxel plus five cycles of gene therapy with 1X10^{13} Adp53 viral particles did not show improved therapeutic effectiveness compared to patients receiving standard chemotherapy only. Therefore this study was terminated.

2.1.1.6. Reasons for failure of p53 gene therapy clinical trial
Possible reasons for the failure of this phase II/III study may have been that malignant transformation is multistep process involving changes in several genes, such as C-ERBB2, C-MYC, K-RAS in addition to p53 (28). Therefore repair of single p53 gene may not be a suitable strategy. Further, epigenetic
dysregulation may have resulted in aberrant gene silencing (29). Moreover, dominant negative cross talk between ectopic wild-type p53 and recently identified dominant p53 mutants and splice variants of p63 and p73 which are frequently over expressed in ovarian cancer could have also seriously compromised the effectiveness of p53 gene therapy (30, 31).

One of the important factors contributing to the failure of this trial could have been the sub-optimal gene delivery by adenovirus due to decreased expression of coxsackie-adenovirus receptors in ovarian tumors cells resulting in lower transduction levels ultimately resulting in lower gene transfer (32-34). In addition to this low receptor expression problem, neutralizing antibodies in the ascites of ovarian cancer patients seems to be another major obstacle in tumor cell targeting and are strongly suspected of adversely affecting the outcome of adenoviral-based gene therapy (35, 36). Another phenomenon that could have compromised the efficacy of adenoviral gene therapy is the innate anti-adenoviral immunity of cells. Infection with adenoviral vector induces the production of cytokines such as interferon gamma and TNFα, which have been shown to significantly inhibit the expression of transgenes.

Development of novel genetically modified adenoviral vectors with enhanced ability to specifically target ovarian cancer cells may be a novel solution to achieve effective p53 gene delivery in quantities sufficient to achieve therapeutic effect. Recent endeavors to develop new recombinant viral vectors with modified tropism and higher transduction rates, increased stability of transgene expression, and the ability to bypass the host-immune response are the most encouraging and meaningful approaches to achieve an effective therapeutic gain.

2.1.2. BRCA1
BRCA1, is a nuclear phosphoprotein which functions as a tumor suppressor and play a critical role in the regulation of apoptosis (37). Lack of or decreased levels of BRCA1 proteins contribute to the malignant transformation. Gene transfer of BRCA1sv (a normal splice variant of BRCA1) into ovarian cancer cells produced growth inhibition in vitro and tumor suppression in nude mouse xenografts. In phase I/II trial, i.p. delivery of BRCA1sv by retroviral vector in ovarian cancer patients was well tolerated with only three of 12 patients developing an acute sterile peritonitis due to vector, which spontaneously resolved within 48 h (38). Out of 12 patients, eight patients showed stable disease for 4-16 weeks, and three patients showed tumor reduction with diminished miliary tumor implants at re-operation (two patients) and radiographic shrinkage of measurable disease (one patient). In a subsequent Phase II trial however, BRCA1sv therapy in patients with less extensive disease showed no response, no disease stabilization, and little or no vector stability suggesting a significant variation in the tumor burden, immune system status, and response to BRCA1 gene therapy between Phase I and II trials (39).

2.1.3. PTEN
PTEN is another tumor suppressor gene, which encodes a phosphatidylinositol phosphatase that antagonizes activation of the phosphatidylinositol 3'-kinase-mediated pathway involved in cell growth. Since a gene encoding the catalytic subunit of phosphatidylinositol 3'-kinase (PIK3CA) is frequently activated in ovarian cancers; over expression of the PTEN product through Ad-mediated PTEN gene transfer significantly inhibited growth and peritoneal metastases of several human ovarian cancer cells in vivo and the degree of inhibition correlated with the efficiency of gene transfer (40, 41). The tumor growth inhibition of PTEN was mediated by two mechanisms, apoptosis and/or arrest in the G1 phase of the cell cycle (40). These findings carry significant implications for adenovirus vector-based PTEN gene therapies for ovarian cancers.

2.1.4. p16
p16 is a tumor suppressor gene which regulates G1 progression in the cell cycle by inhibiting CDK4/6 from phosphorylating the Rb gene product. It is also known to induce transcriptional downregulation of the RB gene (42). Adenoviral transduction of p16 gene into ovarian cancer cells inhibited their growth irrespective of their endogenous p16 status (SKOV3 p16 deleted or OVCA 420 wild type p16) (43).
However, SKOV3 required less amount of gene transfer for growth inhibition. The growth inhibition of p16 gene therapy was also demonstrated in vivo in ovarian cancer murine models (44) suggesting that this could be another potential strategy for ovarian cancer therapy. Unfortunately, only 26-37% of ovarian tumors showed decreased p16 expression. Since it was shown to be more effective than p53 gene therapy (45), p16 strategy needs to be evaluated in ovarian cancer patients to determine the enhanced clinical benefit.

2.1.5. p21
p21cip1/waf1 is cyclin-dependent kinase (cdk) inhibitor, a negative regulator of cell cycle (46-48). It is the downstream target of p53 and it is also inducible in a p53-independent manner (49, 50). The cell cycle inhibitory effects of p21 may be attributed to its ability to bind cdk5 as well as the proliferating cell nuclear antigen (PCNA) (51, 52)], resulting in inhibition of progression from the G1 to the S phase, of DNA replication, and progression through G2 phase (53). The transfection of p21cip1/waf1 cDNA into SKOV3 and OVCAR3 cells led to reduction of tumor cell growth, enhanced susceptibility to cisplatin-induced apoptosis, and abolition of recurrence after cisplatin exposure. Further, p21cip1/waf1 gene transfer allowed a marked reduction of the cisplatin concentration needed to eradicate the tumor cell population and also enhanced the cytotoxicity of cells which were resistant to HSV-tk and ganciclovir therapy (54). Thus suggesting the potential of p21cip1/waf1 to be used as an adjunctive to conventional chemotherapy and molecular chemotherapy in chemoresistant ovarian cancer.

2.1.6. Bax
Bax is a proapoptotic protein, and is a member of bcl-2 family, which inhibits p53-mediated apoptosis. It may partly mediate its apoptotic effects by predisposing to the release of mitochondrial cytochrome c into the cytosol, a critical first step in the activation of an important group of downstream cysteine proteases known as caspases (55). BAX was shown to be under expressed in tumor specimens of ~40% patients with ovarian cancer and that under expression of this protein predicts an inferior response rate to paclitaxel and shortened disease-free survival (56). Adenovirus-mediated Bax gene therapy resulted in a significant cytotoxicity to both chemosensitive and chemoresistant human ovarian cancer cells in vitro (57, 58) and in vivo. A DF3 (muc1) targeted adenoviral-mediated Bax gene delivery intraperitoneally eradicated more than 90% of tumors in nude mice (57). When treated in combination with cisplatin or paclitaxel, it further enhanced almost 10 fold more cytotoxic effect (58, 59). Our group has demonstrated for the first time that over expression of Bax directly induced apoptosis in patient-derived primary cancer cells. However, the sensitivity of these cells to Bax varied and appeared to be independent of both the status of p53 and the endogenous levels of bcl-2 or Bax. Importantly, over expression of Bax significantly enhanced chemotherapy-induced cytotoxicity in primary ovarian carcinoma cells. Bax, when combined with TRAIL as a fusion gene, further prolonged survival of mice with human ovarian cancer xenografts (60) suggesting that adenovirus-mediated Bax induction in combination with other apoptosis inducing agents may be a useful strategy for the treatment of ovarian cancer.

2.1.7. N5
p84N5 is a nuclear death domain-containing protein, which induces apoptosis upon transfection into cells. Adenovirus-mediated N5 gene therapy induced apoptosis and significantly reduced the proliferation and tumorigenicity of breast, ovarian, and osteosarcoma tumor cell lines (61).

2.2. Oncogenes
In addition to the mutations in tumor suppressor genes or their complete loss, many ovarian tumors also exhibit dysregulated oncogenes. Oncogenes function as growth factors, protein kinases, transcription factors, and GTP binding proteins, all of which regulate cell cycle growth. When oncogenes are amplified or over expressed, they cause uncontrolled cellular proliferation. Therefore targeting these genes to either block their expression or to down regulate them is a logical approach.
Dominant oncogenes implicated in ovarian carcinoma include Her2/neu (erbB2) (62-78); erbB1 (70, 72), which encode the epidermal growth factor receptor (EGFR); c-int2, which encodes fibroblast growth factor; c-fms, (79) which encodes the macrophage colony stimulating factor; and MET (80), which encodes the hepatocyte growth factor receptor; and proteins involved in cell signaling such as K-ras (70, 81-88), transcription factors such as c-myc (70, 84).

2.2.1. HER-2/neu.
The most widely studied oncogene for therapeutic targeting in the context of ovarian carcinoma is HER-2/neu. It encodes a 185 kDa transmembrane receptor tyrosine kinase with significant sequence homology to other members of the class I receptor tyrosine kinase (RTK) family consisting of EGFR, HER-3 and HER-4. The HER-2/neu gene is amplified and/or over expressed in 25%-30% of human ovarian cancers, (A recent phase II trial showed only 11.4% of ovarian cancer patients expressed HER-2/neu at significant levels (89)) and is associated with progression of invasive cancer and poor prognosis (74-77) and resistance to chemotherapy (90). The precise mechanism by which Her-2/neu over expression transforms cells remains unknown. However, it may involve tyrosine phosphorylation and activation of the HER-2 receptor leading to activation of specific signal transduction pathways such as the ras/MAP kinase cascade, phosphatidylinositol 3-kinase, and phospholipase C-gamma which results in dysregulated expression of diverse number of genes involved in cellular proliferation and differentiation regulation (78).

2.2.1.1. Anti-erbB-2 single chain strategy to target HER-2/neu
A variety of gene therapy strategies to target this receptor have been endeavored as a therapeutic modality (91-95). Our group has selectively “knocked out” HER-2/neu expression in ovarian cancer cells by adenoviral-mediated delivery of anti-erbB-2 single chain antibody (sFv) gene directed to endoplasmic reticulum (ER). This anti-erbB-2 sFv entraps newly synthesized erbB-2 oncoprotein in endoplasmic reticulum and down regulates cell surface HER-2/neu expression thus abrogating the HER-2/neu mediated signal transduction pathways. Abrogating signal transduction pathway resulted in arrest of anchorage-independent growth and marked cytoidal effect. Most importantly, the ER-directed anti-erbB-2 sFv elicited a significant cytotoxic effect in transfected primary ovarian cancer cells obtained from a patient with malignant ascites (91).

Evaluation of the mechanism of anti-neoplastic activity of HER-2/neu by our group showed that tumor killing effect is due to induction of apoptosis secondary to the intracellular antibody-mediated ectopic localization of the erbB-2 oncoprotein (93). Thus, the strategy of selective oncogene "knock-out" using intracellular antibodies represents a novel anticancer gene therapy strategy that offers the potential to achieve highly specific, targeted eradication of human tumor cells. In this regard, adenovirus-mediated delivery of anti-erbB-2 sFv gene significantly reduced tumor burden, prolonged survival of animals carrying a human ovarian carcinoma tumor intraperitoneally (92) and was shown to be safe in immunocompetent tumor models without any toxicity (94). The expression of anti-erbB-2 sFv is confined to peritoneum and no deleterious effects were observed in non-erbB-2 expressing cells thus indicating the tumor specificity of this gene therapy strategy (94). These results establish that adenoviral-mediated delivery of the anti-erbB-2 sFv in human context can be effectively assayed, can be potentially free of vector-associated toxicity, and can retain tumor specific activity. Therefore, this strategy of gene therapy for ovarian carcinoma offers the potential to achieve highly specific, targeted killing of human tumor cells and establishes the rationale to undertake human clinical trials.

2.2.1.2. Anti-erbB-2 single chain clinical trial
Our group has conducted a phase I trial with recombinant replication incompetent adenovirus vector encoding anti-erbB-2 sFv gene (Ad.21) in ovarian cancer patients. In this trial, a total of 15 patients with recurrent ovarian cancer and positive for erbB-2 were treated with Ad.21 in doses ranging from 1x10^9 to 1x10^10 plaque forming units. No dose limiting toxicities were observed and the most common side effects
were constitutional. Five patients had stable disease and 8 eight patients progressed but one patient with non-measurable disease remained disease free for 6 months (95). Although anti-erbB-2 sFv expression was demonstrated in ascites samples, the effectiveness of this vector may have been limited by low CAR expression of tumor cells and anti-adenovirus immunity.

2.2.2. Other strategies to target HER-2/neu and other oncogenes

In addition to single chain antibody approach, many different strategies have been developed to abrogate HER-2-neu expression as well as other oncogenes involved in cancer. These include ribozyme (96), antisense oligonucleotide (97, 98) (99), triplex forming oligonucleotides, (100, 101), dominant negative approach, and c-neu-specific transcriptional repressors (E1A, SV large T antigen, and PEA3) and a recent siRNA strategy.

2.2.2.1. Ribozyme

A ribozyme is a catalytic RNA that can be used for sequence specific cleavage of target RNA (102, 103). This strategy was used to target HER-2/neu and down regulate its expression (96, 104) in order to reverse the malignant phenotype. A particularly interesting ribozyme is “hammerhead” ribozyme, so named because of its cleavage domain shaped into hammerhead tertiary structure. Many different hammerhead ribozymes targeting to oncogenes (H-ras, N-ras, bcr-abl, c-fos, and human papillomavirus E6 and E7) have been developed with some success (103). In ovarian cancer, a chimeric U6 RNA promoter driving expression of hammer head ribozyme against HER-2/neu, down regulated c-neu mRNA and protein in a dose-dependent manner and growth inhibition of HER-2/neu over expressing human ovarian cancer cells SKOV3.ip1 both in vitro and in vivo (105). Other oncogenes targeted by this ribozyme approach in the context of non-ovarian cancers include H-ras (106) K-ras (107). The potential of targeting these genes is questionable because, one study reported that ras mutations, amplification or over expression have been detected rarely in most common histologic subtypes (70, 81, 82, 108) of ovarian cancer. In contrast, another study reported that elevated levels of mRNA transcripts and protein of K-ras have been noted in almost half of all ovarian carcinomas (109).

2.2.2.2. Triplex forming oligonucleotides

Oligonucleotides have been shown to form interstrand DNA triple helices in a number of polypurine/polypyrimidine rich sequences (110). These triplex forming oligonucleotides bind to DNA sequences of regulatory regions/ promoter regions of genes and inhibit the transcription and expression of genes (111). Several human gene promoters such as the epidermal growth factor receptor, and the dihydrofolate reductase, and IL-2 receptor genes have been successfully targeted with this strategy to inhibit their transcription (112-114). On this basis, triplex forming oligonucleotides were also employed to target dominant oncogenes HER-2/neu, c-myc at the DNA level to prevent their expression (115), which resulted in significant decrease in viable ovarian cancer cells (116, 117). Other promoters which were targeted by this method are EGFR promoter (111) and H-Ras promoter (118). These studies represent a future modality for specific inhibition of oncogenes in the context of ovarian cancer.

2.2.2.3. siRNA Technology

siRNA is a novel technology in which chemically synthesized or in vivo-expressed short interfering RNA (siRNA) are used to specifically and effectively direct homology-dependent post-transcriptional gene silencing. Transfecting these siRNA molecules with cationic lipid complexes not only specifically knocked down their cognate targets such as bcl-2, cdk-2, mdm-2, pck-alpha, TGF-β1, H-ras, VEGF, and GFP mRNAs, but also effectively suppressed the proliferation of cancer cells to different extents (119). Long term expression of siRNA against H-ras by retroviral vector significantly inhibited proliferation, increased G(0)/G(1) arrest and apoptosis, blocked transformation in vitro, and suppressed tumor growth in nude mice (120).
2.2.2.4. Antisense oligonucleotide

Antisense oligonucleotide is a synthetic piece that is complimentary to the DNA or mRNA of the gene to be targeted. These oligonucleotides bind to their target DNA and inhibit transcription or bind to the target RNA and block further processing of genetic information by impaired transport, translational arrest, or initiating RNA degradation. This antisense strategy was used to target erbB-2 (121). A single-dose application of 5 \( \mu \text{M} \) c-erbB-2 anti-sense phosphorothioate oligodeoxynucleotides (S-ODNs) specifically reduced erbB-2 levels in the ovarian cancer cell line SKOV3, which over express erbB-2. This was accompanied by a 60% inhibition of anchorage-dependent cell growth. More strikingly, c-erbB-2 anti-sense S-ODNs almost completely abrogated serum-induced cell spreading (121, 122). In addition, transfection with anti-sense erbB-2 cDNA rendered the ovarian cancer cells significantly, more sensitive to chemotherapeutic drugs (5-fluorouracil, cisplatinum) (122). These studies prove that anti-sense approaches have the potential of providing novel strategies for the therapy of ovarian cancer.

In addition to HER-2/neu, antisense strategies have been used to ablate mutant forms of K-ras (123, 124) and H-ras (125), as well as c-myb (126, 127), c-myc (128, 129), insulin like growth factor (IGF) 1 receptor (130, 131), bcr-abl (132) and p53 genes. A combination of these antisense gene therapy strategies was also endeavored to achieve synergistic therapeutic effect. A combined delivery of antisense phosphorothioate oligonucleotides targeting both c-erbB-2 and c-myc was shown to significantly enhance inhibition of ovarian cancer cell line proliferation and increase apoptosis \textit{in vitro} up to 82 % compared to 61-65 % when targeted with individual genes (133). In contrast, another study targeting both c-myc and p53 phosphorothioate oligos directed against c-myc and p53 in different cell lines were shown to have both antiproliferative and stimulatory activity, as single agents and in combination, at concentrations that are achievable \textit{in vivo}. In CAOV-3 ovarian cancer cell line, c-myc/p53 combinations were synergistic but they were antagonistic in SKOV-3 cell line. Because of these complex patterns of effects, further \textit{in vitro} studies are warranted before advancing these studies with these agents in gynecologic cancers.

2.2.2.4.1. Strategies for enhanced delivery of antisense molecules

To achieve successful antisense therapy approach, effective delivery of anti-sense molecules to each and every tumor cell is required. The ability to deliver antisense oligos to the target tumor cells is one of the major problem inhibiting the success of this approach (134). Towards this end, a number of gene delivery approaches, such as retrovirus vectors, electroporation, transferrin receptor-mediated delivery, liposomes have been explored to achieve effective cellular gene delivery (125, 135, 136) (137) and more recently radiolabelled antisense oligos against c-erbB-2 complexed with dendrimer and or avidin was also explored. This approach significantly enhanced the delivery and facilitated imaging in i.p.-disseminated tumors (138). A new antisense oligonucleotide delivery system based on self-assembled conjugate of antisense c-raf oligonucleotide (ODN) and poly(ethylene glycol) (PEG) and polyethyleneimine hybrid conjugate micelles showed enhanced uptake of ODN and significant inhibition of proliferation of ovarian cancer cells (139).

2.2.2.4.2. Challenges of antisense therapy

One of major problems with antisense oligo approach is their instability. To circumvent this problem, a number of modifications of antisense molecules are being currently developed to enhance their stability (140) (discussion of these modification are beyond the scope of this review). These developments allow targeted delivery to the tumor cells and may enhance the therapeutic efficacy of this approach.

2.2.2.5. Dominant negative approach

Inactivation of oncogenes can also be achieved at the protein level by delivery of mutated forms of oncogenes that produce non-functional form of oncoproteins, which then bind to native oncoprotein to form multimers and inhibit its function. Naturally, this approach works only in the context of protein which must form multimers to become functional. Example of the proteins targeted with this approach are EGFR (141), insulin-like growth factor type 1 receptor (142), platelet growth factor receptor (143) and
vascular endothelial growth factor receptor (144). In the context of ovarian cancer, survivin, an anti-apoptotic protein upregulated in ovarian cancer (145) as well as activator protein-1 (AP-1), a transcription factor which has been linked to chemotherapeutic resistance (146) was inhibited by this dominant negative approach. Thus demonstrating the feasibility of this approach for ovarian cancer therapy.

2.2.2.6. Transcriptional repressors
c-neu specific transcriptional repressors such as E1A, SV large T antigen, and PEA3 have also been employed to down regulate HER-2 neu expression.

2.2.2.6.1. Adenovirus 5 E1A.
Adenovirus 5 E1A, is a well know transcription factor, and the early gene that is expressed after virus infection, was shown to inhibit HER-2/neu transcription, transformation and tumorigenicity induced by HER-2/neu over expression in cancer cells (147-149). Repression of neu expression by E1A reduced transforming ability of the human ovarian cancer cells that over express the neu oncogene and decrease ability to induce tumors in nu/nu mice (150). Liposome mediated delivery of E1A in pre-established intraperitoneal human ovarian cancer xenograft mouse models prolonged survival of approximately 70% of the mice more than one year without showing any detectable tumors in peritoneal cavity or toxicity to normal organs compared to control groups in which all the mice died by day 160 (151). A series of subsequent safety studies conducted in normal mice using 5-40 times the DNA-lipid doses proposed to be used in clinical trials showed no adverse effects in renal, hepatic and hematological parameters.

2.2.2.6.1.1. E1A gene therapy clinical trials
Two E1A phase I studies were conducted using liposomes complexes by two different groups. In the Phase I trial conducted by Hartobagyi et al. (152), 12 patients with advanced ovarian cancer were treated intraperitoneally with E1A gene complexed with cationic liposomes. The treatments were well tolerated with fever, nausea, vomiting, and discomfort at injection site being the most common side effects. Expression of E1A in tumor cells obtained from patients was accompanied by downregulation of HER-2/neu expression, increased apoptosis, and reduced proliferation. Five patients showed a decrease in tumor markers, and three patients showed a transient improvement in performance status but no other clinical response was noted.

In another phase I multicenter clinical trial conducted in patients with recurrent epithelial ovarian cancer over expressing HER-2/neu (153), a total of 15 patients (median age of 57 yrs) were recruited out of which 3 patients received a dose of 1.8 mgDNA/m². Four patients received E1A-lipid complexes at a dose of 3.6 mg DNA/m² and 8 patients received a dose of 7.2 mg DNA/m². All the treatments were given as intraperitoneal infusions for 3 to 4 weeks (cycle) upto a maximum of 6 cycles. Abdominal pain was the dose limiting toxicity and the maximum tolerated dose was 3.6mg/m². E1A gene expression was observed in all patients but HER-2 downregulation was seen in 2 patients only. There was correlation between dose and biological activity. These studies show that E1A gene therapy is safe.

2.2.2.6.1.2. E1A gene therapy for chemoresistant ovarian cancer
The advantage of the E1A gene therapy is that in addition to inhibiting tumor growth directly, it also sensitizes chemoresistant ovarian cancer cells to paclitaxel by activation of caspase-3 pathway. Since over expression of HER-2/neu in ovarian cancer cells was shown to confer resistance to paclitaxel therapy downregulation of HER-2/neu by E1A gene therapy sensitized these chemoresistant ovarian cancer cells to paclitaxel both in vitro and in vivo. (154). Based on this ability of E1A to sensitize cancer cells to paclitaxel, a phase I trial combining E1A administration with chemotherapy needs to be evaluated to determine its potential for clinical therapy.

2.2.2.6.2. Other transcriptional repressors
In addition to E1A, K1 mutant of SV40 large T antigen and ets transcription factor (PEA3) therapy have also shown to down regulate or inhibit the HER-2/neu expression (155-157). Liposome mediated K1
mutant SV40 large T antigen gene transfer significantly inhibited tumor growth and prolonged the survival of mice with pre-established intraperitoneal HER-2/neu over expressing tumors (155). Similar to SV40 large T antigen gene therapy, liposome-mediated PEA3 gene therapy also significantly prolonged the survival of mice with ovarian cancer tumors (157). All these studies demonstrate that targeting HER-2/neu by transcriptional repressors is a feasible approach for therapy of ovarian cancer.

2.2.2.7. Potential problems with mutation compensation
A variety of approaches of mutation compensation have showed that it is a promising strategy for the therapy of ovarian cancer. However, there are some problems which need to be overcome for clinical applications. Human ovarian cancers are remarkably heterogeneous in expression of relevant oncogenes. Therefore, therapeutic targeting of a single oncogene may only have inconsequential effect on clinical management of the disease. Further, these strategies inhibit the tumor cell growth by ultimately modulating intracellular responses. Therefore every single cell must be targeted to achieve a significant clinically effective therapy. Although significant advances have been made in gene delivery vectors to enhance gene delivery, further advances to deliver genes in quantities sufficient to achieve significant therapeutic effect and tumor specific gene delivery will increase the therapeutic index of this strategy. Also other approaches such as molecular chemotherapy and immunopotentiation that do not require that every single cell to be targeted may hold promise for tackling some of the above mentioned limitations.

3. Molecular chemotherapy
In conventional chemotherapy, a toxic drug is delivered to tumor cells to kill them. However, treatment with conventional chemotherapy also results in toxicity to normal cells. Therefore the goal of molecular chemotherapy approach is to selectively kill tumor cells and minimize toxicity to normal cells. This is achieved by an enzyme prodrug strategy. In this strategy, instead of directly delivering a toxin to tumor cells, tumor cells are first transduced with an enzyme gene, which can convert a subsequently delivered harmless prodrug into a toxic metabolite. This approach selectively kills tumor cells, and minimizes the toxicity to normal cells.

3.1. (HSV/tk)/ganciclovir system
(HSV/tk)/ganciclovir system is most commonly and widely studied system in the context of ovarian cancer. Thymidine kinase (TK) is an enzyme that plays a role in the salvage cycle of DNA synthesis. The TK derived from herpes simplex virus (HSV/TK) has high affinity to ganciclovir and preferentially monophosphorylates ganciclovir, which is then phosphorylated further by cellular factors to a triphosphate form. Incorporation of this triphosphate form into DNA during replication inhibits DNA synthesis and RNA polymerase activity resulting in cell death. Although TK is present in mammalian cells, it shows less affinity to ganciclovir and therefore normal cells tend to be resistant to ganciclovir.

3.1.1. HSV/tk and GCV Pre-clinical studies
A number of studies employed HSV/tk/GCV strategy for the therapy of ovarian cancer. Our group employed adenoviral-mediated HSV/TK delivery to significantly sensitize ovarian cancer cell lines and also primary tumor cells derived from patients in vitro (158, 159). Subsequently, a number of in vivo studies by our group and others showed tumor reduction and prolonged survival of immunodeficient mice with human ovarian xenografts (160-165) as well as immunocompetent mice (166) and rats with ovarian cancer (167).

3.1.2. Bystander mechanism of HSV/tk and GCV gene therapy
A surprising finding in HSV/tk/GCV studies was that a greater number of tumor cells were actually killed than initially were transduced with a HSV/TK gene. This phenomenon is called “bystander effect” (168, 169). Due to bystander effect, a tumor mass containing only 10% of HSV/TK positive cells were
sufficient to cause regression of subcutaneous tumors (170). Further, intraperitoneal tumor inoculation containing only 50% of HSV/TK positive tumor cells was able to prolong the survival of mice more than 70 days. The mechanisms underlying this bystander phenomenon in vitro was thought to be due to the transfer of ganciclovir toxic metabolites through gap junctions (171, 172) to the unmodified cells, or induction of cellular apoptosis (which was inhibited by Bcl2 expression) (173), or by phagocytosis of apoptotic vesicles generated from the dying gene-modified cells (170). In addition to these mechanisms, immune system was also shown to play a major role in in vivo bystander effect (174). This includes cytokine release (175), which leads to hemorrhagic necrosis and infiltration of immune cells into tumor after HSV/TK gene therapy (174), up regulation of immune regulatory molecules such as B7.1, B7.2, and ICAM-1 (176, 177), increase in expression of MHC class I molecules and the induction of tumor-specific cytotoxic T- cells. In addition to localized bystander effect, a distant bystander effect was also demonstrated in mice implanted with HSV/TK positive and negative cells. Treatment with ganciclovir not only completely regressed HSV/TK positive cells but also showed regression in a distant HSV/TK negative tumor. This effect was associated with an increase in CD4+, CD8+ and NK cells (178).

3.1.3. Alternate vectors for HSV/tk gene therapy
For efficient delivery of HSV/tk, our group has explored alternate vector systems. One of the vectors was human vascular endothelial cells (HUVEC). These cells were transduced by adenovirus expressing HSV/TK (AdCMVHSV-TK) and mixed with equal number of untransduced ovarian cancer cells. This resulted in killing of > 70% of cells in vitro and significantly inhibited tumor growth and prolonged survival in vivo (179). The advantage of these HUVEC cell vectors is that it can be used to overcome limitations associated with adenoviral vectors such as low transduction efficiency, host immune response to the vector, and vector toxicity (180). Similarly, mesothelial cells were explored as cellular delivery vehicles of HSV/TK to tumor cells. Other vectors which displayed superior delivery of HSV/TK are herpes simplex virus-1 (HSV-1). Gene transfer mediated by HSV-1 is quantitatively superior to adenoviral vector in five of the six ovarian cancer cell lines even at a 100-fold lower dose in vitro, suggesting that HSV-1 may be a promising alternative vector for ovarian cancer gene therapy (181).

3.1.4. Tumor targeted Ad for HSV/tk gene therapy
To decrease the adenoviral dose required for HSV/TK gene delivery, our group has redirected the adenovirus to basic fibroblast growth factor (FGF2) (using a bispecific antibody) which resulted in requirement of 10 fold less dose than regular adenovirus, and significantly prolonged the survival of mice (182). More recently, our group demonstrated the feasibility of not only enhancing the adenoviral mediated HSV/tk efficacy, but also feasibility of non-invasive assessment of HSV/tk gene transfer in vivo (183). This was accomplished by modification of adenovirus by incorporating RGD in its capsid to enhance transduction efficiency and bicistronic expression of both HSV/tk and the human somatostatin receptor subtype-2 gene (SSTR2), to facilitate non-invasive imaging.

3.1.5. Correlation between tumor CAR and integrin levels and HSV/tk therapeutic response
Since Ad infectivity depends on CAR and integrin levels, it was hypothesized that high CAR and integrins levels may positively correlate with high therapeutic effect. On this basis, a study was conducted to determine the feasibility of predicting the therapeutic response of Ad-mediated HSVtk therapy. Determination of correlation between Ad-mediated HSVtk therapeutic response and CAR and integrin levels in tumors revealed that no correlation exists between therapeutic response and CAR and integrin levels (184). This finding was surprising. However, these results may not be applicable to therapies based on other genes.

3.1.6. Transcriptional targeted HSV/tk gene therapy
To achieve tumor specific gene delivery, HSV/tk was expressed under the control of hTERT promoter, which was shown to be as strong as CMV promoter but enabled expression specifically in ovarian cancer cells (185). To further increase the therapeutic effect by using strong promoters to express HSVtk, two
candidate promoters CMV and RSV were compared. Although CMV was better in killing efficiency compared to RSV, no difference in long term survival of mice was noted (186).

3.1.7. HSV/tk and GCV clinical trials
In phase 1 trial conducted by Link et al., (187) intraperitoneal delivery of retroviral producer cell-mediated HSV/Tk resulted in direct tumor cell killing and also bystander killing effect. Four out of nine patients showed an anti-tumor response with acceptable toxicity, but gene transfer efficiency was noted to be low. In a phase I dose escalation study conducted by our group, intraperitoneal administration of replication deficient recombinant adenovirus encoding HSV/tk in 14 patients with doses ranging from 1x10⁹ to 1x10¹¹ pfu, followed by fixed-dose intravenous ganciclovir therapy (5mg/kg bid for 14 days) was shown to be safe with only mild side effects such as transient fever, abdominal discomfort, but the therapeutic response was modest. Of the 13 patients evaluated for response, 5 (38%) had stable disease and 8 (62%) had evidence of progressive disease. Molecular analysis of ascites samples demonstrated the presence of transgene DNA and RNA in most patients 2 days following Ad HSV-TK administration. Ten of 11 evaluated patients had an increase in anti-adenovirus antibody titer. These results suggest that treatment with AdHSV-TK in combination with GCV is feasible in the context of human ovarian cancer and tolerated at the dosages studied.

To further enhance the therapeutic efficacy of this system in clinical trials (188), a combination of HSV/tk (at doses ranging from 2x10¹⁰ to 2x10¹³ intraperitoneally) followed by acyclovir and topotecan (1.0mg/m² daily for 5 days) after 24 hrs, was administered to 10 patients with recurrent ovarian cancer who underwent secondary cytoreduction. No dose limiting side effects were observed, and the most common side effect was myelosuppression, secondary to chemotherapy. These results suggests that this combination therapy was well tolerated without significant toxicities.

3.1.8. HSV/tk in combination with other therapies
The HSV/tk therapeutic approach was combined with other therapies to augment its therapeutic efficacy. Concomitant treatment with HSV/TK gene therapy and chemotherapy (topotecan) resulted in significant enhancement in cell killing in vitro by sensitizing tumor cells to chemotherapy (189). However, combination with either topotecan or paclitaxel did not accord similar therapeutic effect in vivo (190). Similarly, combining p53 gene therapy with HSV/TK gene therapy also failed to enhance its efficacy (191). In contrast, combining caspase-3, a potent executor of apoptosis with TK gene therapy significantly enhanced the efficacy of HSV/TK and GCV therapy (192). In another approach, double transgenic mice expressing SV40 T antigen and HSV/TK showed significant inhibition of tumor growth compared to single transgenic control mice (193). Another study showed transduction of both TK and IL-2 not only killed all the cells upon treatment with GCV in an immunocompetent syngeneic ovarian cancer model, but also developed resistance against tumor challenge with ovarian cancer cells as a result of immune activation in the mice (194). A bicistronic retroviral vector expressing both GM-CSF and HSV/TK genes was shown to enhance the function of immune cells and increase efficacy of HSV/TK gene therapy. These types of combination therapies may be advantageous in the clinical application of gene therapy for human ovarian cancer. Evaluation of these gene therapy strategies in clinical studies may prove useful in overcoming inefficient therapeutic gains achieved by current approaches.

3.2. Other enzyme prodrug systems
Several other enzyme-prodrug systems such as E.coli nitroreductase (NTR) / CB1954, Cytosine deaminase (CD) / 5-fluorocytisine, E. coli purine nucleoside phosphorylase (PNP) / 6methyl purine deoxyriboside (MeP-dR), and Carboxypeptidase / mustard prodrugs were also employed in pre-clinical studies of ovarian cancer therapy.
3.2.1. E. coli nitroreductase (NTR)/ CB1954 system
In E. coli nitroreductase (NTR) and CB1954 system, expression of nitroreductase in tumor cells activates the alkylating agent CB1954 leading to cell death. The mechanism of cell death was suggested to be predominantly caspase-dependent apoptosis (195). Retroviral mediated NTR gene therapy has been shown to increase survival of mice with ovarian tumors including platinum resistant cells and primary ovarian cells derived from patients (196). Similar to the HSV/TK system a marked 'bystander killing' effect was also observed with this system. Further, this system showed synergistic effect when combined with 5 fluorouracil (195). In an effort to enhance the efficacy of this system, a mutant form of NTR was developed and adenovirus-mediated delivery of this mutant F124K NTR resulted in 5-fold more potent in sensitizing the cells to CB1954 (197). This system is currently being evaluated in clinical trials in the context of other carcinomas, evaluation in the context of ovarian carcinoma may prove valuable.

3.2.2. Cytosine deaminase (CD) and 5-fluorocytisine system.
In the cytosine deaminase (CD) and 5-fluorocytisine system, CD, a microbial enzyme, converts 5-fluorocytisine to 5-fluorouracil an anti-tumor agent. Adenovirus mediated CD delivery has been shown to induce apoptosis in vitro (198, 199), and significant tumor growth inhibition in vivo in a murine ovarian carcinoma model (199).

3.2.3. Purine nucleoside phosphorylase (PNP) and 6methyl purine deoxyriboside (MeP-dR) enzyme-prodrug system
E. coli derived enzyme PNP cleaves MeP-dR to liberate 6-methyl purine, which is a potent membrane permeant and a freely diffusible toxin. Transfection of E. coli PNP in as few as 0.1% of the tumor cells showed pronounced bystander killing resulting in substantial antitumor effects (200). Due to its high bystander effect, this system may be useful in overcoming low transduction limitations of current gene delivery vectors (200).

3.2.4. Carboxypeptidase/ mustard prodrug system
In this system, carboxypeptidase, a bacterial (Pseudomonas) enzyme, specifically activates mustard prodrugs such as 4-[2chloroethyl)(2-mesoxyethyl) amino] benzoyl-L-glutamic acid (CMDA) to release L-glutamic acid and the DNA alkylating drug 4-[(2chloroethyl)(2-mesoxyethyl) amino] benzoic acid, which is a potent cytotoxic agent. Transduction of CPG2/CMDA leads to apoptosis of ovarian cancer cells with significant bystander effect. In fact the bystander effect was more pronounced when the expression of enzyme was on the cell surface than when expressed intracellularly (201).

4. Genetic immunopotentiation
Genetic immunopotentiation is a strategy to enhance the immunogenicity of tumors by augmenting the function of pre-formed immunological components in order to abrogate tumor growth (202). Several gene therapy strategies were developed to increase the potency of host’s immune system cells against tumor cells or enhance the immunogenicity of tumor. These strategies focus on two concepts namely, passive immunotherapy and active immunotherapy.

Passive immunotherapy refers to use of pre-formed immunological elements in order to augment immune response to tumor cells. For example, tumor infiltrating lymphocytes or tumor-associated lymphocytes can be isolated and expanded in vitro and subsequently injected back into the patient. Further, these lymphocytes can be specifically targeted to ovarian cancer cells by genetically modifying them to express ligands or receptors which bind to antigens specifically expressed on ovarian cancer cells. In addition, the therapeutic efficacy of these lymphocytes can be augmented by genetically modifying them ex vivo to express cytokines.
Active immunotherapy refers to initiation or augmentation of immune response directed against previously unrecognized or poorly immunogenic tumor antigens by increasing the expression of known tumor antigens or augmenting local concentration of cytokines and co-stimulatory molecules by gene therapy to enhance immune response against tumors. In addition, genetically modified tumor and dendritic cell vaccines are also being used for active immunotherapy.

4.1. Passive immunotherapeutic strategies

4.1.1. Artificially increasing the number of tumor specific immunocompetent cells
In this strategy, immune cells with antitumor reactivity are obtained from a tumor, grown artificially in culture to increase their number, and transferred back into the tumor-bearing host. In the context of ovarian cancer, certain T cell lines and clones derived from ovarian tumor infiltrating lymphocytes (TILs) exhibit in vitro, autologous tumor-specific cytotoxicity and/or cytokine production (interferon-gamma, tumor necrosis factor-alpha) preferentially in response to autologous tumor cells (203). Isolating these tumor-infiltrating lymphocytes and expanding their number artificially in cell culture by means of human interleukin-2 and then administering them back into the bloodstream, along with IL-2, resulted in destruction of the tumor cells.

4.1.2. Targeting lymphocytes to the ovarian tumor cells
Although injection of ex vivo amplified TILs in in vivo exhibits anti-tumor effect, enhancing their specificity to tumor cells may further increase their efficacy. On this basis, an attempt was made to redirect normal human lymphocytes to ovarian cancer cells by transducing peripheral blood lymphocytes (PBL) obtained from patients with a chimeric antigen receptor (MOv-gamma), which acts as a receptor to folate binding protein that is normally over expressed in ovarian cancer cells. Amplification of these ovarian cancer redirected cells by stimulation with anti-CD3, IL-2, and demonstrating the retention of their tumor specificity clearly showed the feasibility of using these tumor specific T cells for ovarian cancer therapy (204). Another strategy used a chimeric antibody/T-cell receptor gene constructed from an anti-ovarian cancer antibody to redirect TILs to ovarian cancer cells and to specifically recognize and lyse them (205).

4.1.3. Genetically engineering the TILs to enhance their cytotoxic ability
In addition to the above-described approaches, therapeutic efficacy of TILs was also enhanced by transducing several cytokine genes into TILs. Introduction of TNF gene, by retrovirus vector into TILs increased the local concentrations of TNF in the tumor microenvironment, enhanced cytotoxicity against autologous tumor cells without inducing systemic toxicity (205, 206). This enhanced cytotoxicity was ascribed to autocrine effects of secreted TNF on TIL, which included augmentation of adhesion molecule (CD2 and CD11a) and interleukin-2 receptor expression, and elevation of production of interferon gamma, lymphotoxin and granulocyte/macrophage-colony-stimulating factor and its paracrine effect on target cells to facilitate them to be more susceptible to TIL. Retroviral transduction of fyn gene into tumor-infiltrating lymphocytes (TILs) (obtained from six patients with epithelial ovarian cancer), augmented T cell receptor-CD3 complex signal transduction, and significantly enhanced the cytolytic activity of the TILs against autologous tumor cells (207).

These gene therapy approaches of amplification, redirection of TILs to tumor cells, and enhancing their cytotoxic ability clearly demonstrated their clinical translational potential for therapy ovarian carcinoma.
4.2. Active immunotherapeutic strategies
4.2.1. Altering the tumor cells to make them more immunogenic

In this approach, weakly immunogenic tumor cells are genetically altered to make them strongly immunogenic. For example, genes coding for MHC I, B7 or for cytokines such as IL-2, or TNF are inserted into the weakly immunogenic tumor cells by means of a vector to make them strongly immunogenic.

Since reduced immunogenicity of tumor can be related to the absence or downregulation of tumor specific antigens or downregulation of major histocompatibility complex class I (MHC I) molecules on the tumor cells, it was hypothesized that either increasing the tumor antigens or MHC I expression may increase the immune response. Although several potential tumor antigens such as HER-2/neu, folate binding protein, MAGE-1, and MUC-1 have been identified towards this end, over expressing these tumor antigens alone is not sufficient to enhance immune response because of development of tolerance to self-antigens and immunosuppression (208, 209). However, combining over expression of tumor antigens with cytokine or co stimulatory molecule gene therapy, enhanced the immune response (208, 210).

It is known that cytokines such as IFN-\(\gamma\) induces MHC I expression. In this regard, enhancing MHC I expression on ovarian tumor cells by IFN-\(\gamma\) gene therapy increased immunogenicity (211). Introduction of allogeneic MHC and ‘foreign’ DNA into tumors also lead to the subsequent rejection of tumors in vivo and the generation of tumor-specific CTL against the ‘wild-type’, parental tumor (212-217). Two phase I/II trials were conducted to examine the toxicities of employing the human HLA-A2, HLA-B13 and the murine H-2K genes to generate tumor regression in patients with different cancer types via DC-Chol/DOPE cationic liposomes. A total of 19 late-stage patients (various carcinomas including ovary) received 4 weekly injections of DNA-liposome mixtures and showed no clinical side effects suggesting that the therapy is safe. Of the eight patients whose cutaneous nodules received HLA-A2 DNA, two completely regressed while four tumor nodules gave a partial local response. A strong local immune response following in situ gene therapy was observed in patients who had relapsed metastatic disease and were refractory to all available therapies.

The rejection of the tumor cells not only requires the presence of tumor antigens, and their appropriate display in an MHC I context, but also co-stimulatory signals provided by antigen presenting cells (APCs). Interaction of the B7 family of molecules on APCs and CD28 receptor of T cells, results in activation of T cells which are then able to destroy tumor cells that express antigens in an MHC I context (218). Based on the understanding of this mechanism, it was hypothesized that introduction of the B7 gene into tumor cells may allow them to present directly their antigens and provide co-stimulatory signals to activate lymphocytes thus leading to the tumor eradication in vivo. In this regard, delivery of B7-1 co stimulatory molecule gene to tumor cells via adenovirus vector induced significantly higher levels of T cell proliferation than tumor cells modified with a control adenovirus (219).

Gene therapy with cytokine genes activates CTL responses and increases the recognition of the immune system to non-transduced adjacent tumor cells. Based on this knowledge, retroviral-mediated IL-2 gene transduction of human ovarian cancer cells followed by intraperitoneal implantation into SCID mice significantly enhanced the survival with 25% of mice totally rejecting their tumors (220). In addition, feasibility of this approach was shown in primary tumor cells derived from patients (221).

To further augment the cytotoxic effect of IL-2 gene therapy of ovarian cancer cells, peripheral blood lymphocytes (PBL) were also transduced by IL-2R gene (222). This significantly increased cytotoxicity of IL-2R transduced PBLs on IL-2 and is thought to be due to increased sensitivity of PBLs to IL-2 as well as increased local IL-2 secretion.

Another cytokine gene used to potentiate cytotoxicity of TILs was Interleukin-7 (IL-7). IL-7 produced by stromal cells performs critical functions during lymphoid development (223) and functions as a stimulator
or co stimulator for activation of mature lymphocytes (224, 225). In addition, IL-7 induces and augments activity of lymphokine-activated killer (LAK) cells. A recent study also showed that IL-7 played a crucial role in regulating the expansion of peripheral T-cell population in states of T-cell depletion (226). In ovarian cancer cells, IL-7 is rarely expressed and therefore its protein level is very low in the peritoneal cavity of patients. On this basis, increasing IL-7 levels in the peritoneal cavity would probably improve host immunity against ovarian carcinoma. Towards this end, IL-7 gene transfected into SKOV3 cells was shown to down regulate TGFbeta1 secretion, up regulate ICAM-1 expression and enhance sensitivity to LAK cells in vitro and reduced the tumorigenicity in SCID mice in vivo (227).

Although cytokine gene transfer into tumor cells was shown to produce significant antitumor effects in several animal models. Some studies have failed to demonstrate systemic antitumor immunity following these forms of cytokine gene therapy [10, 11]. One possible contributing factor may be the expression of immunosuppressive agents like transforming growth factor-β (TGF-β) by the tumor cells (228), which inhibits the activation of various immune effector cells including cytotoxic T lymphocytes and may therefore inhibit the efficacy of immunostimulatory interleukin-2 (IL-2) gene therapy. Based on the recognition of this mechanism, an attempt was made to down regulate TGF-β by TGF-β antisense therapy. When this strategy was combined with immunostimulation via IL-2 gene therapy, in the intraperitoneal model of murine ovarian teratoma (MOT), it generated effective antitumor responses suggesting that tumor cell expression of immunosuppressive factors may inhibit cytokine immunogene therapy and may have potential implications for the development of future clinical immunogene therapy protocols (228).

IFN-β showed potent antiproliferative and immunostimulatory activity. In several pre-clinical tumor models, antiproliferative, immunomodulatory, and antiangiogenic effects of IFN-β have been demonstrated (229-234). Similarly in ovarian cancer model, transfection of human ovarian tumor Hey-A8 cells with mIFN-β (Hey-β) suppressed tumor growth and also suppressed non-transduced tumor cells in an in vivo ovarian cancer murine model (234). This tumor suppression was mediated by up regulation of the inducible nitric oxide synthase (iNOS) gene expression in host macrophages.

4.2.2. Augmenting immune response by cytokine gene therapy
The body's immune responses can be boosted artificially through delivery of cytokines genes such as interleukin-2 (IL-2) (235), IL-6 (236), interferons (IFN-beta, IFN-gamma) (237, 238), and tumor necrosis factor-alpha (TNF-alpha) (239) whose over expression results in enhancing the cytotoxicity to tumor cells.

4.2.2.1. IL-2, and IL-6
Regional delivery of murine interleukin-12 (mIL-12)-secreting fibroblasts in a syngeneic immunocompetent mouse model of ovarian cancer (ID8) showed a significant increase in survival with 88% of mice surviving 120 days post injection but showed no toxicity. Both immunological and anti-angiogenic mechanisms mediated reduction of tumor growth in this model (240). Interleukin-6 (IL-6) functions as a late-acting killer helper factor in the differentiation of CTLs. In an attempt to exploit this function of IL-6 for tumor cell killing, IL-6 cDNA was delivered in vivo by adenovirus vector. This induced CD8+ human CTLs specific for autologous human tumor cells from human precursor T cells and resulted in significant reduction in tumor growth and metastasis (236).

4.2.2.2. IFN-β
IFN-β gene therapy for cancer and its mechanism of action was also studied. Adenoviral-mediated IFN-β gene therapy resulted in strong CD8(+) T-cell-mediated antitumor effects in pre-established murine tumor models (229). Antitumor effects were mediated by large influx of CD4(+) and activated CD8(+) T cells in the peritoneal fluid and within the tumor nodules suggesting that tumor-specific CD4(+) and CD8(+) T cells are the key effector cells for tumor eradication. In another study, a single treatment with adenovirus encoding IFN-β 3.3 x 10(8) plaque-forming units decreased tumor burden and significantly prolonged survival of mice in an immunocompetent murine ovarian teratoma model (241).
4.2.2.3. IFN-γ
In addition to IFN-β, transfection of ovarian cancer cells in vivo with IFN-γ gene resulted in suppressed tumor growth (or killed tumor cells) and increased the long-term survival of animals (at least 80 days) without tumors (237). The expression of inducible nitric oxide synthase (iNOS) and the tumor necrosis factor alpha gene subsequent to IFN γ gene therapy suggested that nitric oxide may play a major part in tumor cell killing or growth. Another study demonstrated stimulation of local and regional immune responses by retroviral-mediated intraperitoneal IFN γ gene therapy in a murine ovarian cancer model (238).

4.2.2.4. TNFα.
Adenovirus-mediated TNFα gene therapy also showed significant antitumor activity but this strategy resulted in systemic toxicity when directly injected into tumors (242). Induction of systemic toxicity is a major factor limiting the use of TNFα gene therapy for cancer.

4.2.3. Genetic tumor vaccines
In this strategy, tumor cells are modified to enhance the immunogenicity of tumors by gene therapy with genes encoding cytokines, co-stimulatory molecules and MHC molecules. Vaccination with these modified tumor cells protected animals against subsequent challenge with unmodified parental tumor cells. These vaccine strategies are already described above as part of altering immunogenicity of tumors. In order to further improve on these strategies, combined gene therapy with B(7 1) (CD80) and IL-12 of tumor cells resulted in enhanced lymphocyte proliferation and induced recognition and cytotoxic effects of CTLs on tumor cells thus prolonging the survival time in syngeneic rat model (243). The enhanced therapeutic effect is a result of synergistic effect of IL-12 and CD80 in stimulating cytotoxic CD8+ T-cell lines.

It is known that CD80 (B7-1) and Interferon-gamma (IFN-gamma) play important roles in antitumor immunity induced by T lymphocyte. A combination gene therapy with human B7-1 and IFN-gamma using bicistronic retroviral vector pLXSN, in a human immune reconstituted SCID mice (hu-PBL-SCID) model showed significant enhancement in the tumor-specific cytotoxic activity. and markedly decreases tumorigenicity (244). These studies support the rationale for use of adenoviral delivered B7-1 in combination with cytokines as a genetic vaccination therapy for ovarian cancer (245).

4.2.4. Dendritic cell vaccines
Dendritic cells are potent antigen presenting cells (246) and they can directly sensitize T cells and stimulate the development of antigen specific immune response (247). Because of their immunostimulatory capacity, immunization with DCs presenting tumor antigens has been proposed as a treatment regimen for cancer. In this regard, strategies that use antigen-pulsed DCs have protected animal models from tumor challenge (247-252). In order to further improve the DCs for vaccination, gene therapy approach was used to express an antigen of interest for a longer period (248, 253, 254). These DCs have been shown to be more potent primers of antitumor immunity both in vitro and in vivo animal models of disease (253). Efforts are currently being directed on optimizing DC activation with polycistronic constructs of cytokine genes, and over expressing the most relevant tumor-associated peptides (255).

One of the most challenging obstacles for DC-based vaccination has been the means by which to deliver tumor antigen-encoding genes to DCs. Although adenoviral vectors are widely used for gene delivery to tumor cells, DCs are refractory to adenovirus infection. In order to overcome this problem, our group developed novel strategies to redirect adenoviral vector to CD40 by means of bispecific antibodies to enhance gene transfer to DCs (256). This redirection of Ad vector carrying the gene for a human papillomavirus (HPV) E7 tumor antigen showed enhanced protection against HPV-16-induced tumor cells in a murine model (254). In addition to efficient gene transfer, by virtue of targeting to CD40, this vector initiated phenotypic changes characteristic of DC maturation. Our group also developed other strategies.
such as genetic modification of adenoviral vector by incorporating RGD motifs in the capsid (257), or retargeting Ad vectors to DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) (258) to enhance their gene delivery efficacy.

5. Antiangiogenesis

Tumor angiogenesis is critical for supporting the growth of tumor beyond 2 cu mm as well as for its metastases. (259). Similar to many carcinomas, ovarian cancer is also critically dependent on tumor growth and metastases (260-263). Multiple pro-angiogenic factors such as basic fibroblast growth factor (bFGF), Vascular endothelial growth factor (VEGF), matrix metalloproteinases, and secreted protein, acidic and rich in cystein, angiopoietin, Thrombospondin 1, IL-8, Platelet derived endothelial growth factor, platelet derived growth factor are involved in the formation of tumor angiogenesis. Among these, VEGF was shown to be most potent and well characterized.

5.1. Vascular endothelial growth factor

VEGF is overexpressed in ovarian cancer cells (260, 261, 264-269) and is associated with increased tumor growth, ascites fluid accumulation (260, 264, 270-273), metastases, poor prognosis, and shorter survival periods in ovarian cancer patients (263, 264, 268, 269, 274, 275). On this basis, inhibition of VEGF function is a logical approach to inhibit tumor angiogenesis. Several gene therapy approaches have been developed to target VEGF. Since major pro-angiogenic effects of VEGF are mediated through the endothelium-specific VEGF receptors, Flt-1 and Flk-1/KDR and Flt-4 (276-279), Our group has employed a novel soluble FLT-1 gene therapy approach to inhibit binding of VEGF to its receptors. Soluble FLT-1 is an alternatively spliced version of VEGF receptor, which prevents VEGF binding to its receptors by sequestering it or by heterodimerizing with either FLT-1 or FLK-1 and thereby inhibiting its angiogenic function. Adenovirus-mediated sFLT-1 gene therapy significantly inhibited tumor growth and prolonged survival of mice when given intraperitoneally in a human ovarian cancer intraperitoneal xenograft mouse model (280). This approach proved to be efficient in loco-regional inhibition of tumor growth. However, systemic delivery of Ad.sFLT-1 in order to inhibit metastases resulted in significant hepatic toxicity and early death of mice compared to controls. This hepatic toxicity is a result of ectopic localization of Ad.sFLT-1 mainly to the liver (281). So liver toxicity is the major limitation of this systemic delivery approach. Tumor specific expression of sFLT-1 using tissue specific promoters which exhibit “liver off” and “tumor on” profile may help in overcoming this limitation.

To achieve an effective inhibition of tumor angiogenesis, long-term expression of antiangiogenic agents is desired. Towards this end, we and others have employed adeno-associated viruses (AAV) (which accord long term gene expression) for sFLT-1 gene delivery (282) and demonstrated significant reduction in ascites and tumor growth in an in vivo ovarian cancer mouse model. In this approach, unlike systemic delivery of sFLT-1, intramuscular delivery of sFLT-1 did not result hepatotoxicity. The reason for this variation could be that adenoviral-mediated systemic delivery results in high sFLT-1 expression due to preferential ectopic localization of Ad.sFLT-1 to liver whereas with intramuscular delivery, this ectopic localization is not observed.

An alternate way of inhibiting VEGF is downregulation of VEGF production by anti-VEGF hairpin ribozyme gene therapy (283). Thi strategy resulted in proliferation inhibition in vitro and decreased rate of tumor growth in vivo (284). A more recent study used a novel RNA interference (RNAi) approach to specifically "knock-down" VEGF (285). RNA interference (RNAi) is a sequence-specific posttranscriptional gene silencing mechanism, which is triggered by double-stranded RNA (dsRNA) and causes degradation of mRNA homologous in sequence to the dsRNA (286-289). Targeting VEGF by RNAi resulted in significant reduction in VEGF expression levels and tumor growth suggesting that specific “knock down” of VEGF by RNAi gene therapy approach may provide a more powerful strategy for inhibition of tumor angiogenesis.
than ever before to inhibit tumor angiogenesis in ovarian cancer (285). Further, use of stable expression vectors such as AAV, lentiviral vectors may enhance the efficacy of this approach for ovarian cancer.

5.2. Angiostatin and Endostatin
Angiostatin is a naturally occurring proteolytic fragment of plasminogen composed of four functional domains known as kringles out of which, first three kringles were shown to suppress angiogenesis (290). Endostatin is a proteolytic fragment of collagen XVIII, was also shown to inhibit tumor angiogenesis (291, 292). Adenoviral-mediated gene therapy with either angiostatin, or endostatin, angiostatin and or endostatin fusion genes in an intraperitoneal human ovarian xenograft model of ascites resulted in statistically significant down regulation of ascites formation, tumor growth, vascularity, and prolongation of animal survival (293). Further gene therapy with mutated form of endostatin (point mutation at position 125, proline substituted with alanine) significantly improved antiangiogenic activity by virtue of its improved binding to endothelial cells and inhibited tumor growth in athymic mice (294). Endostatin gene therapy was also shown to enhance the therapeutic efficacy when combined with DDP in an i.p. ascites model (295).

5.3. Platelet factor-4 (PF4)
Platelet factor-4 (PF4) (296) as well as a peptide fragment of PF4 (amino acids 47–70) were shown to inhibit functions of VEGF and FGF-2 by preventing their binding to their respective receptors (297, 298). Adenoviral-mediated PF4 gene therapy down regulated ascites formation, inhibited tumor growth, vascularity, and prolong animal survival (293) and is associated with significant downregulation of VEGF. Although the mechanism of down regulation was not clear, it was thought that intracellular binding of PF-4 to VEGF may have resulted in inhibition of VEGF secretion.

5.4. HGF/NK4
Hepatocyte growth factor (HGF) regulates migration, invasion, and angiogenesis in cancer. It can be inhibited by NK4, which is a large part of the α-chain of HGF, containing four kringle domains. NK4 competitively inhibits the specific binding of HGF to the receptor (299). Plasmid mediated expression of NK4 inhibited ovarian cancer cell growth and migration both in vitro and in vivo and suppressed intraperitoneal dissemination and extended the survival of mice with ovarian cancer (300).

5.5. IL-10
Interleukin-10 (IL-10) is produced by T lymphocytes, which shows immunosuppressive, and antiangiogenic effect. Transfection of IL-10 significantly inhibited tumor angiogenesis, growth and metastasis and resulted in prolonged survival of mice injected with ovarian cancer cells (301).

5.6. Thymosin-β
Thymosin-β is a monomeric actin sequestering protein that regulates actin dynamics (10). It also acts as a potent inhibitor of angiogenesis and tumor growth by its interaction with Ras. Interaction with Ras results in inhibition of the downstream mitogen-activated protein kinase/extracellular signal-regulated kinase-signaling pathway, leading to decreased vascular endothelial growth factor production. Over expression of thymosin-β (10) significantly inhibited vascular endothelial growth factor-induced endothelial cell proliferation, migration, invasion, and tube formation in vitro and markedly inhibited tumor growth and reduced tumor vascularity in vivo in a xenograft mouse model of human ovarian cancer (302).

5.7. Bikunin
Bikunin is a Kunitz-type protease inhibitor (303). It plays a key role in the inhibition of tumor cell invasion and metastasis, possibly through the direct inhibition of cell-associated plasmin activity and suppression of metastasis-associated molecules urokinase-type plasminogen activator (uPA) and uPAR expression (304). It also inhibits several signaling cascades including MAPK and PI3K/Akt (305). Transfecting the highly invasive human ovarian carcinoma cell line HRA, with bikunin cDNA
significantly reduced uPA expression resulting in reduced invasiveness, and suppressed ERK1/2 activation. In an intraperitoneal ovarian cancer mouse model, bikunin gene transfection prevented ascites and peritoneal disseminated metastasis (306).

5.8. Other molecules (HER-2/neu, p53, and ras)
Recent evidence suggests that alterations in oncogene expression stimulate angiogenesis by increasing endothelial mitogenesis, increasing production of proangiogenic factors, and increasing endothelial cell resistance to apoptosis and by down regulation of antiangiogenic factors (307). In this regard, HER-2/neu, which is over expressed in ovarian carcinoma, as well as ras oncogene, was shown to increase VEGF expression. Loss of wild type p53 was shown to augment hypoxia inducible factor (HIF-1)-mediated VEGF expression. In addition, p53 and ras stimulate thrombospondin-1. Therefore targeting these oncogenes and apoptosis regulatory genes not only inhibits tumor growth by direct tumor cell killing, but also by inhibition of tumor angiogenesis. Down regulation of HER-2/neu by retrovirus-mediated anti-HER-2/neu siRNA gene therapy resulted in slower proliferation, increased apoptosis, increased G0/G1 arrest, and decreased tumor growth of ovarian cancer cells (308) and is associated with decreased expression of the pro-angiogenic VEGF, suggesting that Her-2/neu stimulates tumor growth at least in part by regulating angiogenesis. Similar studies showing anti-angiogenic effects with p53 and ras have not been conducted so far in the context of ovarian cancer.

Although many anti-angiogenic approaches using recombinant IFN-α, -β, -γ, IL-12, thrombospondin 1, TIMPS and other antiangiogenic proteins have been endeavored for ovarian cancer therapy, gene therapy strategies using their genes and other angiogenic genes such as IL-8, TNF-α, angiopeititin have not been conducted so far. Direct comparison studies of these molecules may help in determining the extent of role played by these genes in tumor anti-angiogenesis.

The advantage of inhibiting tumor angiogenesis for the therapy of cancer is that unlike cancer cells, endothelial cells are genetically stable, so it is easy to target them. Killing tumor vasculature results in blockade of nutrient supply to a large number of tumor cells thus results in amplified cell killing compared to approaches that target individual tumor cells. Another main advantage of anti-angiogenesis approach is that it is possible to partially overcome current limitations of low gene transduction efficiency of vectors to tumor cells by targeting tumor vasculature because it does not require every tumor cell to be transduced to achieve tumor cell killing. On this basis, anti-angiogenesis gene therapy approaches may show promise in clinical studies particularly after cytoreduction by surgery to eliminate any residual tumor. Thus far therapy clinical trials employing anti-angiogenic gene therapy strategies for ovarian cancer have not been conducted.

6. Gene expression profiling of ovarian tumors
Studies understanding the ovarian cancer biology have identified many genes which are dysregulated in ovarian cancer and have been targeted for ovarian cancer therapy. However, a comprehensive knowledge of all the genes that are involved in ovarian cancer will provide a better understanding of the development and progression of ovarian cancer. Identification of all genes involved in ovarian cancer simultaneously was not possible previously due to lack of available methods. Recent development of cDNA microarray technology made this possible. In this regard, a recent study which analyzed gene expression profiles of nine ovarian tumors identified 55 genes that are commonly up regulated and 48 genes that are down regulated in ovarian cancer compared to normal ovarian tissues (309). Further they have shown that 115 genes are differentially regulated between serous and mucinous adenocarcinomas. Investigation of these genes will help in further understanding the molecular mechanisms involved in ovarian carcinogenesis thus defining new genetic targets for effective management of ovarian cancer. This cDNA array technology can also be exploited for predicting metastatic ability, invasiveness, and chemosensitivity as
well as diagnosing disease at an early stage, and defining effective therapies. Most importantly, this technology will become an essential source for the development of personalized treatments in the future.

7. Gene delivery vectors for ovarian cancer therapy

A variety of vectors both viral and non-viral vectors have been employed for gene therapy of ovarian carcinoma (Table 2).

<table>
<thead>
<tr>
<th>Table 2. Vectors For Gene Therapy Of Ovarian Cancer</th>
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<tbody>
<tr>
<td><strong>Viral Vectors</strong></td>
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<tr>
<td>Adenovirus</td>
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<tr>
<td>Adeno-associated virus</td>
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<tr>
<td>Retrovirus</td>
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<tr>
<td>Moloney leukemia virus</td>
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<tr>
<td>Harvey murine sarcoma virus</td>
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<tr>
<td>Avian spleen neurosis virus</td>
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<tr>
<td>Lentivirus, eg., HIV, SIV, FIV.</td>
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<tr>
<td>Herpes simplex virus</td>
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<td>Polyoma virus</td>
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<td>Vaccinia virus</td>
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<tr>
<td>Papilloma simian virus</td>
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<tr>
<td><strong>Non-viral vectors</strong></td>
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<tr>
<td>Calcium phosphate</td>
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<tr>
<td>Ultrasound</td>
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<tr>
<td>Electroporation</td>
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<tr>
<td>Polymers</td>
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<tr>
<td>Cationic lipids/liposomes</td>
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<tr>
<td>Cell-based vectors (mesothelial, HUVECs, Fibroblasts and lymphocytes).</td>
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</table>

7.1. Viral vectors

Viruses have the natural ability to infect cells. This natural ability of viruses has been exploited for delivering genes to the tumor cells by first rendering them non-pathogenic by deleting the genes responsible for replication. Cloning therapeutic genes in place of deleted genes allowed them to carry gene of interest. Some of the major viral vectors systems studied in the context of ovarian carcinoma are 1) Adenovirus, 2) Adeno-associated virus, and 3) Retrovirus,

7.1.1. Adenovirus

7.1.1.1. Ad Vector biology

It is a non-enveloped icosahedral particle that encapsulates a 36 Kb double stranded DNA genome, which encodes upto 70 gene products. The viral genome contains 5 early transcription units (E1A, E1B, E2, E3, and E4), two early delayed transcription units (pIX and 1Va2) and 5 late units (L1-L5), which mostly encode structural proteins for the capsid and internal core. Inverted terminal repeats (ITR) at the end of the
gene function as replication origins. The E1A region is activated shortly after infection and is essential for the activation of other promoters and the replication of the viral genome. E1 region is deleted to render them non-replicative. This replication deficient virus is propagated in E1 transcomplementing cells such as 293 cells (310), or 911 cells (311). E3 region encodes proteins which modulate host defense but are not required for viral replication can also be deleted to increase the packaging size limit of therapeutic gene of interest. Normally therapeutic gene of interest upto 8.1 to 8.2 Kb can be cloned in place of deleted E1 region.

The capsid structure of adenovirus is made up of three major proteins that make up the viral particle. Hexon is the most abundant structural component and constitutes bulk of mature virion. Five subunits of the penton base are found in each of the twelve vertices of the capsid and form the platform for the twelve fiber homo-trimers that protrude from the virion. At the distal tip of each linear fiber is a globular knob domain through which it binds to the cells and infects them.

There are more than 50 serotypes of adenoviruses. Of these, serotypes 2 and 5 have been widely employed for ovarian cancer gene therapy mainly due to their advantages such as high in vivo stability, superior gene transfer efficiency to numerous dividing and non-dividing cell targets in vivo, feasibility of production in high titers, and not being linked to any severe disease in humans.

7.1.1.2. Challenges of employing adenoviral vectors for gene therapy
Although good therapeutic effect and safety have been shown by Ad vectors, in numerous gene therapy studies, clinical translation of the same studies failed to show similar therapeutic benefit and safety (312). Insufficient gene delivery by vectors to tumor cells and transduction of normal cells was believed to be one of the key reasons behind this limited clinical efficacy. By enhancing tumor cells transduction, and limiting normal cell transduction, both high therapeutic gains and low or limited toxicity to normal cells can be achieved. Towards this end many strategies have been developed to achieve tumor specific gene delivery. These strategies can be broadly classified as “transductional strategies” and “transcriptional strategies”. Transductional strategies are aimed at improving the infectivity/ transductional ability of vectors to cancer cells in order to improve the gene delivery specifically to cancer cells. Transcriptional strategies are aimed at restricting the therapeutic gene expression specifically to cancer cells thereby limiting toxicity to normal cells.

7.1.1.3. Targeting strategies to improve gene transfer
7.1.1.3.1 Transductional strategies
Adenoviral vectors serotypes 2 and 5 are widely used and continue to show increasing promise in the context of ovarian cancer gene therapy. Therefore improving the tumor targeting capacity of these serotype Ads is a logical approach. For designing and developing transductional targeting strategies, understanding of the adenovirus infection pathway is critical. The mechanism of adenovirus cellular entry involves initial high affinity binding of the fiber knob domain of adenovirus to its cognate cellular receptor known as coxsackie and adenovirus receptor (CAR) expressed on cell surface (313), followed by interaction of penton base Arg-Gly-Asp (RGD) motifs with cellular integrins $\alpha_\text{V} \beta_3$, $\alpha_\text{V} \beta_5$, $\alpha_\text{V} \beta_1$, or other integrins which results in endocytosis of the virion (314, 315). So the rate limiting step is initial CAR binding and cells expressing low levels of CAR are refractory to adenovirus infection (32, 316, 317). Since human ovarian primary cancer cells express low levels of CAR, clinical efficiency of adenovirus mediated gene therapy is low (32, 317-319). Therefore redirecting adenovirus to other receptors or tumor-associated antigens over expressed in cancer cells for initial binding may help in overcoming low gene transfer efficiency. Based on this hypothesis, several targeting strategies have been endeavored. These approaches can be broadly classified as 1) Adapter molecule-based targeting 2) Genetic modification of the adenovirus capsid.
Adapter molecule-based targeting

In adapter-molecule based targeting approach, a “molecular bridge” between the adenovirus vector and a cell surface receptor is formed by “bi-specific” molecules or molecular conjugates that link the Ad vector and target cell surface receptor. This approach bypasses the requirement of CAR binding for cell entry. Several bi-specific adapter molecules have been used which include but are not limited to bi-specific antibodies, chemical conjugates between antibody fragments (Fab) and cell-selective ligand such as folate, Fab-antibody conjugates using antibody against target cell receptors such as epidermal growth factor receptor (EGFR), Fab-peptide ligand conjugates such as basic fibroblast growth factor-2 (FGF-2) and recombinant fusion proteins that incorporate Fabs and peptide ligands.

Adenovirus retargeting by the adapter-based approach was first demonstrated in vitro by our group using bi-specific conjugate consisting of an anti-knob neutralizing Fab chemically linked to folate to target folate receptor which is over expressed in cancer cells (320). Furthermore, retargeting adenoviral vector carrying the gene for herpes simplex virus thymidine kinase, using the Fab-folate conjugate resulted in specific killing of cells that over expressed the folate receptor. In a similar targeting approach, adapter comprising of the anti-knob Fab fused to FGF-2 was utilized to target Ad vectors to FGF receptor positive ovarian cancer cells. Retargeting Ad to FGF resulted in more than 9 fold increase in reporter transgene expression in ovarian cancer cells and in an intraperitoneal ovarian cancer mouse model, retargeting Ad encoding HSV.tk to FGF resulted in prolonged survival of mice to 44 days compared to 36 days of controls (182). Importantly, this targeting system reduced hepatic toxicity (321).

Other Fab-ligand conjugates targeted against Ep-CAM, Tag-72, EGF receptor, CD-40, and other cell markers have been employed in a similar manner with promising results (256, 322-325). Although these bi-specific chemical conjugates showed the feasibility of retargeting Ad, producing such retargeting molecules is relatively complex and expensive to develop. Therefore, a simple and efficient method of targeting Ad to specific cells was developed by our group (316). We constructed a single fusion adapter molecule by genetically fusing truncated, soluble form of CAR (sCAR) to epidermal growth factor (EGF) (326). Retargeting Ad using this molecule resulted in up to 9 fold increase in gene expression in several EGFR over expressing cancer cells and in an intraperitoneal ovarian cancer mouse model, retargeting Ad encoding HSV.tk to FGF resulted in prolonged survival of mice to 44 days compared to 36 days of controls (182). Importantly, this targeting system reduced hepatic toxicity (321).

Several other bi-specific adapter based targeting strategies have been reported but they are not in the context of the ovarian cancer. Adapter-based Ad targeting studies provide compelling evidence that Ad tropism modification can be achieved by targeting alternate cellular receptors and that this modality augments gene delivery to CAR negative or low CAR expressing target cells. Although two-component targeting has shown promising results for retargeting Ad to new receptors over expressed in cancer cells, these Ad based delivery systems have more complex pharmaco-dynamics and kinetics, and their stability in humans as not been studied yet. Therefore one-component systems may be more easily applicable to human gene therapy trials.

Targeting by genetic modification of the adenovirus capsid

Based on a clear understanding of native Ad cell recognition, development of genetically targeted vectors has rationally focused on the fiber knob domain, the primary determinant of Ad tropism. Three primary modes of genetic tropism modification via structural alterations to the Ad fiber have emerged. 1) Ligand incorporation into the fiber knob, 2) Fiber pseudotyping, 3) De-knobbing of the fiber. In addition to the fiber, other capsid locales have been utilized for inclusion of targeting moieties.

Incorporation of targeting ligands into the Ad knob domain without ablating native CAR-binding has resulted in Ad vectors with expanded cell recognition. Based on the rigorous structural analysis of the
knob domain two separate locations C terminus and HI loop have been identified that tolerate genetic manipulation without loss of fiber function (329, 330). Recent study by our group (330) demonstrated that the Ad5 will tolerate peptide insertions up to 100 amino acids with minimal negative effects on virion integrity, thus providing enormous potential for ligand incorporation at this site.

Our group incorporated an RGD peptide sequence into the HI loop of the Ad knob (316). The resulting vector Ad5.lucRGD, which uses the RGD/cellular integrins interaction dramatically augmented gene transfer efficiency to ovarian cancer cells up to 2 to 3 orders of magnitude (316). In a subsequent study conducted by our group, the enhancement of gene transfer in ovarian cancer cell lines ranged from 2.5- to 471.6-fold in cell lines. More importantly, this genetic modification enhanced the gene transfer in primary tumor cells obtained from patient ascites 26.1- to 64.0-fold, and in tumor explants 1.6- to 11.1-fold. Although gene transfer to normal mesothelial tissue was slightly augmented by RGD retargeting, the level of gene transfer was much lower than that seen in ovarian cancer cells (317). Based on these results a number of in vitro and in vivo studies have employed RGD modified vector for in vitro and in vivo studies which showed enhanced therapeutic effect in ovarian cancer and other cancer cells including enhanced infectivity in Ad refractory dendritic cells (33, 183, 257, 280, 281, 327, 331-337). In fact combining genetic modification strategy (RGD) with adapter based strategy (single-chain bi-specific antibody directed against the human EGFR and against the fiber knob of the Ad) further increased gene transfer efficiency in glioma cells (331). A very important finding was that RGD incorporation by genetic modification allowed efficient gene transfer to cell lines and primary ovarian cancer cells in the presence of ascites fluid containing high-titer neutralizing anti-Ad antibodies suggesting that genetic modification of Ad by incorporating RGD targeting ligand not only enhanced the gene transfer, but also circumvented inhibitory effect of neutralizing Ad antibodies present in ascites (36). Further, in vivo studies to investigate the effect of pre-existing neutralizing antibody (NAbs) in naive mice and the effect of induced NAbs in mice immunized with either an RGD or non-modified Ad5 vector on the transduction efficiency of adenoviral vectors revealed that the RGD-modified human Ad vectors appear to be less recognizable than unmodified Ad to preexisting NAbs in mouse models. RGD-modified Ad vectors also appear to elicit a relatively lower level of NAbs that may also contribute to the higher gene transduction efficiency of these modified vectors. These results suggest that RGD-modified Ad vectors may be reagents of great clinical utility in the context of preformed anti-Ad immunity and in the setting of repetitive dosing for ovarian carcinoma (338).

Other peptides incorporated into HI loop locale of Ad fiber knob include, the 59 amino acid c-terminus of the Fc-binding domain of Staphylococcus aureus Protein A. This vector accorded a 35-fold increase in gene delivery to human monocyte derived dendritic cells when coupled to a soluble anti-CD40 single chain antibody fusion protein (339). In addition, an endothelial cell-binding motif SIGYLPLP has been inserted into the HI loop. Of note, incorporation of this ligand was accompanied by CAR-blocking knob mutation (340).

Incorporation of targeting ligands at C terminus of the fiber knob to redirect the Ad was also endeavored. Incorporation of polylysine pK7 motif at C-terminus, which binds to heparan sulfate-containing receptors normally over expressed in ovarian cancer cells, showed significant increase in gene transfer. In fact double modification by incorporation of pK7 at C-terminus and RGD in HI loop showed additive effects in gene transfer in both ovarian cancer cell lines and primary tumor cells (341).

7.1.1.3.1.3. Ad fiber psuedotyping
This strategy involves genetic replacement of either entire fiber or knob domain with its structural counterpart from another human Ad serotype that recognizes a cellular receptor other than CAR. These pseudotyped vectors display CAR-independent tropism by virtue of their natural diversity in receptor recognition and showed enhanced infectivity and gene transfer in various clinically relevant cell types including B cells, CD34+ hematopoietic cells, synovial tissue, human cardiovascular tissue and others.
Our group created an Ad5 vector that expressed a chimeric fiber displaying the Ad serotype 3-knob domain (Ad5/3) that demonstrated the same CAR-independent cell recognition, as does Ad3. Ovarian cancer cells are refractory to Ad infection due to low level of CAR expression but they are relatively high in serotype 3 receptors which are now identified as CD80 (B7.1) and CD86 (B7.2) compared to CAR. Therefore use of Ad5/3 chimeric vector will overcome this limitation of low CAR levels and enhance gene delivery (33). On this basis, evaluation of Ad5/3-mediated gene delivery to ovarian carcinoma cells showed 5.18-, 16.5-, 5.63-, and 12.5-fold higher transgene expression respectively in OV-4, SKOV3.ip1, PA-1, and Hey cell lines and also showed significant increase in gene transfer in human primary ovarian tumor samples (33, 350) but biodistribution, liver toxicity, and rate of blood clearance of Ad5/3 was not different from that of Ad5 when administered intravenously or intraperitoneally, suggesting an excellent safety profile for Ad5/3 (33). However, clinical trials are needed to determine these parameters in human.

Additional studies by our group showed that a mosaic virus possessing both the Ad5 knob and the Ad3 knob on the same virion was able to utilize either primary receptor, resulting in expanded tropism in both CAR dominant and Ad3 receptor dominant cells (351).

As previously mentioned, the success of gene therapy depends not only on enhancing gene transfer to tumor cells but also decreasing gene delivery to normal tissue particularly liver, in case of adenoviruses to reduce toxicity. In this regard, our group (352) developed a novel strategy to address the problem of liver uptake and improve the tumor/liver ratio by genetic replacement of the Ad fiber shaft. The authors constructed an Ad5.Ad3.SH.luc1 an Ad5-based vector that contains the fiber shaft from Ad serotype 3 but the fiber knob from Ad serotype 5. Intravenous and intraperitoneal administration of this vector with short fiber in a human ovarian cancer xenograft mouse model displayed significantly lower liver tropism but increased tumor gene transfer thus resulting in high tumor/liver ratio (352). This enhanced targeting strategy could improve the effective tumor dose and reduce side effects, and thereby increase the bioavailability of therapeutic agents.

The above studies showed the advantages of enhanced gene delivery by both approaches of targeting ligand incorporation and vector chimerism. To determine the possibility of further enhancing these approaches, our group developed a novel "complex mosaicism" approach for fiber modification, which combines serotype chimerism with peptide ligand(s) incorporation in a single-fiber molecule. We incorporated integrin-binding peptide RGD-4C in the HI-loop, at the carboxy (C)-terminus, or at both locales of the Ad3 knob of Ad5/3 chimera fiber in order to retarget simultaneously the Ad vector to integrins and Ad3 receptors. These complex mosaic Ad vectors bearing chimeric fiber (F.5/3), with or without C-terminal RGD-modification of Ad3 knob, demonstrated up to 55-fold gene transfer increase in bladder cancer cell lines (353). Although this augmentation was primarily due to Ad3 receptor targeting, some contribution of RGD-mediated integrin-targeting was also observed, suggesting that complex mosaic modification can function in a dual-receptor targeting via a single Ad3 fiber knob.

Further advances in developing targeted Ads was based on the feasibility of replacing native fiber with knobless fiber (354, 355), which allows complete ablation of CAR binding. The addition of a targeting ligand to this knobless fiber can overcome size and other structural problems associated with knob modification; for example, addition of foreign trimerization motif fused with six histidine targeting motif resulted in 100 fold increase in gene transfer to tumor cells (356) and addition of CD40L to these fiberless knobs, resulted in 100 fold increase in gene transfer in dendritic cells (357). Evaluation of these vectors for enhanced infectivity in the context of ovarian cancer is worth investigating.

In addition to the modification strategies incorporating at HI loop and C-terminus, incorporating targeting ligands at other sites such as hexon and pIX are also showing promise (358).
7.1.1.3.2. Transcriptional targeting strategies
Advances in Ad tropism modification and transductional targeting have shown enhancement in gene delivery to cancer cells and further studies will continue to improve the tumor specific gene delivery. An additional layer of tumor specificity can be gained by restricting transgene expression to the target tumor cells. This can be achieved by transcriptional targeting strategy, which seeks to exploit the unique transcriptional profile of the target cell by employing target cell-specific transcriptional control elements (promoters) to restrict the therapeutic transgene expression to target cells. Transcriptional targeting strategy has dual advantage. In addition to enhancing the therapeutic efficacy, it also helps in decreasing the toxicity to normal tissues by virtue of restricting the gene expression to tumor cells only. Thus this strategy is valuable for achieving a high therapeutic index.

Table 3. Promoters For Transcriptional Targeting Of Ovarian Cancer

<table>
<thead>
<tr>
<th>Promoter</th>
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<tbody>
<tr>
<td>L-plastin</td>
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<tr>
<td>MUC1/DF3</td>
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<tr>
<td>COX-2</td>
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<tr>
<td>Midkine</td>
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<tr>
<td>HER-2/neu</td>
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<tr>
<td>OSP-1</td>
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<tr>
<td>KDR/flk-1</td>
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<td>SLPI</td>
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<td>hTERT</td>
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<td>HE4</td>
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<td>CXCR4</td>
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<tr>
<td>Survivin</td>
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<tr>
<td>Metallothionein</td>
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<tr>
<td>Mesothelin</td>
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<tr>
<td>beta catenin-dependent</td>
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<td>synthetic promoter</td>
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</table>

7.1.1.3.2.1. The leukocytes plastin (L-Plastin) promoter
A number of promoters have been explored to determine their specificity and activity in ovarian cancer (Table 3). The leukocytes plastin (L-Plastin) is a member of the actin-binding protein which is known to be aberrantly active in a wide variety of cancer cells (359). This promoter driving a reporter gene lacZ was compared to the ubiquitously expressed cytomegalovirus (CMV) promoter which is widely used for gene expression (360). CMV driven gene expression was seen in both tumor cells as well as in normal cells. In contrast, L-plastin driven LacZ expression was observed only in tumor cell lines and ascites samples with negligible expression in normal human skin fibroblasts and normal mesothelial cells suggesting the utility of this promoter for tumor specific gene expression in ovarian cancer. On this basis, an adenovirus constructed using this promoter to drive CD expression in ovarian cancer cells was shown to suppress ovarian tumor growth in mice (361) when treated with 5-fluorocytosine (5FC).

7.1.1.3.2.2. MUC1/DF3 promoter
The MUC1/DF3 gene encodes the polymorphic epithelial mucin (PEM). Over three quarters of human breast and epithelial ovarian carcinomas over express this protein but not normal peritoneal mesothelium (362). The DF3/MUC1 promoter showed ovarian cancer specificity when driving the expression of BAX in vitro. Intraperitoneal injection of adenovirus with DF3/MUC1 promoter driving BAX into ovarian
tumor bearing mice, showed most prominent expression of BAX in tumor tissue resulting in greater than 99% eradication of ovarian tumors (57).

7.1.1.3.2.3. COX-2 and Midkine promoters
Other promoters which were evaluated in ovarian carcinoma include, cyclooxygenase-2 (COX-2) promoter and the midkine (MK) promoter. Midkine is a heparin-binding, growth and differentiation factor, highly expressed in many malignant tumors (363). Cox-2 is an inducible form of enzyme, which synthesizes prostaglandins from arachidonic acid is associated with cellular growth and inflammatory processes is shown to be highly expressed in a number of epithelial tumors (364, 365). Our studies demonstrated that both promoters were active in a panel of ovarian cancer cell lines, as well as primary tumor cells, with a reduced level of activity in normal primary mesothelium and liver. In addition, a clear decrease in toxicity in normal tissue following injection of Ad vectors expressing HSVtk driven by COX-2 and or MK promoters was demonstrated (366).

7.1.1.3.2.4. HER-2/neu promoter
HER-2/neu is over expressed in 25-30% human of ovarian carcinomas. Using a retroviral vector encoding GFP under the control of HER2 promoter, Zheng et al., demonstrated tumor-specific expression of the reporter gene in ovarian cancer cell lines (367).

7.1.1.3.2.5. Ovarian-specific promoter 1 (OSP-1)
Ovarian-specific promoter 1 (OSP-1) is part of a retrovirus-like element specifically expressed in the rat ovary. The expression of herpes simplex virus thymidine kinase (HSV-TK) under OSP-1 control resulted in approximately 50-fold more antitumor activity in the A2780 human ovarian cancer cell line upon injection of GCV compared to control tumor cells and showed market anti-tumor efficacy in vivo tumor models (368, 369).

7.1.1.3.2.6. KDR/flk-1 promoter
The KDR/flk-1 gene encodes for a receptor for VEGF. Although it was originally considered as an endothelial cell-specific gene, it is expressed in murine L1 sarcoma and OVP-10 human ovarian carcinoma cell lines as well. KDR promoter-driven cytosine deaminase gene can be specifically expressed in these cells leading to sensitization to 5-fluorocytosine, both in vitro and in vivo (370).

7.1.1.3.2.7. The secretory leukoprotease inhibitor (SLPI) promoter
The SLPI has been shown to be over expressed in ovarian carcinoma as well as other carcinomas but its expression in normal organs such as liver is low (371) which makes it an ideal candidate promoter. On this basis, SLPI promoter was utilized to drive transgene expression in ovarian cancer cell lines and primary tumor cells obtained from ovarian cancer patients in an Ad context in vitro (372). A murine orthotopic model of peritoneally disseminated ovarian cancer was used to demonstrate high tumor transgene expression vs low liver expression with the SLPI promoter; Ad-delivered HSVtk under the control of the SLPI promoter was able to increase survival in combination with GCV in this model (372). To further increase the tumor targeted gene expression, our group (373) combined this transcriptional targeting approach using SLPI promoter with transductional targeting approach using bi-specific adapter molecule targeting erbB-2. This dual-targeting strategy increased the efficiency and specificity of transgene expression in reporter and cell-killing assays in vitro and in vivo (373).

7.1.1.3.2.8. Human telomerase reverse transcriptase (hTERT) promoter
hTERT is the catalytic subunit of human telomerase (374), which is highly active in immortalized cells and more than 90% of human cancers, but is stringently repressed in most normal somatic cells with the potential exception of stem and hematopoietic cells (375-377). In ovarian carcinoma, more than 90% patient tumor samples showed telomerase activity (376). Adenoviral-mediated CD expression under the control of hTERT demonstrated 18-fold sensitization to the prodrug CB1954 specifically in ovarian cancer
cells including chemoresistant cells but not in normal epithelial cells (378). **In vivo** administration resulted in significant therapeutic effect in a mouse cancer model. Similar studies using hTERT driving HSVtk showed tumor specific cytotoxicity but not in normal ovarian epithelial cells when injected with GCV (185, 379). A comparison of hESE1 (human epithelium-specific ets transcription factor promoter, SLP1, and OSP1 with hTERT in ovarian carcinoma cells showed that hTERT is highly tumor selective and exhibited the strongest transcriptional activity compared to the other three promoters (380); This suggests that hTERT is an optimal promoter for ovarian cancer transcriptional targeting.

7.1.1.3.2.9. Human epididymis protein 4 (HE4) promoter
HE4 is frequently over expressed in ovarian cancer. Berry et al., showed that the HE4 promoter is highly transcriptionally active in 5 out of 7 ovarian cancer cells tested; additional studies exploiting this promoter for transcriptional targeting for ovarian cancer are in progress (381).

7.1.1.3.2.10. Ceruloplasmin promoter
A recent study showed that ceruloplasmin is another promising promoter based on its ability to selectively enhance gene expression (up to 30-fold higher) in ovarian cancer compared with immortalized normal cells (382). Further studies are being conducted to explore its potential for ovarian cancer targeting.

7.1.1.3.2.11. CXCR4 and survivin promoters
Our recent studies evaluated two novel promoters, CXCR4 and survivin. CXCR4 is an alpha-chemokine receptor gene, which is markedly up regulated in breast cancer cells, but undetectable in normal mammary epithelial cells. Survivin is a novel member of the inhibitor of apoptosis (IAP) protein family, which is expressed in human cancers but undetectable in normal differentiated tissues. Our results showed good “tumor on” and “liver off” profile in both **in vitro** and **in vivo** studies (383).

7.1.1.3.2.12. Metallothionein promoter
In cisplatin resistant cells, metallothionein promoter is activated. Vandier et al., showed the feasibility of using this promoter to drive HSVtk expression to kill cisplatin resistant ovarian carcinoma cells (384). Thus treatment responsive promoters can also be used for transcriptional targeting.

7.1.1.3.2.13. Mesothelin (MSLN) promoter
Mesothelin is a cell surface glycoprotein, overexpressed in ovarian cancer but not in normal tissues with the exception of mesothelial cells. Our group exploited mesothelin for both transductional as well as transcriptional targeting. In this study, adenovirus encoding reporter gene driven by mesothelin promoter was constructed and targeted to mesothelin protein in ovarian cancer cells using anti-mesothelin antibodies (385). This double targeted approach significantly enhanced gene expression in ovarian cancer cell lines and primary cells. At the same time, gene expression was lower in the liver, which is the major target organ of Ad-mediated toxicity (385). Thus, a high therapeutic index was achieved.

7.1.1.3.2.14. Human chorionic gonadotropin promoter
Lidor et al., (386) employed a retrovirus encoding diphtheria toxin A chain gene under the control of the human chorionic gonadotropin promoter, to demonstrate specific gene expression and cytotoxicity in tumor cells but not in normal ovarian epithelial cells or fibroblasts (386).

7.1.1.3.2.15. Synthetic promoter
In an effort to develop a tightly regulated promoter for tumor specific expression of toxin genes, Lipinski et al, for the first time constructed a synthetic beta-catenin-dependent promoter, CTP4. This promoter showed virtually undetectable expression in normal cells with normal beta-catenin regulation but high level expression in tumor cells deregulated for beta-catenin (387).
7.1.1.3.2.16. Potential candidate promoters

Many genes including oncogenes, growth factors and their receptors, and other genes such as ras, epidermal growth factor receptor, fibroblast growth factor receptor, VEGF, flt-1, PIK3, akt2, and fms are over expressed in ovarian carcinoma. Promoters of these genes can be potential candidates for use in transcriptional targeting for ovarian cancer. Studies evaluating some of these promoters are currently being pursued in our lab and others.

7.1.2. Retroviral vectors

7.1.2.1. Retroviral vector biology

Retroviral vectors (Rv) are modified retroviruses, and have been widely employed for gene therapy. Some of the well-studied retroviral vectors are Moloney murine leukemia virus, Harvey murine sarcoma virus, and avian spleen neurosis virus. Rvs were the first genetic vectors to permit an efficient and stable gene transfer into mammalian cells (388) and also the first vectors used in a gene therapy clinical trial (for adenosine deaminase (ADA) deficiency) (389). Retroviral vectors are lipid-enveloped particles containing two identical copies of a linear single-stranded RNA genome of around 7–11 kb. After binding to a specific membrane-bound receptor for viral entry, Rv enters the cytoplasm. Viral reverse transcriptase in the cytoplasm retro-transcribes the viral genome into double-stranded DNA (dsDNA), which binds to cellular proteins to form a nucleoprotein preintegration complex (PIC), (which contains karyophilic elements that facilitate its migration to the nucleus) and integrates into host genome. The therapeutic gene of interest is cloned into the viral genome by replacing its structural genes, (gap, pol and env) and placing it under the control of promoter sequences located at the 5’ and 3’ ends in and around the long terminal repeats (LTR). Cloning the gene of interest in place of structural genes makes it replication deficient and at the same time allows expression of the gene of interest.

7.1.2.2. Advantages and disadvantages

The advantages of using these vectors for gene therapy are the relative simplicity of their genomes, the ease of use and their ability to integrate into the cell genome permits long-term transgene expression in the transduced cells or their progeny. The disadvantages are the inability of retroviral vectors to infect non-dividing cells, low efficiency of gene transfer, the low stability, and the difficulty in large scale production and obtaining high titers. Furthermore, tumor targeting for specific gene delivery, and tumor expression are major challenges.

7.1.2.3. Retroviral vector development strategies to enhance gene transfer efficiency

To overcome the problem of low gene transfer efficiency, regulatory regions of retroviral vectors were engineered to incorporate either hybrid regulatory sequences (FMEV vector family) (390), or LTR derived from the other viruses such as myeloproliferative sarcoma virus (MND vectors) (391). These modifications resulted in higher levels of transgene expression than conventional retroviral vectors. Tumor specific gene expression was achieved by employing promoters which are induced specifically in tumor cells.

To enhance tumor specific gene delivery, tumor transduction of retrovirus was enhanced. Some of the strategies that have been employed include, modification of the envelope structure by pseudotyping, or incorporating targeting ligand, or using bi-specific adapter molecules including single chain antibodies (392-395). Retroviruses pseudotyped with the envelope of the gibbon ape leukemia virus (GALV) or the feline endogenous retrovirus (RD114) transduced HSCs more efficiently than amphotropic RVs (394, 395). Further, retroviruses pseudotyped with G protein from the vesicular stomatitis virus (VSV-G) showed a very broad spectrum of infectivity (396). Pseudotyping with VSV-G or RD114 also enhanced physical stability and allowed the concentration of virus by ultra centrifugation to obtain high titers. Incorporation of a collagen-binding peptide from von Willebrand factor into the retroviral envelope protein also increased gene transfer to tumors upon intravenous administration (392). In addition, single
chain antibodies against CEA or folate receptors were used to target retroviral vectors to tumor cells (397, 398).

7.1.2.4. Gene therapy strategies based on retrovirus vector

Employment of retroviral vectors for ovarian cancer gene therapy has been very limited compared to adenoviral vectors. Retroviral vectors have been mainly used to deliver genes to either lymphocytes for immunotherapy or ovarian cancer cells to directly kill them in pre-clinical studies. These genes include, fyn, IL-2, IL-12, TNF-alpha, INF-gamma, B7.1, P16, HSVtk, and nitroreductase. In clinical trials, so far retroviruses have been used in two early clinical trials. The objective of the trials were: 1) To transfer MDR-1 gene to hematopoietic stem cells and progenitors in bone marrow as a means of providing resistance to these cells to the toxic effects of ovarian cancer chemotherapy. 2) To transfer BRCA1sv gene to ovarian tumors, which proved to be to be safe and effective (38, 39). However, the recent incidents of leukemia development (due to insertional activation of oncogenes) in X-SCID patients treated with retroviral gene therapy raised the safety concerns regarding the use of retroviral vectors in clinical gene therapy (399). Studies are underway to reduce these risks by re-engineering strategies such as targeting integration to inactive regions of the host genome (400).

7.1.3. Adeno-associated viruses (AAV)

7.1.3.1. Vector biology

AAV is a small single stranded DNA virus belongs to the group of human Parvovirus family. This virus is not known to cause any clinical disease. AAV genome is about 4.679 Kb, which contains rep, cap and two ITRs. Rep encodes for a non structural protein, which is involved in rescue and replication of the virus whereas cap encodes for icosahedral capsid. ITRs are sole elements required for rescue, replication, packaging and integration of AAV. In recombinant AAV, the rep and cap genes are deleted and therapeutic gene of interest is cloned in place of rep and cap genes. Due to deletion of rep and cap genes, rAAV are less immunogenic than other commonly used viral vectors. Recombinant AAV (rAAV) vectors are showing great potential as an alternative to more commonly used adenovirus and retrovirus-based vectors for cancer gene therapy (401-405) (406). rAAV readily transduces both dividing and non-dividing cells. The transduction pathway of AAV involves binding to its receptor heparan sulfate proteoglycan (407) and co-receptors fibroblast growth factor-1 [FGFR1 (408), αVβ5 integrin (409) for viral entry. Subsequent to viral entry, AAV is transported to the nucleus within a short time (410, 411) and uncoating of the capsid releases the vector genome in the host cell nucleus where it integrates into the host genome at chromosome 19.

7.1.3.2. Advantages and disadvantages of AAV

The major advantages of rAAV vectors include stable integration, low immunogenicity, long-term expression, and ability to infect both dividing and non-dividing cells. The major limitations include variations in infectivity of AAV among different cell types, the limited size of the recombinant genome (4.5Kb) that can be packaged, and difficulty in producing large quantities of high titer virus.

7.1.3.3. AAV vector based gene therapy strategies

Although rAAV use for gene therapy of various diseases is increasing, so far very few studies reported use of rAAV for the therapy of ovarian cancer. rAAV was mainly used either for ex vivo gene delivery to lymphocytes or tumor cells for immunogene therapy or for direct intramuscular gene delivery of secreted antiangiogenic protein genes. Genes for immunostimulatory proteins such as B7-2 (CD86), p35 subunit of IL-12, p40 subunit of IL-12 and IL-2 were delivered by rAAV to both cultured and primary ovarian tumor cells in vitro for immunotherapy (221, 412, 413). Our group as well as other groups have successfully delivered soluble FLT-1 a VEGF antagonist to inhibit angiogenesis and tumor growth in ovarian cancer xenograft mouse model (282, 414). Similarly, anti-angiogenic angiostatin and endostatin and mutant endostatin genes were delivered by rAAV which inhibited ovarian tumor angiogenesis and tumor growth.
in xenograft mouse model of ovarian cancer (294, 415). Other examples of rAAV mediated gene therapy are delivery of DT-diaphorase (NQO1) gene which encodes for an enzyme used in combination with prodrug EO9 for molecular chemotherapy of ovarian cancer (416), and delivery of nm23H1 gene in orthotopic implantation model to reverse metastatic potential of ovarian cancer (417).

7.1.3.4. Comparison of Ad vs. Raav
Although the above reviewed studies showed rAAV as potential vector for ovarian cancer gene therapy, its efficiency compared to adenoviral vectors is relatively low. Comparison of the gene delivery efficiency by adenoviral and adeno associated viral vector for ovarian carcinoma showed that transduction efficiency of rAAV in ovarian cancer cells was <1% whereas adenovirus transduction efficiency was >50% (418). Repeated injection of the vector improved the transduction efficiency slightly (3%) but when combined with adenovirus, transduction efficiency of rAAV improved upto 15% suggesting that low transduction is a major problem with rAAV. Therefore rAAV is not suitable for therapeutic gene transfer in ovarian cancer cells unless combined with adenovirus or other agents which enhance its infectivity.

7.1.3.5. AAV vector development strategies to improve gene transfer efficiency
To overcome limitation of flow transduction ability, several strategies have been endeavored. Cationic liposomes were used to facilitate adeno-associated virus (AAV) plasmid transfections of primary and cultured cell types. Using this technique, several fold higher gene expression was achieved compared to complexes with standard plasmids in primary T lymphocytes and primary and cultured tumor cells (419). Other strategies used are modification of the tropism of the virus to abrogate non-specific infectivity of rAAV due to widespread distribution of heparan sulfate proteoglycan (HSPG), the primary cellular receptor for the virus. In this regard, incorporation of 15-amino acid peptide, which binds to the human lutenizing hormone receptor (LH-R) into the capsid (420) was able to transduce ovarian cancer cells (OVCAR-3) which express LH-R in a HSPG-independent manner. Similarly, RGD peptide incorporation increased transduction of ovarian cancer cells in HSPG independent manner (421).

Although these tumor targeting strategies increased the therapeutic utility of rAAV vectors for ovarian cancer gene therapy, their limited (4.5Kb) carrying capacity of transgene and difficulty in producing large quantities of high titered virus for in vivo gene therapy are still the major limitations inhibiting their application in clinical studies. Strategies to overcome these limitations will enhance the exploitation of this vector’s advantages such as long-term gene expression, low immunogenicity for clinical gene therapy of ovarian cancer.

7.1.4. Other viruses
In addition to the vectors described above, other viral vectors such as lentiviral vectors for HSVtk gene therapy (16, 422), vaccinia, and polyomavirus were used in ovarian cancer therapy. However, low efficacy, undesirable side effects, inability to penetrate tumors, high cost of production are major limitations preventing these viruses for application in cancer gene therapy.

7.2. Non-viral vectors
Although viral vectors are widely used for gene transfer both in pre-clinical and clinical studies based on their remarkable levels of transduction and transgene expression, safety concerns related to generation of host immune responses, possibility of inserted genes activating oncogenes (423), and their low capacity to carry large-sized therapeutic genes, and high production cost led to research in non-viral vectors systems (424).

Various non-viral methods/systems have been developed for gene delivery. These include injection of naked DNA, electroporation (425-429), ultrasound (430, 431), gene gun, calcium-phosphate precipitation, polymers, peptides, and liposomes and cell-based vectors. Non-viral vectors have several advantages over
viral vectors such as relative non-immunogenicity, ability to carry large size therapeutic genes, and ease of mass production, and feasibility of repeated injection without causing immune response.

7.2.1. Naked DNA, Ultrasound, and electroporation methods
Injection of naked DNA is the simplest form of non-viral gene delivery. Although injection of naked DNA directly into certain tissues, particularly muscle (217), or into tumors results in significant levels of gene expression, it is comparatively lower than those achieved with viral vectors. To facilitate higher gene transfer efficiency, electroporation (425-429) and ultrasound (430, 431) techniques subsequent to the injection of DNA have been employed. Although in vivo gene transfer in murine models improved using these methods, their efficiency and feasibility in clinical studies needs to be evaluated to determine if they can be superior to viral vector systems. The disadvantage of this naked DNA injection is that it is inefficient at in vivo transfer of genes to tumor cells when injected systemically due to various factors such as low stability, low half-life, and nuclease degradation and lack of tumor specificity. Thus far use of these methods for gene delivery in the context of ovarian cancer has not been reported.

7.2.2. Gene gun and calcium phosphate methods
The gene gun technology uses DNA coated gold particles, which act as high velocity projectile when fired from the gene gun to deliver DNA into cells. Whereas in calcium phosphate method, co-precipitates of DNA and calcium phosphate are formed, which are then taken up by cells by phagocytosis (432), resulting in gene delivery. However, these two methods are relatively inefficient for in vivo gene delivery compared to viral vectors.

7.2.3. Cationic lipids/liposomes
More efficient non-viral gene transfer vectors are polycationic polymers (433, 434), and cationic lipids (424). Cationic lipids are most commonly used for gene delivery because they display good transfection efficiency, biodegradability, and low toxicity (435). Cationic lipids such as DOTMA (condense DNA through ionic interactions) and a neutral phospholipid, DOPE produce lipid-DNA complexes (lipoplexes) that are capable introducing into cells at high efficiencies (436, 437). A variety of cationic lipids have been synthesized to enhance gene transfer efficiency these include DMREI, DOTAP (438, 439) which have structures analogous to DOTMA, as well as cationic lipids containing polylysine (440, 441), and cholesterol 3 beta [N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), (442, 443), and lipopolyamides (444, 445). Neutral lipids such as monooleoglyceride (MOG) and cholesterol have also been used in the place of DOPE (446, 447). To further enhance the efficiency and reduce toxicity, an advanced cationic liposome 3 beta[l-ornithinamide-carbamoyl] cholesterol (O-Chol) was developed which displayed higher level of gene expression, reduced susceptibility to serum inhibition, and achieved selective transfection into tumor cells in a peritoneal disseminated ovarian cancer model (447), compared to other commercially available liposomes such as lipofectin, lipofectamine, DC-Chol, and FuGENE 6. Recently, another novel cationic liposome DDC was synthesized by mixing DOTAP, DOPE and cholesterol (Chol) in a ratio of 1:0.7:0.3 which showed 4 fold increase in GFP gene expression compared to DC-Chol/DOPE or lipofectin in OVCAR-3 cells.

A new type of liposome system containing N-acylphosphatidylethanolamine was also developed for delivery of spermine condensed plasmid DNA and was showed to be much less toxic than cationic liposomes and showed significant gene transfer even in the presence of ascites (448).

7.2.3.1. Cationic lipid based gene therapy strategies
In ovarian cancer, cationic lipids have been used in several pre-clinical studies for delivery of various genes such as E1A [DC-Chol, (DC-Chol/DOPE)] (151, 449, 450), mutant SV40 large T antigen (DC-Chol ) (155, 450), antisense HER-2 oligonucleotides (451), p53 (452), antisense erbB-2 oligonucleotides (453-455), interferon gamma (456), thymidine kinase (384). These studies demonstrated efficiency of cationic liposomes as gene delivery vehicles to deliver genes at sufficient levels to achieve therapeutic efficacy
both in \textit{in vitro} and \textit{in vivo}. Further these [DC-Chol, or (DC-Chol/DOPE)] were shown to be safe for E1A gene delivery in murine models of ovarian cancer (449, 457).

Liposomes as delivery vehicles were also shown to be safe in phase I clinical trials of ovarian carcinoma for delivery of E1A (152, 153, 458) and xenogenic MHC (DC-Chol/DOPE)(459).

7.2.3.2. Targeted cationic liposomes
A disadvantage of the liposomes is that they interact non-specifically with any cell membrane having a negatively charged surface resulting in gene delivery to non-target tissues. To specifically target the liposomes to the ovarian cancer cells, various targeting motifs are coupled to these liposomes such as HIV-1 derived TAT-peptide, (460), folate receptor (461) monoclonal antibody against HER-2/neu, and EGP-2 (MOC31) (462). These motifs on the liposomes bind to their respective antigens overexpressed in ovarian cancer cells thus facilitating tumor specific gene delivery.

7.2.3.3. Comparison of adenoviral vectors and liposomes
Comparison of efficacy of adenovirus and liposomes as delivery vehicles showed that adenoviruses-mediated E1A therapy is better than liposome–mediated therapy which was demonstrated by more than 80% of the mice surviving longer than one year compared to 70% with liposome mediated-delivery (463). Another study which used adenoviral-mediated E1A delivery also confirmed these findings (367).

7.2.3.4. Challenges of employing liposomes as gene delivery vectors
Although clinical studies with liposomes showed promise as gene delivery vectors, relatively low gene transfer efficiency, shorter half-life, and the cytotoxicity are still the major challenges which needs to be addressed for the development of vectors that are clinically superior better than viral vectors.

7.2.4. Cell-based vectors
Cell-based vectors derived from human cells are easy to culture, they do not elicit immune response, or toxicity.

7.2.4.1. Mesothelial cells and HUVEC cells
As a proof of concept, mesothelial cells were transduced \textit{ex vivo} with HSVtk to use them as delivery vehicles to ovarian tumor cells. Injecting these cells \textit{in vivo} in mice bearing ovarian cancer tumors (180) significantly reduced tumor growth as a result of bystander mechanism upon injection of GCV. These results demonstrate the feasibility of using a mesothelial cell-based therapy for treatment of ovarian cancer gene therapy. Similarly, human vascular endothelial cells were also used as delivery vehicle for HSVtk in immunodeficient mice ovarian cancer mouse model (179).

7.2.4.2. Fibroblasts and lymphocytes
Although above strategies showed proof of concept of using cell-based vectors, utility of these vectors can be better judged when evaluated in immunocompetent mice. Towards this end, syngeneic immunocompetent mouse model of ovarian cancer was used for evaluation of autologous fibroblasts expressing murine IL-12 (240). Intraperitoneal delivery of these fibroblasts significantly reduced tumor growth and increased survival upto 120 days compared to controls suggesting that ovarian tumor biopsy-derived fibroblasts are a safe and efficacious vehicle for i.p. delivery of IL-12 as a regional secondary treatment for minimal residual disease of ovarian cancer (240). Other cells used are: lymphocytes stably expressing cytokines for immunomodulation therapy of ovarian cancer, normal bone marrow cells, for delivery of MDR1, as a means of providing resistance to these cells to the toxic effects of cancer chemotherapy (464).
8. **Virotherapy.**

Viruses have the natural ability to infect cells, replicate and lyse the cells releasing progeny. This natural ability of viruses has been exploited to kill tumor cells. This strategy is called “virotherapy”. Currently viruses which are used for virotherapy in ovarian cancer include, adenoviruses, herpes simplex viruses, and edmonston strain measles virus out of these, adenoviruses are most widely employed for virotherapy.

**8.1. Conditionally replicative adenoviruses (CRAds)**

Conditionally replicating adenoviruses are genetically engineered to control viral replication specifically in cancer cells but not in normal cells thus killing cancer cells specifically.

**8.1.1. Strategies used in developing CRAds**

There are two main strategies to control viral replication selectively in tumor cells. One strategy involves:

1) Introduction of deletions in the viral genome that require tumor-specific cellular factors to functionally compensate for these deletions, thereby allowing selective replication (deletion type CRAds). The other strategy involves:

2) Use of tissue specific promoters (TSP) to drive viral replication specifically in cancer cells (TSP CRAds).

**8.1.1.1. Deletion type CRAds**

To develop deletion type CRAds, two approaches were utilized. In this first approach, two mutations were introduced in the viral gene coding for the E1B 55-Kd protein. The resulting virus initially reported as dl 1520 was named as ONYX-015. One of the functions of this E1B protein is binding and inactivation of p53 in infected cells for induction of S-phase required for viral replication. Thus, this virus will not be able to inactivate p53 in normal cells and therefore cannot replicate in normal cells. Alternatively, in cancer cells, lack of functional p53 allows replication of this virus resulting in oncolysis. Initial studies suggested that this virus replicates more effectively in tumor than in normal cells. However, the function of E1B55Kd is not limited to the p53 binding, which causes inefficient replication of the virus compared to wild type. In addition, recent studies have suggested replication of ONYX-015 in non-target normal tissue.

In the second approach, a 24 bp deletion (\(\Delta 24\)) was introduced in the constant region 2 (CR2) of the E1A gene to confer tumor-specific replication. This domain of the E1A protein is responsible for binding the retinoblastoma tumor suppressor/ cell cycle regulator protein (Rb), thereby allowing the Ad to induce S-phase entry. As a result, CRAds with the \(\Delta 24\) E1A deletion have reduced ability to overcome G1-S checkpoint in normal cells and therefore cannot replicate. Whereas in most tumor cells, because Rb-p16 pathway is defective, binding of Rb is not required therefore viruses can replicate. It has been shown that replication of CR2-deleted viruses is attenuated in non-proliferating normal cells. Employment of this \(\Delta 24\) CRAd showed significant oncolysis comparable to wild type virus in tumor cell lines, and primary cells derived from patients in vitro. In vivo treatment with this CRAd completely eradicated i.p. tumors in mouse model (332, 334).

**8.1.1.2. TSP CRAds**

In this strategy, Ad replication regulators such as the viral early gene E1 is placed under the control of tissue specific promoters (TSP) so that viral replication occurs only in tumors in which this promoter is induced but not in normal cells. Although many CRAds were developed using various promoters for cancer therapy, promoters that have been used for development of CRAds for ovarian carcinoma include, COX-2 (336), IAL3B (465), L-plastin promoter (466), SLPI promoter (467) and VEGF (468). All these CRAds demonstrated excellent tumor specific replication and showed good therapeutic activity both in cell lines and primary tumors in vitro and ovarian cancer. In addition, SLPI CRAd significantly decreased liver toxicity (467).
8.1.1.2.1. Candidate promoters for development of TSP CRAds

Although several CRAds have been developed using various promoters for the therapy of ovarian cancer, there are many more potential candidate promoters such as OSP1 HTERT, Midkine, hESE1, Flk-1 and endoglin which can be exploited for CRAd development for ovarian cancer therapy. Development of targeted CRAds employing these promoters and comparing them with existing CRAds may help in determining the best CRAd for ovarian cancer therapy.

8.1.1.3. Tumor targeted CRAds

To further enhance the efficacy of CRAds, tumor targeting strategies described earlier in this review have been employed to improve transduction of CRAds. These include incorporation of RGD targeting ligands in the HI loop, (332, 336), using bi-specific single chain antibody adapters to target EGFR on cancer cells (469, 470), and using Ad5/3 chimeric knobs (467, 468, 471). Surprisingly, CRAd expressing EGFR targeting molecule impaired its oncolytic potential (472).

To compare the efficacy of the various CRAds, our group evaluated AdRGDcox-2R, Ad5VEGFE1, Ad5/3VEGFE1, Ad5-Delta24RGD, and Ad5/3-Delta24 CRAds. All five CRAds tested showed robust DNA replication, oncolysis, in cell lines, as well as primary tumor cells derived from patients in vitro and showed high in vivo therapeutic efficacy in ovarian cancer xenograft murine models (468). Suggesting that each virus has potential for clinical testing, and such further testing will ultimately determine its safety and relative usefulness.

8.1.1.4. CRAds in combination with other therapies

Combining CRAd with conventional chemotherapy, and radiotherapy enhanced its therapeutic efficacy (473). Another study showed synergistic therapeutic effect in ovarian cancer when treated with Ad5/3-Delta24 CRAd and gemcitabine. This synergistic effect may be due to chemosensitizing activity of E1A and/or altered replication kinetics. Although this combination therapy increased the survival and cured almost 60% of treated mice, treatment-related hepatic or bone marrow toxicity was also observed in some mice (474).

Combining CRAd therapy with apoptosis inducing p53 gene therapy (470, 473), cytosine deaminase/ or carboxylesterase /CPT-11(475, 476) also showed promised. However, combination of HSVtk and CRAd was reported to be less efficacious (475). A recent study showed that combining tumor targeting to EGFR with p53 expressing CRAd further improved the oncolytic efficacy (470).

8.1.1.5. ONYX-015 clinical trial

ONYX-015 virus has shown safety and efficacy in clinical trials in the context of other carcinomas either alone or in combination with chemotherapy (477-481). A phase I trial conducted in patients with recurrent ovarian cancer using ONYX-015 (482) showed that viral therapy was well tolerated at doses ranging from 1 x 10(9) plaque forming units (pfu), to 1 x 10(11) pfu with most common significant toxicities related to virus administration were flu-like symptoms, emesis, and abdominal pain. One patient receiving 1 x 10(10) pfu developed common toxicity criteria grade 3 abdominal pain and diarrhea, which was dose-limiting. The maximum-tolerated dose was not reached at 10(11) pfu, and at this dose level patients did not experience significant toxicity. There was no clear-cut evidence of clinical or radiologic response in any patient.

8.2. Other oncolytic viruses

In addition to Ad based CRAds, herpes simplex viruses were also used for ovarian cancer virotherapy. Two HSV-1 replication competent mutant viruses, hrR3A and HR522; were evaluated in a mouse model. Hr3A prolonged survival of mice compared to paclitaxel group but not HR522. However, when combined with HSVtk/GCV, HR522 significantly prolonged the survival (483). Further, incorporation of membrane fusion capability into an oncolytic HSV viruses eradicated all tumor masses in 75% of the animals, when
injected i.p. in an ovarian cancer tumor model (484). Other viruses such as recombinant oncolytic measles virus (MV-CEA), and two commercially available live attenuated vaccines, Moraten measles and Jeryl-Lynn mumps, also showed potential for ovarian cancer therapy (485).

8.3. Tumor models for evaluation of CRAds
Current animal tumor models are inadequate for the evaluation of toxicity and efficacy of conditionally replicative adenoviruses because human serotype CRAds do not replicate productively in murine tissues therefore meaningful safety data is difficult to obtain. A novel model system that will provide insight into the anticipated therapeutic index of conditionally replicative adenoviruses pre-clinically is needed. Towards this end, we have developed a novel method of \emph{ex vivo} evaluation of replicative adenoviral vectors, which utilizes thin precision cut slices of human ovarian tumor or liver obtained from patients. These thin slices which can be maintained \emph{ex vivo}, are infected with CRAds and their replication in these tissues is determined by quantitative PCR. Replication data thus obtained provides an indirect method of replication-associated toxicity. This method allows stringent assessment of human liver replication relative to human tumor replication, which provides a powerful tool to determine therapeutic index for clinical translation of conditionally replicative adenoviruses (486). In an effort to develop an immunocompetent tumor model for CRAds evaluation, CRAds based on canine adenovirus serotype 2 were employed to evaluate toxicity in syngeneic canine osteosarcoma tumor models (487).

9. Conclusions
Gene therapy is a promising therapeutic modality for ovarian carcinoma. Advances in understanding of the ovarian tumor cell biology have identified a number of genes involved in development of ovarian cancer. To target these genes, novel gene targeting approaches based on mutation compensation, molecular chemotherapy, genetic immunopotentiation and most recently antiangiogenesis and virotherapy have been developed. These approaches showed promising results in pre-clinical studies. Translation of these approaches in clinical trials have shown not only the feasibility but also identified the limitations. The major limitations were sub-optimal gene delivery and toxicity. Main reasons behind these limitations are inefficient gene delivery vectors, immune response against vectors, and non-specific therapeutic gene expression. To overcome these problems, significant advances are currently being made in development of tumor targeted vectors which display enhanced tumor specific gene delivery and low immunogenicity as well as development of tumor specific promoters with increased fidelity for ovarian cancer. These endeavors may enhance the therapeutic index and significantly advance the applicability of gene therapy approaches for treatment of ovarian cancer.
New high-throughput differential display technologies and the recently completed sequencing of the human genome will result in the defining of a plethora of new target molecules for ovarian cancer. Investigating these molecules will provide a deeper understanding of molecular mechanisms involved in development of ovarian cancer, which will form a basis for development of more specific gene targeting approaches for ovarian cancer therapy.
<table>
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* Denotes the studies that are not published but listed at GEMCRIS gene therapy clinical trial web site.
References.


240. Sanchez, R., Kuiper, M., Penault-Llorca, F., Aunoble, B., D'Incan, C., and Bignon, Y. J. Antitumoral effect of interleukin-12-secreting fibroblasts in a mouse model of ovarian cancer:


259. Folkman, J. What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst, 82: 4-6, 1990.


