Winter 2005

Tumor Response TCF-4/β-Catenin Regulatory Elements for Enhancing Cancer Gene Therapies

Saurabh Kumar Gupta
Old Dominion University

TUMOR RESPONSE TCF-4/β-CATENIN REGULATORY ELEMENTS FOR ENHANCING CANCER GENE THERAPIES

by

Saurabh Kumar Gupta
B.Sc. June 1995, University of Delhi
M.Sc. June 1997, Indian Institute of Technology

A Dissertation Submitted to the faculty of Old Dominion University and Eastern Virginia Medical School in Partial Fulfillment of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES

OLD DOMINION UNIVERSITY
December 2005

Approved by:

Richard R. Drake (Chair)

Richard Britten (member)

Richard Ciavarra (member)

O. John Semmes (member)

ABSTRACT

TUMOR RESPONSE TCF-4/β-CATENIN REGULATORY ELEMENTS FOR ENHANCING CANCER GENE THERAPIES

Saurabh Kumar Gupta
Old Dominion University, 2005
Chair: Dr. Richard R. Drake

Mutations in the adenomatous polyposis coli gene are frequently associated with progression of colon carcinoma and most other types of epithelial carcinomas. This usually results in stabilization of β-catenin protein levels, followed by transactivation of Tcf-4/ β-catenin responsive genes. The effectiveness of a Tcf-4/ β-catenin transcriptional enhancer element in combination with a c-fos or carcinoembryonic antigen promoter was tested for it's ability to act as a tumor specific regulator of gene expression in a panel of human tumor and normal cell lines. Luciferase reporter assays indicated enhanced activity of the Tcf-4/ β-catenin transcriptional element only in tumor cell lines, with minimal activities in normal colon cell lines. The Tcf-4/ β-catenin enhancer and c-fos promoter linked with the herpes virus thymidine kinase suicide gene in combination with ganciclovir was further evaluated in the normal and tumor cell lines. The Tcf-4/β-catenin elements conferred tumor specific expression of HSV thymidine kinase (HSV-TK) gene, resulting in selective metabolism of ganciclovir and cell killing of only tumor cell lines. There was no detectable expression of HSV thymidine kinase expression in normal colon cell lines. Additionally, recombinant adenoviral constructs were made to deliver the gene expression cassette, containing the HSV-TK expressed from a Tcf-4/c-fos enhancer/ promoter combination, to the tumor cells. No expression was detected in the normal colon cells as opposed to siginificant levels of HSV-TK gene expression observed in

tumor cells. Furthermore, various chemical and genetic modulators were also screened in an effort to identify newer methods to regulate the activity of the proposed recombinant enhancer/promoter combinantions. These data suggest that Tcf-4/ β-catenin enhancer can be effectively coupled with a suitable tumor-specific or tumor-related mammalian promoter for selective expression of therapeutic genes for gene therapy of epithelial cancers. This approach also offers a potential promoter cassette approach linked with the Tcf-4/ β-catenin enhancer to better individualize treatment to cancer patients.

This dissertation is dedicated to my family, especially my parents. Their help and support was very crucial in the completion of my studies.

ACKNOWLEDGMENTS

I would like to thank my committee members, Dr. John Semmes, Dr. Richard Ciavarra, Dr. Richard Britten, and especially my advisor Dr. Richard Drake for his mentoring which allowed me to conduct my research. He has been more than a mentor and his continuous support and faith in me was very instrumental in finishing the presented study. A special thanks to Dr. Julie Kerry for taking an interest in reviewing my work. Besides, those named above several others helped me in different ways and I want to thank them for their support.

TABLE OF CONTENTS

CHAPTER Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Colorectal Tumorigenesis

Colorectal cancer is one of the most common malignancies among populations in the United States and Western Europe, and one of the leading causes of worldwide morbidity and mortality due to cancer. The life time colorectal cancer risk in the general population is 5%, but this figure dramatically rises with age: by the age of 70, approximately half of the Western population will have developed an adenoma (1). In general, the incidence of colorectal cancer is high in developed countries, with incidence rates varying up to 20-fold between high- and low-risk geographical areas throughout the world (1). These variations in colorectal cancer incidence are most likely to be a result of environmental and dietary modifying factors. Colorectal carcinomas arise through a series of well characterized histopathological changes that result from specific mutation 'hits' of a select set of oncogenes and tumor suppressor genes. However, it is still a matter of debate regarding the exact sequence of events that leads intestinal epithelial cells to develop into aggressive carcinoma. Currently, it is estimated that 85% of all sporadic colorectal tumors have inactivating mutations in the APC (Adenomatous Polyposis Coli) gene (2) and the other 15% are believed to harbor defects in DNA mismatch repair pathway genes (3). Therefore, in general there are two widely accepted basic models of colorectal tumorigenesis, one driven by mutations in the tumor uppressor APC gene, and the other caused by defects in DNA mismatch repair pathway genes.

This dissertation follows the format of the journal *Cancer Research*.

Histopathology of Colorectal Cancer

Throughout the entire length of the large intestine, tubular glands or crypts are lined by a monolayer of columnar epithelial cells. These invaginations effectively increase the surface area of colon and rectum. At the base of each crypt, a small number of stem cells are present which asymmetrically divide to give rise to four different types of epithelial cells: columnar absorptive cells, goblet cells, neuroepithelial cells and paneth cells. Dividing cells are present in the lower third of the crypt, whereas the differentiated types are present in the upper two-thirds. The cells continuously migrate upwards and are eventually exfoliated into the lumen by an apoptotic mechanism. This process of epithelial renewal takes 3-6 days. Therefore, a delicate balance between mitotic rates and cell loss exists in these cells. The earliest signs of colorectal neoplasia are aberrant crypt foci, or ACF, which are only visible by microscopy. ACF usually include a small number of crypts and can be composed of cells with either normal morphology (nondysplastic), or dysplastic cells. The latter cells are more likely to develop into a benign tumor mass (polyp) protruding into the lumen from the intestinal epithelium. Polyps can also be classified into two types: hyperplastic (nondysplastic) and adenomatous (dysplastic). Hyperplastic polyps show normal cellular morphology whereas adenomatous polyps are characterized by abnormalities in both inter- and intracellular organization. The epithelium, in such adenomatous polyps, is organized in multiple layers, nuclei are enlarged, and their alignment at the basal membrane is lost (4).

It is widely accepted that these ademato us polyps generally arise as a result of genetic changes initiated by mutations in either APC gene or in genes involved in DNA mismatch repair pathway.

Genetic Model for APC-Driven Colorectal Tumorigenesis

Mutational inactivation of APC gene is considered a key initiating event which results in cascade of genetic alterations results in development of majority of colorectal carcinomas. Therefore, it is very important to understand the role the basic biology of APC gene in context of colorectal carcinoma.

Biology of APC

The APC gene was initially identified by positional cloning of the FAP (familial adenomatous polyposis) locus (5). Later, clues regarding APC functional came from studies which identified β-catenin as a binding partner of APC (6). β-catenin was originally identified as an essential intracellular component of the cadherin adhesion complexes. It is now well established that β-catenin is an important component of the wingless/wnt signal transduction pathway. The canonical wnt signaling pathway has been characterized from combined work in flies, frogs and mammals. In unstimulated cells, i.e. in the absence of the extracellular wnt signal, free β-catenin is bound and phosphorylated in a so-called destruction complex, consisting of the scaffolding proteins axin and conductin, glycogen synthase kinase 3 β (GSK-3β) and APC (7). Phosphorylation of β-catenin by this complex labels it for ubiquitination and subsequent proteolytic degradation. In the presence of a Wnt signal bound to frizzled receptors (8), GSK-3β is inactivated in the destruction complex by a still poorly defined mechanism involving an intracellular protein termed Dishevelled (9). As a consequence, unphosphorylated β-catenin becomes stabilized and can shuttle to the nucleus. Once in the nucleus, β-catenin can bind to DNA-binding proteins of the T-cell factor (TCF) family, to serve as an essential co-activator of transcription (10). β-catenin not only functions as a Wnt

transducer, but it also plays an important role in cellular house-keeping functions. For example it is an essential component of the adheren junctions, where it provides the link between E-cadherin and α-catenin (11). Together these studies demonstrate that APC can regulate cell adhesion and gene transcription by regulating the stability and sub-cellular localization of β-catenin. In addition, APC directly associates with microtubule cytoskeleton (12). This function is apparently unrelated to its capacity to regulate the Wnt signaling pathway.

APC, β-catenin and Cancer

Disruption of any of the several physiological roles played by APC can be potentially linked with cancer; it seems that the main tumor suppressing function of APC resides in its capacity to properly regulate intracellular β-catenin levels. Moreover, although the vast majority of colorectal tumors carry mutations in APC, most tumors with an intact APC gene were found to contain activating mutations in β-catenin that alter functionally significant phosphorylation sites (13). In addition mutations in other members of the Wnt pathway have been shown to be associated with cancer including conductin (14) and axin (15).

Mutational activation of APC gene is regarded as one of the earliest events in initiation of colorectal tumorigenesis (16-18), ultimately resulting in a nuclear β-catenin/TCF-4 transcription complex. The first two identified downstream targets of this transcription complex, MYC and Cyclin-D1, are clearly relevant for tumor formation because of their role in proliferation, apoptosis and cell-cycle progression (19-21). Changes in the level of expression of MYC and cyclin-D1 are likely to affect the rate of renewal of normal colon epithelium by increasing the overall proliferation rate. Other wnt

target genes such as matrilysin (22), CD44 (23) and urokinase-type plasminogen activator receptor (24) appear more likely to play a role in tumor promotion rather than initiation. Similarly, genes like Gastrin (25) and Cox-2 (26) which are believed to have tumor promoting roles are targets of wnt signaling pathway.

In epithelial cells lining the intestinal crypts, nuclear β-catenin expression is higher in the lower proliferative compartment of colon crypts, while it is decreased in the upper two-thirds of the crypt. In contrast, APC staining is dramatically increased in post replicative cells in the upper portions of the crypts as the migrating cells mature upwards on the crypt-villi axis. This inverse relationship between levels of expression of APC and β-catenin is in agreement with the function of β-catenin in maintaining stem cell properties and controlling differentiation in the intestine (27). In the bowel, Tcf-4 is the main transcription factor which transduces β-catenin signaling in the nucleus (28). Tcf-4 -/- mice cannot sustain an intestinal stem cell compartment, strongly suggesting that activation of downstream targets such as MYC and cyclin-D1 are required to maintain proliferative capacity (27, 29-31). Moving upwards along the crypt villus axis, an increase in APC mutations is therefore likely to result in the enlargement of the stem cell compartment and diminished differentiation. Indeed, it is generally accepted that colorectal tumors arise from the stem cells that are located at the base of the crypt.

APC driven colorectal tumorigenesis

The most widely accepted model of colorectal tumorigenesis is that tumors initiate as the result of mutation in a single gene in a single progenitor cell. Subsequent mutations in the progeny of this cell and waves of clonal expansion give rise to daughter cells that have the growth advantage typical of cancer. This whole process is driven by

clonal selection. In accordance with Knudson's hypothesis, the mutational targets of this multi-step process are oncogenes and tumor suppressor genes known to directly affect the rate of cell growth or cell death (32). Besides these growth regulating genes, other gene classes may be affected by mutations, including nucleotide excision repair genes (33), the Bloom's syndrome gene BLM (34) and DNA mismatch repair pathway genes (35). The common function for all the above wild type genes is to maintain genetic stability. Alterations of these genes increase the effective rate of mutations in cancer which in turn fuels tumorigenesis by providing variation for the forces of selection to act upon. Different types of genomic instability results in inactivation of different set of oncogenes and tumor suppressor genes. The net outcome of inactivation of different set of genes always results in targeting of a crucial growth regulating pathway. Colorectal tumorigenesis provides a classic example of this phenomenon, in that the inactivating mutations in APC cause β-catenin to transactivate downstream growth regulatory genes, giving cells with APC mutations a growth advantage compared to non-mutated neighboring cells (32, 36).

Assuming APC mutations are a primary initiating event in colorectal tumorigenesis, and that this provides some growth advantage to the initiator cell, this single mutation is not sufficient to initiate the waves of clonal expansion needed for a tumor to mature. Colorectal tumors are known to harbor hundreds of mutations, but current technology does not allow the dissection of the evolutionary history of a tumor and sequentially arrange one mutation after another with reference to tumor metamorphosis. However, with the knowledge of known mutations in oncogenes and tumor suppressor genes, a model has been constructed by Vogelstein and colleagues.

According to this model, at least four sequential genetic changes need to occur to ensure colorectal cancer evolution. One oncogene (K-RAS) and three tumor suppressor genes (APC, TP53 and SMAD4) are the main targets of these genetic changes. At least seven genetic hits are required according to this model: one in oncogenic K-RAS and six additional ones to inactivate both the alleles of the mentioned tumor suppressor genes (32, 37). All the mentioned tumor suppressor genes are found mutated in most of the colorectal tumors whereas K-RAS mutations are found in approximately 50% of cases (38-40). It appears that a synergistic action of the mutated APC and K-RAS genes underlies clonal expansion and dysplasia in colorectal tumors. However, because only 50% of colorectal tumors are observed to have K-RAS mutations, other unidentified genes might be able to substitute for K-RAS functions.

Another important concept for describing colorectal tumorigenesis is 'loss of heterozygosity' (LOH): In cells that carry a mutated allele of a tumor suppressor gene, the gene becomes fully inactivated and loses a part of the chromosome carrying the wild type allele. LOH studies have been very instrumental in identifying tumor suppressor genes which are involved in colorectal tumors (40-43). LOH in chromosome 5q, 17p and 18q are found with high frequencies and are most likely to be associated with the following tumor suppressor gene: APC (5q), TP53 (17p) and SMAD4(18q). Chromosome 17p is lost in 75% of the colorectal cancers but infrequently in benign tumors, suggesting that loss of p53 occurs later in tumor progression and is not crucial for initiation (40, 44). The other TP53 allele is almost invariably found to have mis-sense mutation, in line with Knudson's two hit hypothesis. Chromosome 18q is also frequently found lost in 50% of adenomas and 75% of malignant colorectal cancers (43). Several

candidate tumor suppressor genes map to this location, including SMAD2 and SMAD4. SMAD4, also known as DPC4 (deleted in pancreatic cancer) (45) encodes a key signaling molecule with in tumor growth factor-β pathway (46). SMAD4 loss has been observed in colorectal and pancreatic cancer (45, 47). SMAD2 also maps to chromosome 18q locus and encodes another member of the TGF-β signaling pathway. It is also found mutated in a subset of colorectal tumors. However, the precise role of SMAD2, SMAD4 and other genes mapping to the 18q locus in tumor progression is not properly understood. Additional studies are needed to elucidate the mechanism of involvement of these genes in colorectal tumorigenesis.

The model for colorectal tumorigenesis proposed by Vogelstein and colleagues states that inactivation of both alleles of the APC gene triggers the adenomatous process by providing the tumor cell with a growth advantage to allow clonal expansion. Additional mutations, e.g. K-RAS, are needed to support adenoma growth and expansion. Subsequent malignant transformation and clonal expansion is driven by additional mutations and allelic loss in TP53, SMAD4 and other 18q tumor suppressor genes. Although, additional unidentified mutations might be necessary for colorectal cancer development, this model predicts that at least seven genetic hits are needed. This is reflected in the fact that it takes about 20-40 years for the whole process of developing an aggressive colorectal tumor from a smallest observed lesion, the aberrant crypt foci.

Genetic Instability

Thus, natural selection with waves of clonal expansion seems to be a major force underlying clonal evolution of a tumor. However, it has been argued that endogenous mutation rates are not sufficient to achieve the high number of genetic alterations found

in human cancers. This implies that another selection force in the form of genetic instability might be crucial in tumor development. Indeed, it has been hypothesized that 'genomic instability' in the form of a mutator phenotype is an essential requirement for cancer to occur (48). Two forms of genetic instabilities have been described in colorectal cancers: Microsatellite instability (MIN or MSI) and chromosomal instability (CIN). MSI is widely present in sporadic colorectal tumors having impairments in DNA mismatch repair (MMR) genes. In contrast, 85% of colorectal tumors that include APC mutations, no MSI is evident (49). The reasons behind this difference will be described in detail below. CIN is a hallmark of most colorectal cancers, but is not very well understood at molecular level. Most colorectal cancers can be characterized for the presence of abnormal chromosomal content that is they are aneuploid. There is evidence that supports the idea that aneuploidy arises in these cancers because of CIN due to an accelerated rate of gain or loss of chromosomes. CIN is postulated to allow cells to rapidly acquire genetic changes that are required for tumorigenesis (50). How CIN originates in a cell is not clearly understood, and there are several different mechanism proposed to explain how cells might achieve aneuploidy. One proposed view is that CIN is not genetic and propagates the idea that CIN is result of aneuploidy (51). According to this hypothesis, following a random tetraploidization event, the chromosomal complement is inherently unstable and CIN is a trivial consequence of the near doubling of the genetic content. Another hypothesis is that CIN is genetic in origin, and there are at least two lines of evidences supporting it. First, the CIN phenotype is dominant as it can be conferred on a chromosomally stable diploid cell which is fused with a CIN cell (52). Second, a fraction of cancer contains mutations or abnormal expression patterns of spindle checkpoint genes

such as BUB1 and MAD2 (53-57). Targeted mutations in these genes in non-CIN lines can convert them to CIN phenotype with resultant aneuploidy, presumably because of disruption of the cell's normal error checking mechanisms which prevent mis-segregation of DNA during mitosis.

In addition, it has recently been reported that APC mutations can give rise to CIN (58, 59). Experimental evidence have shown that the C terminus of APC is involved in maintaining chromosomal stability during mitosis (58, 59). During metaphase, APC localizes to kinetochores of chromosomes, where in association with the microtubule associated protein EB1, it facilitates the binding of the spindle microtubule to the kinetochore. In cells that express a truncated form of APC; the interaction between kinetochores and spindle microtubules is disrupted leading to chromosomal instability. However, the idea of APC being the cause of CIN has been challenged by the Vogelstein group. They argue that APC mutant cells actually tend to polyploidize in whole genome increments rather than show the increased rates of loses and gains of one or few individual chromosome that is a characteristic of cancers. Further, they advocate that some well characterized human cancer cell lines with APC mutations have chromosome complements that have remained stable and invariant over thousands of cell divisions *in vitro*.

Although the molecular basis of CIN is yet to be elucidated, it does not undermine the fact that genetic instability in the form of CIN and clonal selection go hand in hand to promote tumorigenesis. An important issue in the current discussion is at what point in tumor developmental history does CIN originate? Does genetic instability precede mutations in rate-limiting oncogenes and tumor suppressor genes or does it simply

underlie tumor progression? There is evidence to support the idea that genetic instability occurs in very early stages of tumor progression (60). An important study by Shih, et al evaluated 32 tiny adenomas with an average diameter of 2 mm. Using highly quantitative methods they found that more than 90% of these lesions had allelic imbalances of one or more of the five chromosomes tested. In this study chromosome 5q, where APC resides, was excluded because of the expectations that alterations at this locus initiated the tumor. More than two-thirds of the tiny tumors showed at least one allelic imbalance of the other chromosome tested. Interestingly, in some tumors, the allelic imbalances were not found in the entire population of neoplastic cells whereas chromosome 5q allelic imbalance generally seemed to affect most neoplastic cells in the tumor. This study suggests that APC mutations may precede genomic instability. However, it does not necessarily conclude that an APC mutation result in genomic instability, but it does strengthen the viewpoint that APC plays a multifunctional pivotal role in colorectal tumorigenesis.

Alternative Pathway for Development of Colorectal Cancer

Over the past decade, researchers have compared colorectal cancers from two inherited disorders: familial adenomatous polyposis (FAP) and the hereditary non-polyposis colorectal carcinoma (HNPCC). Several distinct differences were observed. HNPCC tumors do not necessarily show APC deletions and are chromosomally stable. In addition, they have characteristic defects in DNA mismatch repair (MMR) machinery resulting in microsatellite instability (MSI). Subsequently it was shown that 15% of sporadic colorectal cancers exihibit the presence of MSI (61, 62). This feature arises through defective DNA mismatch repair or due to failure to express a mismatch repair gene. These observations prompted the idea that there is an alternative pathway of

colorectal tumorigenesis which is characterized by the presence of MSI, in contrast to CIN found in majority of colorectal tumors. Microsatellites are tandem repeats of small DNA sequences showing length polymorphisms and are found throughout the human genome. Because of their repetitive nature, microsatellites are prone to strand slippage and replication errors. (63). The principal task of MMR is to remove nucleotides that have been mis-incorporated into the newly synthesized DNA strand by DNA polymerase. Thus a disruption in the DNA mismatch repair system can result in MSI.

DNA Mismatch Repair Systems and Mutations in Colorectal Cancer

The DNA mismatch repair system is responsible for correcting base pairing errors during replication of DNA. It also repairs any mismatches post replicatively which escape the proof reading mechanism of DNA polymerase. The principal players in mammalian MMR are the homologues of the bacterial mutS and mutL proteins. The repair system requires the co-operation of many genes from the mutS (hMSH2, hMSH3, hMSH6) and mutL families (hMLH1, hMLH3, hPMS1, hPMS2) (64). Briefly, hMSH2 serves as the scout that recognizes and binds directly to the mismatched DNA sequence (65, 66). It forms a heterodimeric complex with hMSH6 if a single base pair mismatch is recognized or with hMSH3 if there is a two to eight nucleotide deletion of insertion. A second heterodimeric complex of hMLH1 and hPMS2 is then recruited to excise the mismatched nucleotides. Although heterodimers of hMLH1/hPMS1 and hMLH1/hMLH3 also form, their specific roles remain to be defined. Genetic alteration in mismatch repair genes results in defective DNA mismatch repair. The severity of defect is co-related with the specific gene altered. hMSH2 or hMLH1 mutations result in high levels of MSI

(MSI-H), whereas, inactivation of hMSH6 leads to partial impairment of the mismatch repair machinery (MSI-L) (67, 68).

Mutations in mismatch repair genes may occur in germline or somatic DNA. It has been observed that an inherited germline mutation does not result in widespread tumor formation in most organs (69). One possible scenario is that the wild type allele may provide sufficient DNA mismatch repair function. However, individuals with inherited germ line mutations in a mismatch repair gene can show a predisposition to early onset tumors primarily in the colon and endometrium. The biological basis for this organ specificity is unknown. The direct consequence of mismatch repair is the so called mutator phenotype which promotes tumor formation by providing a favorable environment for mutations to occur. The subsequent mutations which possibly occur in oncogenes and tumor suppressor genes provide a selective growth advantage to the budding tumor. Ensuing accumulation of mutations is the result of MSI and selection pressure working synergistically. For colon, HNPCC is defined as an MSI-H phenotype and is characterized by germ line mutations in hMSH2 or hMLH1 (61, 62). The MSI-H phenotype also occurs sporadically in 15% of colorectal tumors. In sporadic cases, the MMR deficiency is due to epigenetic and bi-allelic silencing of hMLMh1 by de novo methylation of its promoter site in more than 90% of the colorectal tumors showing MMR deficiency. (70-72).

Model for Colorectal Tumorigenesis Characterized By MSI-H Phenotype

MSI-H cancers, like other cancers, are thought to develop through accumulation of inactivating or activating mutations in genes that regulate cell growth and/or cell death. Inherited or sporadic mutations in MMR genes results in the development of a certain

type of instability widely accepted as MSI, since they are unable to repair spontaneous errors that normally occur at microsatellite sequences during DNA replication (61, 62, 73). Besides mutations, epigenetic mechanisms which silence the MMR gene express can also result in impairment of MMR machinery. As a consequence, thousands of mutations are accumulated in these cells at such repetitive sequences that are known to be widely distributed throughout the genome (74).

The nature of genetic instability in MSI-I tumors predict that genes with repetitive coding sequences might be possible targets for inactivating and activating mutations in tumors with MMR deficiency. In line with this idea, it was reported that the TGF-β growth factor receptor II gene, which has 10 poly (A) coding repeat, was mutated in MSI-H tumors. A number of studies have reported mutations in a series of other target genes with possible roles in human carcinogenesis that have been found to be altered in colorectal MSI-H tumors by the same underlying mechanism. These include alterations in cell growth genes such as IGFIIR (75) and pro-apoptotic genes such as BAX (76) or caspase-5 (77). In addition, mutational inactivation of APC or p53 have also been reported in such tumors (3, 78)

All these alterations are known to occur with in the context of a high background of genetic instability. In MSI-H tumors it is very complex to build a sequential model for colorectal carcinogenesis. It is very difficult to establish which mutations really play a role in MSI-H carcinogenesis and at what point in the evolutionary history of the tumor they occur. This is because genetic alterations are also expected to occur in non-specific genes which do not have any expected role in tumorigenesis. However, a generalized model has been proposed (79). hMLH1 or hMSH2 mutations in normal colon epithelium

results in establishing a high basal level of genomic instability. This initial underlying genomic instability, combined with subsequent rounds of clonal selection, results in accumulation of mutations in cell growth and/or death related genes. For example, mutations in the TGF-β receptor and APC can directly aid in selection by providing a growth advantage to mutator cells. Similarly, inactivation of BAX and FAS promote cell survival. Simultaneously, mutations in genes which directly or indirectly affect genomic instability get selected which effectively raises the level of genomic instability in these tumors. The cumulative result of all these events carries the normal epithelial cell through the stages of adenoma and early carcinoma. In the final stages, early carcinoma picks up hMSH6 or hMSH3 mutations to develop into advanced carcinoma. This model has not been experimentally verified. Nevertheless, it does conform to generally accepted pathway for tumorigenesis in which natural selection and an underlying genetic instability contribute in tumor development.

Conventional Therapies for Cancer

Radiotherapy for Cancer

Radiotherapy is now commonly accepted as one of the effective forms of cancer treatment. Currently, it is being used to treat a variety of malignant tumors of different origin and stage. Ionizing radiations (IR), from various sources like linear accelerators, brachytherapy etc. are primarily used for the purpose of radiotherapy. Such IR is defined as corpuscular or electromagnetic radiations which are capable of producing ions, directly or indirectly, in its passage through matter. The absorbed dose of ionizing radiation is measured as the Gray (Gy, 1 joule of energy absorbed by 1 kilogram of material).

Radiation therapy is primarily delivered with high-energy photons (GAMMA-RAYS and X-RAYS) and charged particles (electrons). All IR causes ionization of atoms in the biological target material by electrons that travel through the target material colliding with atoms and thereby releasing packets of energy. The success of IR for treating cancer depends on its ability to selectively kill tumor cells. This is mediated by IR-induced DNA damage, which also explains the mutagenic and carcinogenic activities of IR. The damage of DNA is induced directly by ionization of DNA or indirectly by the generation of free radicals, and is manifested by single- and double-strand breaks in the sugar-phosphate backbone of the DNA molecule. In addition IR also causes cross linking between DNA strands and chromosomal proteins The IR caused genotoxic stress cumulatively results in activation of DNA repair, coupled with arrests at cell-cycle checkpoints. This allows the cells to repair the DNA damage before they can proceed to mitosis. In majority of tumors the cellular machinery responsible for detecting and repairing DNA damage is impaired. This is due to mutations in p53 gene and other genes involved in DNA damage repair pathway. Tumors are generally highly sensitive to gamma radiations because of loss of negative growth regulation and genomic stability. Following IR exposure, tumor cells do not usually die of apoptosis as most tumors lose the ability to apoptose. The antitumor effect of radiation is realized through mitotic catastrophe or in senescence-like irreversible growth arrest. IR induced tumor mitotic catastrophe is a collective result of a series of pathological events that occur after aberrant mitosis and usually result in cell death. Such mitosis does not produce proper chromosome segregation and cell division but leads to the formation of large non-viable cells with several nuclei, containing fractions of broken chromosomes.

Radiation therapy has been very helpful in treating patients with tumors limited to rectal walls and it has been proposed that radiotherapy may be used as single modality treatment approach in elderly patients with rectal tumors. In a series of rectal tumor cases, radiotherapy has been shown to significantly improve patient survival and the authors have proposed that conservative treatment of rectal cancer may be a valid alternative to radical surgery (80). However the use of radiotherapy alone for rectal cancer treatment has been questioned and it has been proposed that adjuvant radiotherapy in combination with surgery may be a better alternative in some patients (81). Local reoccurrence and metastasis of colorectal cancer following surgery has led to the development of the adjuvant cancer therapy concept. Adjuvant therapy is commonly used as a broad term encompassing all types of treatment used in conjunction with surgery and aimed at destroying microscopic metastatic disease which cannot be removed by surgery. Many adjuvant therapy trials, for treatment of colorectal cancer, have been conducted over the past 30 years and it is highly recommended in patients who are risk of developing recurrence following surgery.

IR can kill tumor cells effectively, but the dose that can be given is limited by the fact that damage to normal tissue must be minimized. Effective clinical schemes of radiation therapy and years of experiences have resulted in development of effective radiotherapy regimens. However, even with all the precautions taken to minimize exposure of normal cells to IR, it is impossible to have absolutely safe radiation treatment because the invasive nature of cancer growth means that IR must also be delivered to the areas that immediately surround the tumor. This eventually results in normal tissue toxicity and death of normal healthy tissue. Moreover, exposure to IR during treatment

can result in development of secondary cancers and the risk of cancers increases as the total dose of radiation increases. These secondary cancers can be very aggressive and respond poorly to therapy. For example Radiation associated bone tumors and sarcomas develop with-in the radiation field typically after a latency period of ten years and are refractory to therapy (82).

Chemotherapy for Colorectal Cancer

In addition to radiation and surgical treatments, there are several drugs which are currently being used as chemotherapeutic agents for the treatment of colorectal cancer. These chemical agents are broadly categorized on the basis of the chemical nature and mode of action.

5-FU remains the most widely used chemotherapeutic agent for the treatment of colorectal cancer. It is a pro-drug, an analogue of uracil, that is converted to 5-fluoro-2'-deoxyuridine monophosphate (5-FdUMP) through a de novo pyrimidine pathway. 5-FdUMP combines with methylenetetrahydrofolate to form a ternary complex with thymidylate synthase (TS), thus interfering with DNA synthesis by inhibiting the conversion of deoxyuridylate to thymidylate. Additional mechanisms of action include direct incorporation into RNA to interfere with RNA transcription and, to a lesser extent, direct incorporation into DNA (83). The therapeutic efficacy of 5-FU can be further enhanced by the addition of leucovorin calcium (LV, also known as folinic acid). The half-life and stability of the 5-FdUMP –thymidylate synthase– methylenetetrahydrofolate ternary complex is increased in the presence of greater concentrations of reduced folates, such as LV (84). Therefore LV/5-FU combination is increasingly being used in adjuvant chemotherapy for colorectal cancer treatment.

However, the therapeutic benefits of 5-FU are severely limited by a liver enzyme named dihydropyrimidine dehydrogenase (DPD). It is estimated that more that 80% 5-FU is inactivated by DPD (85) reducing the biological availability of 5-FU to target cancer cells. In addition, expression of DPD also determines the 5-FU related toxicities. DPD activity varies between 8-21 fold among individuals. Patients with lower DPD activities cannot efficiently inactivate 5-FU and form excessive amounts of active metabolite lead to severe haematopoietic, neurological and gastrointestinal toxicities that can be fatal (86-88). Therefore in the context of using 5-FU for colorectal cancer chemotherapy, DPD can be a double edged sword, when present in excessive amount it reduces the bioavailability of 5-FU by detoxification and lower amounts of DPD promote widespread toxicities.

Oral fluoropyrimidines are considered as a more acceptable alternative to 5-FU. Due to its poor bioavailability and rapid catabolic clearance by dihydropyrimidine dehydrogenase, 5-FU is unsuitable for oral delivery. Alternative oral fluoropyrimidines have been developed using a 5-FU prodrug (capecitabine) or combinations with inhibitors of DPD. Capecitabine is well absorbed by the gastrointestinal tract and undergoes a three-step enzymatic conversion to 5-FU. First metabolized in the liver by carboxylesterase to 5'-deoxy-5-fluorocytidine, capecitabine
is converted in the liver and tumour tissues by cytidine deaminase to 5'-deoxy-5-fluorouridine. A tumour-selective phenomenon is facilitated by higher intra-tumoral levels of thymidine phosphorylase, the enzyme responsible for the final conversion step to 5-FU (89)

Irinotecan (also known as CPT-11) is a semi-synthetic derivative of the natural alkaloid camptothecin which targets the activity of topoisomerase I. Topoisomerase I

plays a key role in replication by relaxing the supercoiled DNA helix with reversible and transient single stranded DNA breaks. The conversion of irinotecan to its active metabolite, SN-38, is mediated by irinotecan carboxylesterase-converting enzyme. SN-38 then stabilizes the DNA–topoisomerase complex which results in replication arrest and apoptosis (90). The major toxicity of irinotecan is delayed-onset diarrhoea. Intestinal β-glucuronidase hydrolyses a detoxified SN-38 metabolite to active SN-38, which causes intestinal epithelial damage and diarrhoea. Another characteristic toxicity is an acute cholinergic syndrome characterized by diaphoresis, salivation, lacrimation, abdominal cramps and bradycardia (91)

Oxaliplatin is a third-generation platinum with a 1, 2-diaminocyclohexane carrier ligand which forms DNA adducts and resultant strand breaks. Unlike other platinums, oxaliplatin has activity in colorectal cancer. It is thought that this may be due to the bulky 1,2-diaminocyclohexane ligand which, when retained by the oxaliplatin-formed DNA adducts, may alter the mechanisms of DNA repair (92, 93). Oxaliplatin has a distinctive neurotoxicity which can be categorized into two types. The first is an acute, cold-induced, sensory neuropathy characterized by dysaesthesias and paraesthesias during or soon after infusion. The second is a delayed-onset, dose-dependent neuropathy occurring hours to days after treatment. The latter typically occurs in 10–15% of patients after a cumulative dose of 780–850 mg/m2, with at least partial reversibility in 75% of affected patients within 3–5 months of treatment discontinuation. Both the acute and delayed neurotoxicity may be attenuated by prolonging the infusion time from 2 to 6 h (94).

Chemotherapy, like radiotherapy, is also associated with occurrence of secondary cancers. Exposure to alkylating agents have been shown to increase the risk of bone (95)

and bladder cancer (96). DNA topoisomerase II inhibitors are known cause chromosomal translocations which increase the risk of occurrence of leukeamia (97). Given the toxicities associated with conventional therapies, it is crucial that other strategies like gene therapy should be given consideration. One of the advantages in pursuing gene therapy for cancer treatment which aids in reducing the normal cell toxicity is its flexibility to limit the therapy to a loco-regional context of the tumor. Thus, undesired systemic effects of the therapy are avoided. In addition, gene therapy, by its innovative vector designing, has the potential for selective targeting of cancer cells which would further help in minimizing the therapy associated injuries.

Cancer Gene Therapy

In the past decade several advances have been made in the area of human gene therapy. In particular, gene therapy for the treatment of cancer has emerged as a promising approach. At present, gene therapy for cancer amounts to more than 63% of all the human gene therapy trials (Journal of gene medicine website). Most tumors are the result of a continuing process of accumulation of mutations in the backdrop of genomic instability. Therefore tumors are genetically unstable and thus they are extraordinarily adaptable to environmental changes. In addition tumors are very heterogenous in many respects, including genetic mutations, expression of oncoproteins, immunogenicity, response to environmental changes etc. Given extreme tumor heterogeneity, conventional therapies like radiotherapy and chemotherapy often have limited success in treating cancers. Gene therapy has the potential to address different obstacles in curing cancer as it has several advantages over conventional therapies. Various issues such as toxicity,

drug resistance and inability to target widely disseminated tumors, are associated with conventional chemo and radiation therapies. Chemotherapeutic toxicity mainly occurs because of the inability of the therapeutic drug to distinguish between normal and tumor cells. Also, tumors are genetically unstable and are extraordinarily adaptable to drug insult, which results in acquired resistance to cellular toxins and chemotherapeutic agents. Moreover, tumors induce immunological tolerance or inhibit the effecter mechanism of the immune response (98). In contrast, gene therapy strategies have the potential to target the tumor cells specifically, which in turn would reduce the toxicity load on the normal cells. Further, therapeutic vectors can be designed to deliver and express the desired therapeutic genes specifically in the tumors, independent of the tumor cell division and cell cycle stages. A variety of gene therapy strategies can be designed by incorporating different therapeutic genes, providing the flexibility of either 1) killing the cell 2) substituting the mutated gene responsible for neoplastic transformation or 3) immunomodulating the host immune response to target and eliminate the tumor.

There are several factors that make colorectal cancer in particular an attractive target for gene therapy. Most cancers disseminate early in natural history, in contrast colorectal carcinoma is often confined to specific organs (such as the liver) or compartment (such as peritoneal cavity). As many as 30% of the patients suffering from CRC have macroscopic relapses in liver (99), compared with for example, breast cancer, in which metastases in multiple sites (such as bone, liver and lung) are much more likely to occur. This could offer an advantage through regional delivery of therapeutic vector to maximize tumor exposure and minimize systemic toxicity. There is more information of the sequence of genetic and epigenetic events that characterize the transformation of

colon epithelium to premalignant adenomas and then invasive carcinoma than there is for the development of any other tumor. These data can be used to construct rational gene therapy vectors for example tumor suppressor pathway genes such as p53 and retinoblastoma (RB) can be potentially used for designing tumor selective replicating adenoviruses. Similarly, there is a large database of CRC associated onco-fetal antigens (100). The promoters of which might be used to restrict transgene expression to tumors and allow a further means of assessing tumor response to treatment. In addition, one the most important argument justifying gene therapy approach for treatment of colorectal cancer is; wnt signaling pathway is frequently found selectively activated in majority of colorectal carcinomas due to mutations in APC/β-catenin genes resulting in transactivation of the promoters of TCF-4/ β-catenin responsive genes. These promoters are characterized by the presence of a TCF/ β-catenin enhancer which binds to the heterodimeric complex of TCF-4/ β-catenin. These downstream target genes of, wnt signaling in colorectal carcinoma, are fairly well known. Therefore characterization of such promoters which are a target of aberrantly activated wnt signaling in colorectal carcinoma cells can be very helpful in designing of colorectal cancer selective gene expression promoters for the purpose of gene therapy. In particular, by using Tcf-4 enhancer elements, colorectal carcinoma selective gene expression promoters could be designed for targeting the therapeutic gene to CRC cells and increasing the therapeutic efficacy. Finally, CRC and its adenoma precursors are more often amenable to biopsy of both early and late disease than other tumor types, which can add an important mechanistic element to proof-of principle clinical trials, such as demonstration of viral

replication, transgene expression, induction of apoptosis and tumor infiltration by lymphocytes.

Gene Therapy for Colorectal Cancer

Gene Correction or Replacement

The multi-step development of CRC from a single stem cell to a malignant tumor is well described in literature (32). This involves sequential mutations in tumor suppressor genes and several oncogenes. At its most basic level, gene replacement therapy would involve the use of viral vectors to re-introduce wild type genes for phenotypic correction. There are obvious stoichiometric flaws in this reasoning in that it would be impossible to correct all mutant genes (as many of them are yet to be discovered) in every cancer cell. However, common mutations in CRC, such as those of the tumor suppressor TP53 (45-60% of CRC cases) or the proto-oncogene KRAS (50-60%) have been assessed as therapeutic targets.

There are *in vitro* and *in vivo* studies which show that adenovirus mediated delivery of p53 gene has an antiproliferative affect on colorectal cancer cells. This effect of p53 can be hypotheticaly explained in several ways. For example p53 could be anti-angiogenic and down regulate the vascular endothelial growth factor expression and up regulate thrombospondin (a known anti-angiogenic fector), generating a by stander effect which kills the tumors cells due to lack of nutrients (101). Also, p53 has been shown to Further, p53 gene transfer could lead to increased survival following intratumoral administration of p53 gene carrying adenovirus (102). Replacement of the p53 gene has also been show to sensitize CRC cells to conventional cytotoxic agents such as 5-fluorouracil (5-FU), presumably by reconstituting the apoptotic pathway (103). KRAS

has been implicated in maintaining uncontrolled proliferation of CRC cells; therefore it is a logical strategy to target KRAS for CRC treatment. In a rat model of liver metastasis, developed by using a KRAS mutated CRC cell line, significant tumor regression was shown to occur after hepatic arterial administration of a replication deficient adenovirus encoding an intracellular antibody specific for KRAS (104)

Virus-Directed Enzyme-Prodrug Therapy (VDEPT)

In this approach an enzyme encoding gene which can convert an inactive, non-toxic prodrug to an effective cytotoxic species is used as the therapeutic gene to target cancer cells. Currently there are VDEPT strategies being pursued for the purpose of CRC gene therapy: 1) HSV-tk/GCV 2) cytosine deaminase/5-fluorocytosine 3) nitroimidazole reductase (NTR)/CB1954. The use of HSV-tk/GCV system for cancer gene therapy has been explained in detail on page 23.

Cytosine deaminase (CD) is an enzyme that catalyzes the deamination of cytosine to uracil. This reaction converts an inert prodrug, 5-fluorocytosine (5-FC), to a highly toxic chemotherapeutic agent, 5-fluorouracil (5-FU) (105). This enzyme is found in many bacteria and fungi but not in mammalian cells (106-108). Consequently, mammalian cells are resistant to the toxic effects of 5-FC. The cytotoxic effects of 5-FU occur following its conversion to 5-fluoro-2-deoxyuridine-5-monophosphate (5-FdUMP) (through a de novo pyrimidine pathway). 5-FdUMP is an irreversible inhibitor of thymidylate synthase and thus inhibits DNA synthesis by deoxythymidine triphosphate (dTTP) deprivation. The CD–5FC suicide gene system has been studied as a potential gene therapy strategy in a number of solid tumors. The CD gene was transferred to colon carcinoma cells in vitro, and these altered cells were implanted in mice as xenografts. The mice were then treated

with systemic 5-FC. High local concentrations of 5-FU were produced by conversion of 5-FC to 5-FU in the tumors (109). Adenovirus-mediated transfer of the CD gene together with systemic administration of the prodrug 5-FC caused suppression of colon cancer metastatis to the liver (110-112). Phase I trials have been undertaken with direct intratumoral inoculation of a replication deficient adenovirus encoding cytosine deaminase and oral administration of 5-FC. Initial reports indicate that the virus is well tolerated, and biopsies have indicated cancer-associated expression of cytosine deaminase (113).

One of the most promising suicide gene systems is the *Escherichia coli* enzyme nitroreductase in combination with the prodrug CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide]. CB1954 is a weak, monofunctional alkylating agent that was synthesized during the 1960s. The nitroreductase enzyme DT diaphorase converts CB1954 to its 4-hydroxylamino derivative (114), which after acetylation via thioesters such as acetyl coenzyme A (CoA), becomes a powerful bifunctional alkylating agent. The activated prodrug is then capable of forming poorly repaired DNA crosslinks. Human DT diaphorase is not capable of performing this conversion, thereby limiting toxicity to transformed cells. The advantage of the nitroreductase–CB1954 suicide gene system is that killing mediated by activated CB1954 is not dependent on cell cycle phase, potentially allowing quiescent tumor cells to be killed. Selective killing of nitroreductase-expressing cells following CB1954 administration in vitro (115, 116) and in vivo (117) has been demonstrated. In addition, the bystander effect seen with the HSV-TK gancyclovir suicide gene system is also seen with the nitroreductase–CB1954 system. Mixed populations of nitroreductase-expressing and nitroreductase-none expressing cells

were killed after treatment with CB1954 (115). One study has also demonstrated a synergistic effect when cells expressing both nitroreductase and TK were treated with a combination of CB1954 and gancyclovir (116). In a phase I clinical trail, involving patients with CRC, Nitroreductase/CB1954 suicide gene system was tested with out any significant side effects (118).

Immunogenetic Strategies

The idea of Immunogenetic therapy for CRC, either antibody based or cell mediated, is gaining a lot of attention these days. As opposed to most of the gene-therapy approaches which involve regional targeting of the tumor, immunogenetic approaches have the potential to modify the tumor microenvironment for generating an effective anti-tumor immune response. Further, the promise of immunotherapy lies in the fact that although it can be delivered locally, it can generate a signal that can be amplified (for example, by T-cell proliferation) and disseminated through the vasculature and tissues by lymphocyte trafficking, thereby exposing the tumor cells to the cytotoxic mediator at sites distant from the area of gene vector inoculation. There are several immunogenetic approaches which have been used to target CRC.

Manipulating MHC expression: It is well known that most cases of advanced CRC downregulate MHC class I molecules as one means of escaping immune surveillance. In a phase I clinical trial, involving 15 CRC patients negative for HLA-B7, a plasmid encoding HLA-B7 was injected directly into the hepatic metastatic nodules of CRC. HLA-B7 expression was shown to be present in tumor biopsies of 12 patients and a total of 50% patients mounted an HLA-B7 specific cytotoxic T-lymphocyte response. There were side effects but no tumor regression was reported (119).

Delivery of Interleukin-2 (IL-2): IL-2 has been used extensively as a single agent or in combination of other cytokines to target cancer. IL-2 administration in patients has shown very limited anti-tumor benefits which are associated with toxicity. There are reports which suggest IL-2 related activity upon genetic introduction of IL-2 into autologous tumor cells, fibroblasts or immune effector cells in syngeneic mouse models (120, 121). IL-2 has also been explored in humans for its anti-tumor effects. Clinical trials have involved *ex vivo* transduction of autologous fibroblasts (122) with IL-2 (which were then mixed with autologous irradiated CRC cells before subcutaneous injection), transduction of autologous cytokine induced killer cells with IL-2 and direct intratumoral injection of IL-2-encoding plasmid–lipid complexes (123). These trials have been characterized by evidence of increased immune reactivity (IL-2 detection, CTL responses, and induction of cytokines such as IFN-γ and GM-CSF (granulocyte–macrophage colony-stimulating factor), but no evidence of tumour regression.

Carcinoembryonic antigen (CEA) vaccines: CEA is a cell surface glycoprotein which is found overexpressed in 85% of CRC cases. CEA is tumor specific onco-fetal antigen and has the potential to be used as an antigen for making anti-tumor vaccines. The gene encoding CEA has been incorporated into several vectors for the purpose of making a vaccine for CRC. In a phase I clinical trial patients were immunized with vaccinia-CEA vaccine involving CEA vaccine and generation of cytotoxic T lymphocytes specific for CEA was demonstrated (124). Several other clinical trials involving CEA vaccines have been carried out and in most cases the vaccine was shown to well tolerated (125-128). However, despite of these ongoing efforts CEA encoding tumor vaccines have not, so far, shown any clinically significant anti-tumor effects.

Adenovirus for Therapeutic Gene Delivery

One of the major challenges in gene therapy is efficient delivery of the therapeutic gene to the target cells. The biggest physical hurdle that gene therapy technologies have to overcome is the cell membrane which is impermeable to charged macromolecules such as DNA and RNA (129) While there a multitude of biological, physical and chemical procedures being used to facilitate the delivery of trans-genes, adenoviral vectors, in particular, have shown an enormous amount of potential for clinical delivery of the trans-gene to the target cells. Adenoviruses are double stranded DNA viruses belonging to the parvovirideae family and are known to cause upper respiratory ailments in humans. The virion is a small, non-enveloped particle with a spike, icosahedral morphology (130). Serologically, adenoviruses have been classified into nearly 50 distinct serotypes (131). The different serotypes have been subgrouped (A through E) based on genomic size, organization, %GC content etc (132). Majority of the adenoviral vectors commonly used as gene delivery vehicles, are based on human adenovirus of serotypes 2 and 5 of the subgroup C (133). There are several major advantages of adenoviral vectors, which make them suitable for efficient transfer of trans-gene: high titer (10^{12}-10^{13} virus particles per ml) of recombinant viruses can be produced; ability to infect postmitotic cells; ability to infect a wide variety of cell types; ability to accommodate up to -8 kb of foreign DNA, including expression cassettes or other regulatory sequences; and safety precedents exist, as adenovirus based vaccines have been used in humans without any serious life threatening side effects (134). In addition, adenoviruses infect a variety of cell types including fibroblasts, epithelial cells, stromal cells, endothelial cells and hepatocytes of human, canine, rodent origin. Most of these adenoviral vectors have been rendered

replication deficient by deletion of their essential genes (135). To generate a defective adenovirus for gene transfer application, the E1 gene, important for viral gene expression and replication, can be removed. Thus, E1-deleted viruses can be propagated only in a cell line that provides the E1 gene products in trans, such as 293 cells (136). The E1–deleted adenoviral vectors, however, express low levels of viral antigens following infection and result in a low level of DNA replication, especially at a high multiplicity of infection. Synthesis of adenoviral gene products often stimulates an immune response to the infected cells and results in a loss of therapeutic gene expression 1–2 weeks after injection (137, 138). To overcome the problems of reduced therapeutic gene expression and host cell toxicity, newer generation adenoviruses with E3 and E4 regions deleted have been constructed (139). Such replication incompetent vectors are widely used for gene delivery in vivo and are in clinical trials for cancer and other diseases like cystic fibrosis (140). There are, however, some limitations of adenoviral vectors: lack of sustained expression, as the viral DNA does not integrate into the host genome; antigenicity against viral proteins by both humoral and cytotoxic T-lymphocytes (CTLs); and possible toxicity at high doses (134). The lack of sustained expression is not likely to present a problem for acute applications like cancer as transient expression of therapeutic genes may be favored. The humoral response may be blocked or reduced by co-administration of immunosuppressive agents or cytokines. Alternatively, the use of adenoviruses of different serotypes may allow for repeated administration, even in the presence of neutralizing antibodies (141).

Herpes Simplex Virus Thymidine Kinase Gene/ Ganciclovir and Suicide Gene Therapy

Frederic Moolten was the first to conceptualize the idea that HSV-tk / GCV system can be potentially used for gene therapy (142). This was followed by work done by the same group showing that HSV-tk/GCV treatment generated enough phosphorylated GCV to inhibit mammalian DNA replication(143). Thereafter, a few years later many researchers started to apply this system and other prodrug activating enzymes for gene therapy (108, 144). In this chapter I will summarize: 1) how the HSV-tk/GCV system works 2) HSV-tk/GCV system mediated bystander affect 3) what are the current strategies being followed to enhance the therapeutic efficacy, 4) various clinical trials undertaken.

Mode of Action of the HSV-Tk/GCV System

GCV is an acyclic analog of the nucleoside 2'-deoxyguanosine. It is used as an antiviral agent for treating human herpes infections (herpes simplex type 1 and type 2, varicella zoster virus and Epstein-barr virus) (145). The antiviral property of GCV is dependent on its intracellular phosphorylation by a viral encoded thymidine kinase. The triphosphate derivatives of GCV act as a competitive inhibitor of deoxyguanosine triphosphate, inhibiting viral DNA synthesis (146, 147). In cells infected with HSV or varicella zoster virus, viral thymidine kinase catalyzes the production of ganciclovir mono-phosphate (147, 148). Subsequent conversion of GCV-monophosphate to GCV-triphosphate is aided by cellular guanylate kinase (149) and several other cellular kinase enzymes, such as phosphoglycerate kinase (150). Tri-phosphorylated forms of GCV are analogous to 2' deoxyguanosine and bind to DNA polymerase δ. Once bound to DNA

polymerase GCV triphosphate is either incorporated into DNA or inhibits the polymerase itself. Although GCV has hydroxyl groups found in endogenous nucleosides permitting chain elongation, the lack of a complete sugar ring makes GCV a poor substrate for chain elongation. As a result, chain termination almost always occurs immediately after GCV incorporation into DNA and leads to cell death (151).

Mechanisms of HSV-Tk/GCV Mediated Cell Killing

In reference to cancer gene therapy mediated by HSV-tk/GCV system there is evidence which supports that, depending on the cell type, tumor cell killing can occur via both apoptotic (152) and non-apoptotic mechanism (153). Support for the involvement of the apoptotic pathway in HSV-tk/GCV mediated cell killing comes from the fact that expression of Bcl-2 is able to inhibit HSV-tk/GCV caused cell death (154). In addition apoptosis has been shown to occur in human hepatocarcinoma cells upon treatment with HSV-tk/GCV (155). Moreover, the level of apoptosis was observed to be dependent on the p53 status of the cell as it was found to be reduced in p53 negative cells as compared to p53 positive cells. In contrast, HSV-tk/GCV mediated apoptosis as seen in CHO cells is shown to be dependent upon activation of mitochondrial damage pathway, independent of p53 status (156).

The effect of HSV-tk/GCV mediated cell killing on cell machinery has also been explored by researchers. It has been shown in B16F10 murine melanoma cells, that the cause of cell death, upon expression of HSV-tk and followed by GCV treatment, seems to be due to the irreversible cell cycle arrest at the G_2-M transition and is independent of apoptosis (153). On the contrary in similar model systems it was demonstrated that cell

killing by the HSV-tk/GCV strategy showed an S-G2 phase arrest with signs of apoptosis (157).

The HSV-Tk/GCV Bystander Effect

In addition to direct cell killing, a bystander effect (BE) has also been observed. BE is the ability of the HSV-tk expressing cells to induce sensitivity, upon GCV treatment, in neighboring non HSV-tk expressing cells. BE is one of the most important aspects of using HSV-tk/GCV system for cancer gene therapy. It was originally described by Moolten and Mells in 1990 when they observed that cells transduced with HSV-tk gene and treated with GCV induced sensitivity in neighboring cells (143). Preliminary experiments, analyzing BE demonstrated that for some cell types in culture, when only 10% of the cells were expressing HSV-tk, treatment with GCV could result in 100% cell death. This was soon validated by in-vivo experiments showing that 10-50% of the total cell population expressing HSV-tk is enough to produce complete tumor regression upon GCV treatment (158-160).

Almost immediately it was established that BE requires cell to cell contact between cells expressing and non-expressing the HSV-tk gene (158). This observation was strengthened by experiments which demonstrated that conditional media from cells expressing HSV-tk and treated with GCV did not confer cytotoxicity to non-expressing HSV-tk cells (161). On the whole these experiments suggested that the BE of the HSV-tk/GCV system results from direct cell to cell contact.

The mechanism of BE caused by HSV-tk/GCV is fairly well understood. It is generally accepted that BE occurs by transfer of phosphorylated GCV from HSV-tk expressing cells to non-HSV-tk expressing cells via cell to cell gap junctions. This idea is

further reinforced by the fact that the magnitude of the BE is correlated with the extent of gap junctional intercellular communication (GJIC) present between two cells (162). Gap junctions are semi-channel like structures (called connexons) present in the cell membrane. Connexons are made up of proteins belonging to a multigene family of distinct but functionally related proteins called connexin (Cx) (163). Expression of connexins in cells which do not form gap junctions has been shown to induce GJIC and resultant BE (164). In addition, there are certain drugs such as retinoic acid (165), apigenin (164) or lovastatin (166) which are known to positively regulate the formation of gap junctions and increase the extent of BE when added to tumor cells. In contrast, drugs like 18-α-glycirrethinic acid (AGA) (167) which negatively regulate gap junctions are known to inhibit BE. Further, the role of gap junctions in HSV-tk/GCV system has also been shown in vivo. Subcutaneous tumors derived from Cx43 (connexin 43 gene) transfected glioma cells showed that after GCV treatment only 25% of the HSV-tk expressing cells were enough to eliminate the tumor (168). Although it is clear that gap junctions play the major role in mediating the HSV-tk/GCV BE, there are in vitro studies which do not correlate between gap junctions and the extent of BE. For example studies have shown that in some instances it is not possible to inhibit BE by inhibiting GJIC either in cells lines derived from lung cancer (169) or in colon tumor cell lines (170). Further there is evidence that supports that GJIC may not be solely responsible for BE. This phenomenon has been illustrated in SW620 colon cancer cells. These cells when expressing the HSV-tk are able to efflux phosphorylated GCV metabolites to the extra cellular medium (171). It's been postulated that the efflux phenomenon could either be due to the presence of ATP dependent anionic transporters or to the presence of proteins

which confer drug resistance. The authors propose that two recently identified multi-drug resistance proteins MP-4 and MRP-5, which efflux phosphorylated nucleotides may be responsible for effluxing phosphorylated GCV. In addition, another mechanism to explain HSV-tk/GCV mediated BE have also been proposed. According to this hypothesis, apoptotic vesicles generated in HSV-tk expressing cells containing toxic metabolites are phagocytated by neighboring non-HSV-tk expressing cells (158).

The *in vivo* mechanism of HSV-tk/GCV mediated BE has also been explored. Other than GJIC, the immune response generated due to the expression of non human proteins or due to the death of transduced cells have been proposed to cause BE. It is hypothesized that non-human proteins can stimulate recognition of tumor antigens, trigger the immune response and finally end up with the death of non-transduced cells (172). In addition, some studies suggest that the immune response generated against the HSV-tk/GCV system can induce a distant BE. Treatment with GCV of tumors expressing HSV-tk can mediated regression of tumors which do not express HSV-tk and which are located in other part of the animal (173). This distant BE suggest that local treatment with HSV-tk system could prevent the growth of dissemination and possible metastasis (152).

Strategies to Enhance the Therapeutic Response of HSV-Tk/GCV System

Over the past decade several efforts have been made to improve the efficacy of HSV-tk/GCV system for gene therapy purposes. These strategies can be broadly divided into two categories: 1) HSV-tk mutants 2) Combined approaches.

HSV-Tk Mutants

One limitation of the HSV-tk therapy is the low sensitivity of cells to GCV or other nucleoside analogs. A popular strategy is to improve potency of the suicide effect,

by engineering the HSV-TK protein. The basic rationale behind this approach is to construct and characterize an HSV-TK mutant that preferentially phosphorylates specific nucleotide analogs. Black et al have created several mutants of HSV-tk which were screened for GCV or ACV phosphorylation in mammalian cells and have identified mutants which are more sensitive to ACV or GCV (174). One particular mutant Sr39 when evaluated in a xenograft model prevented tumor growth at prodrug dosages that did not affect wild type HSV-tk (175). Further, using a HSV-tk mutant generated by Black et al in 1999 it was shown that tumor cells expressing this mutant resulted in an increase of GCV sensitivity. This sensitivity was nine to five hundred folds higher when compared to wild type HSV-tk (176)

Combined therapies

It is generally accepted that in cancer therapy multidrug regimens are superior to single agent therapy. This has prompted various investigators to test different combinations of strategies to enhance the cytotoxic effects of the HSV-tk/GCV system. The HSV-tk/GCV system has been used in combination with other gene directed enzyme prodrug therapy (GDEPT) strategies in search of enhanced anticancer effects. HSV-tk/GCV when used in combination with the cytosine deaminase / 5- fluorouracil (CD/5-FU) system to treat 9L gliosarcoma cells reduced cell survival both in vitro and in vivo. The effects of the two components appeared to be synergistic and related mechanistically to the enhancement of CV phosphorylation by thymidine kinase following 5-Fluorouracil treatment (177). The combination of these suicide genes has also been shown to sensitize the tumor cells to radiation with potent antitumor effects (178).In another study the HSV-tk/GCV system has been combined with cytochrome P450 2B1 / cyclophosphamide

GDEPT. It's been postulated that these two GDEPT act synergistically. HSV-tk/GCV gene therapy inhibits the repair of cellular cross-linking of DNA caused by P4502B1/cyclophosphamide (179). In addition this synergism has been shown to require co-expression of both genes in the same cell. In an attempt to achieve enhanced tumor cell killing, several chemotherapeutic agents have been combined with the HSV-tk/GCV system. Drugs like topotecan (topoisomerase I inhibitor) results in synergistic cytotoxicity in HSV-tk expressing murine MC38 and human HT29 colorectal cancer cells, both in vitro and in vivo (180). Moreover, a synergistic effect was observed when GCV and topotecan were administered in combination with a replication competent adenovirus expressing HSV-tk (181).

UCNO1 (a protein kinase c inhibitor) when used in combination with HSV-tk/GCV resulted in increment of apoptosis and tumor cell killing in human colon cancer cells (182). This apoptotic enhancement was associated with the increased levels of proapoptotic proteins Bcl-XL. However, when HSV-tk/GCV was combined with Taxol of Campthecin, an antagonistic effect was observed. Furthermore, in glioblastoma model a synergism between the HSV-tk/GCV and temozoloamide (TMZ, and alkylating agent used to treat gliomas, has been shown (183).

These results clearly show that the combination of certain, but not all chemotherapy drugs and HSV-tk/GCV may result in increased cytotoxic effects.

HSV-Tk/GCV and Radiotherapy

The combination of HSV-tk/GCV prodrug therapy and radiotherapy has been attempted in several studies. The rationale behind these approaches is that radiation induces membrane damage which may facilitate the bystander effect of HSV-tk/GCV

gene therapy whereby cytotoxic nucleotides analogs diffuse from HSV-tk expressing cells to neighboring non HSV-tk expressing cells. In addition, cells would not be able to repair radiation-induced DNA damage in the presence of cytotoxic nucleotide analogs. One of the earliest studies showed that the addition of radiation improves the effectiveness of HSV-tk/GC gene therapy for the brain tumors (184). Similar synergistic effects were observed when a fusion construct containing both suicide genes HSV-tk and cytosine deaminase, was used to transfect 9L glioma cells followed by drug treatment (178, 185). Combination of HSV-tk/GCV therapy and radiation was shown to be synergistic when used to target head and neck cancer xenografts in nude mice (186). Further, in prostrate cancer cells, it was demonstrated that HSV-tk/GCV system and radiotherapy act synergistically in tumor cell killing which increases with higher doses of radiation (187).

HSV-Tk/GCV System and Induction of Immune Response

There are findings suggesting the existence of an antitumor immune response when HSV-tk/GCV therapy is used for in vivo treatments. The phenomenon of the bystander effect which is characteristic of HSV-tk/GCV system is also believed to have an immunologic component (172). There are two important features of this HSV-tk/GCV mediated antitumor immune response: 1) the bystander effect is mediated by the immune /inflammatory system, 2) the killed tumor cells can generate an antitumor response since GCV is not immunosuppressive (188, 189). The other crucial part of bystander effect which occurs through GJIC involving transfer of toxic drug metabolite has been explained earlier on page 25. Findings supporting a role for the immune system include demonstration of cytokine release leading to the haemorrhagic necrosis and to an

infiltration of immune cells into the tumor after the HSV-tk/GCV therapy (172, 190, 191). This cytokine release with in the tumor also leads to up-regulation of immune regulatory molecules such as B7.1, B7.2 and ICAM (192). These findings have led to the hypothesis that the bystander effect changes the tumor microenvironment from one that is immuno-inhibitory to one that is immunostimulatory. This is because HSV-tk expressing cells die through apoptosis, during which they release soluble factors such as IL-1, which further affect the tumor microenvironment.

One of the potential ways of increasing the BE is to use biological response modifiers such as cytokines to augment the immune / inflammatory response generated by the HSV-tk/GCV therapy. In one particular study, immunotherapy in combination with HSV-tk/GCV suicide gene therapy was tested in a experimental metastatic breast cancer model. Adenoviral vectors were used to transfer transgenes for HSV-tk along with either cytokine gene: GCM-CSF and IL-2 to the established breast tumors. Upon 10 days of GCV administration an enhanced reduction in tumor volumes was observed (193). IL-12 has been demonstrated to be highly efficient in enhancing the antitumor response in combination with HSV-tk/GCV therapy. Studies in colon carcinoma using adenoviruses as vectors for gene delivery have proven to be highly efficient in inhibition of tumor growth and in the increase of survival time (194).

Clinical Trials

Several phase I and phase II clinical trials have been implemented in the last decade using the HSV-tk/GCV gene therapy strategy. Results from only few of these studies have been published.

1) Mesothelioma: Adenoviral vectors encoding the HSV-tk gene under the control of sarcoma virus promoter were administered in variable doses to patients with mesothelioma. Transient fever and minor changes in liver function tests were the most common adverse effects noticed. In addition, local skin reaction due to adenovirus infection was observed in 60% of the patients. Tumor cell transduction was confirmed by PCR and immunoblotting. Furthermore, in this study authors did mentioned the existence of humoral and cellular immune responses against the adenoviruses but it did not produce any clinical effects (195). Further results were not reported.

2) Melanoma: Patients with malignant melanoma were treated with intratumoral injections of retroviral vectors encoding for HSV-tk which was followed seven days later by administration of GCV. Adverse effects like transient fever and skin reaction were noticed. A moderate decrease in tumor size was constantly observed for the period of administration of GCV but all tumors progressed there after (196)

3) Brain tumors: Gene therapy for malignant gliomas utilizing HSV-tk/GCV system is currently a very hot area in the field of cancer gene therapy. Glioblastomas have one of the highest mortality rates among cancers and remains very challenging to manage as patients have an average survival rate of 32-56 weeks after being first diagnosed. The earliest clinical trials involving treatment of brain tumors with HSV-tk/GCV suicide gene therapy did not meet with any significant success. In one study, malignant glioma patients received multiple injections of a retroviral vector (coding for HSV-tk) producing cells. A follow up assessment of gene transfer by in situ hybridization showed that effective transfer was very limited and did not produce any antitumor effects (197). A clinical trial following a similar therapy protocol involving patients with primary or

recurrent malignant gliomas did not result in any conclusion which could advocate the use of HSV-tk/GCV suicide gene therapy to treat brain tumors (198). A French group evaluated the effect of injecting retroviral producing cells after debulking surgery for recurrent gliomas. Almost two weeks after GCV treatment, tumors showed necrosis in three of eight patients. However all patients showed disease progression on long term follow up. Limited antitumor response was attributed to poor transfer of the therapeutic gene to the tumor (199).

Recently partial success in treating malignant gliomas using HSV-tk/GCV suicide gene therapy has been reported. Eleven patients with recurrent malignant gliomas after surgical resection and radiotherapy were included in this clinical trial. Adenoviral vectors expressing HSV-tk were used for the gene delivery and no adenoviral vector shedding or systemic toxicity was noticed in this study. Additionally, HSV-tk expression as assessed by PCR was negative after 3 months of adenoviral injections. Ten of eleven patients survived for an average of 112.3 weeks from the day of diagnosis (200).

In summary HSV-tk gene transfer to a variety of neoplasms can be safely achieved in vivo by intratumoral injections using adenoviral or retroviral vectors. GCV administration after HSV-tk gene transfer does not result in significant toxicity. Although, significant success in brain tumor regression has not been achieved, nevertheless recent studies have shown interesting results.

Imaging Gene Expression

There is another aspect of HSV-tk suicide gene therapy which is worth mentioning. One of the hurdles in human gene therapy is the inability to establish in each patient whether gene transfer has been achieved, what tissues have been transduced and

are actively expressing the transgene and what is the magnitude of the transgene expression? In combination with positron emission tomography (PET), the HSV-tk gene has shown the potential to resolve the above mentioned problems. PET is a non invasive imaging technique which follows the fate of a previously administered, radiotracer to obtain accurate, quantitative and dynamic information about the location of the radionucleotide in the patient. The principle behind the approach is HSV-tk once successfully expressed in a cell, is able to phosphorylate the radiotracer (for e.g. 8-[18F]fluoroganciclovir) which gets trapped inside the HSV-tk-expressing cells only and can be visualized externally with a PET camera (201). Several radio labeled thymidine and ganciclovir derivatives have been proposed as probes for imaging of HSV-tk enzyme activity with PET, including [124I]FIAU (202), and 9-[(1-[18F]Fluoro-3-hydroxy-2-propoxy)methyl]guanine ([18F]FHPG) (203). These radiopharmaceuticals are better substrates for the HSV-tk enzyme than for human thymidine kinases. PET imaging, offers a potential method of high resolution and sensitivity which allows noninvasive quantification of HSV-tk expression (204).

Wnt Signaling And β-Catenin/Tcf-4 Transcriptional Regulation

The APC driven model for colorectal tumorigenesis suggests that wnt signaling pathway is aberrantly activated in majority of colorectal tumors. Besides, there is evidence indicating that wnt signaling pathway is also found to be activated in other tumors for example, prostrate and breast (205-207). The differential activation of this pathway in cancer cells makes it an attractive target for designing tumor specific gene expression promoters. The canonical Wnt signaling pathway has been shown to be well

conserved in evolution and is currently best described in invertebrate systems like *Xenopus* and *Drosophila* where it plays a significant role in development. The pathway is comprised of a family of secreted Wnt glycoproteins which signals by binding to cell surface co-receptors, frizzled and LRP5/6, (low-density lipoprotein receptor related protein) (8, 208). One of the major effectors of this pathway is β-catenin which forms transcriptionally active complexes with the Tcf/Lef (T cell / Lymphoid enhancer factor) family of proteins when a Wnt signal is received at the cell surface. This heterodimeric complex transactivates various Wnt responsive genes, which contribute in making developmental decisions. In parallel, the Wnt signaling pathway has been shown to be activated in a majority of human cancers of epithelial origin and is marked by a transcriptionally active β-catenin / Tcf-Lef complex. Genes transactivated by Wnt signaling are considered to contribute to the pathogenesis of human cancers (24). β-catenin is a multifunctional protein, and apart from it's oncogenic role, it is known to associate with proteins like cadherins and other catenins, participating in processes like cell adhesion (209). The Wnt signal increases the levels of monomeric β-catenin in cells by destabilizing the complex of proteins which are responsible for glycogen synthase kinase-3β mediated phospholyration of β-catenin. This is well documented in *Xenopus* and *Drosophila* (210-212). However, in human cancers it has been shown that, mutations in the key proteins responsible for β-catenin degradation can also lead to upregulation/stabilization of β-catenin, even in the absence of a Wnt signal. Stabilized β-catenin forms a transcriptionally active complex with Tcf-Lef and consitutively transactivates Wnt responsive genes (10). A variety of proteins are involved in regulation of β-catenin levels including the tumor suppressor gene APC (Adenomatous Polyposis

Coli), which is found to be associated with β-catenin and mutated in more than 85% of colorectal tumors (13) In these carcinomas elevated levels of β-catenin coupled with transcriptionally active complex of β-catenin/Tcf-4 were found (213).

In vitro studies done on cell lines derived from colon tumors have shown that introduction of wild type APC effectively reduces the levels of β-catenin in conjunction with disruption of transcriptional activity of β-catenin / Tcf-4 complex (213). APC is also associated with at least two other proteins, Axin and glycogen synthase kinase-3β (GSK-3β), which contribute towards regulation of β-catenin. Axin has binding sites for APC, GSK-3β and β-catenin and it functions as central scaffolding in the multiprotein complex (214). The APC-Axin complex is believed to associate and regulate the activity of GSK-3β, which in turn phosphorylates APC-Axin increasing their affinity for β-catenin (215). GSK-3β, a serine threonine kinase, associates and phosphorylates β-catenin on the N-terminal serine/threonine residues. Phosphorylated β-catenin is recognized and tagged by the β-Trcp protein; β-catenin further gets covalently modified by addition of ubiquitine. Ubiquitinated β-catenin is degraded by the proteasome destruction pathway (216, 217). Therefore, an N terminal serine/threonine residue mutation in β-catenin renders it refractory to APC's regulatory tumor suppressive effect resulting in stabilization of β-catenin (218). Hence the ability of GSK-3β to phosphorylate β-catenin is crucial for its destruction. How the wnt signaling pathway communicates with GSK-3β is not clearly understood. However, studies from invertebrate model systems have suggested that at least two proteins, disheveled (Dvl) and FRAT provide a link between Wnt signal and GSK-3β. FRAT is associated with Dvl which is turn is bound to Axin, Dvl / FRAT complex, upon receiving a Wnt signal, inhibits GSK-3β resulting in non-phosphorylation

and stabilization of β-catenin (219). The mechanism of inhibition of GSK-3β by Dvl /FRAT complex is not clearly understood; but possibly occurs by disruption of the Axin-APC scaffolding (7).

Therefore, with the underlying principle that canonical Wnt signaling pathway is constitutively activated in a large number of adenocarcinoma, it is reasonable to hypothesize those recombinant promoters incorporating Tcf-4 enhancer elements will be specifically active in such Wnt signaling positive tumors and not in normal cells.

β-catenin/Tcf-4 Transcriptional Regulation

The APC and β-catenin mutations result in the formation of a constitutively active β-catenin/Tcf-4 complex, which transactivates a number of cancer promoting genes. This occurs by binding of the β-catenin/Tcf-4 complex to the Tcf-4 enhancer regions in the promoters of these target genes. A few genes activated by this complex have been identified and include c-Myc (19), c-jun and fra-1(24), cyclin D (20, 215) and matrilysin (MMP7) (22). It appears that LEF-1 family of transcription factors, of which Tcf-4 is a member, act as DNA scaffolding proteins that alone do not affect transcription (220). They must interact with a partner protein (like β-catenin), and this complex leads to gene regulation via interaction with basal transcription machinery (221). β-catenin has been shown to translocate into the nucleus independent of any association with Lef/Tcf binding (222), and other non-DNA binding proteins may be involved in forming or stabilizing the β-catenin and Tcf-4 interactions (222). Because the Lef/Tcf interactions appear to interact with DNA as a true enhancer element and do not activate transcription independently (220), it is possible for gene therapy purposes to couple the Tcf-4 enhancer with other promoter elements that may not affect overall gene expression activities.

Another unique aspect of Tcf-4/ β-catenin regulation is the modulation of responsive genes by chemical modulators like butyrate, phorbol esters, doxorubicin and retinoids. Short chain fatty acids like butyrate and trichostatin A have been reported to increase Tcf-4/ β-catenin mediated gene expression (223, 224). This is consistent with the reported role of these compounds in the homeostasis of the colonic mucosa through induction of cell maturation pathways, cell cycle arrest, differentiation and apoptosis (224). Recent microarray analysis of butyrate and trichostatin A treated colon tumor cells indicated activation of a distinct subset of genes in cell differentiation processes, including elevation of Tcf-4/ β-catenin responsive genes (224). Addition of the phorbol ester PMA has also been reported to increase Tcf-4 mediated gene expression (225). Conversely, the addition of doxorubicin (226) or retinoids (227) led to decreased Tcf-4/ β-catenin mediated gene expression. Therefore, modulation of the transcriptional activity of the recombinant Tcf-4/ β-catenin enhancer /promoters may provide an opportunity to regulate the level of expression of therapeutic genes under clinical situations.

Promoters for Design of Recombinant Tumor Specific Gene Expression DNA Elements

There are several oncogenes which are found up-regulated in cancers and are believed to give direct or indirect growth advantage to the cancer cell. Promoters of such oncogenes are usually found to be the target of mitogenic signaling pathways resulting in their constitutive transactivation. Further, these oncogenic promoters are not expected to be activated in normal cells. Therefore, such promoters are an ideal candidate for designing of tumor specific gene expression promoters. However, most of these

promoters are weak activators of transcription and may not allow achieving desired levels of therapeutic gene expression in the target cell. This issue could be effectively solved by coupling an enhancer element to such promoters to increase their activity. In this study Tcf-4 / β-catenin enhancer element are coupled with the minimal oncogenic promoters described in this section.

c-fos Promoter

c-fos is a transcription factor belonging to the AP-1 family of proteins, which are known to play an important role in cell proliferation and survival (228). AP-1 (activating protein -1) is a collective term referring to dimeric transcription factors composed of Jun, Fos or ATF (activating transcription factor) subunits that bind to a common DNA site, the AP-1 binding site. AP-1 activity is induced by a variety of biological, chemical and physical stimuli. These include growth factors, cytokines, neurotransmitters, polypeptide hormones, cell matrix interactions, bacterial and viral infections, and a wide variety of physical and chemical stresses. This multitude of stimuli, largely regulates the abundance and the activity levels of AP-1 proteins (229). The abundance of AP-1 proteins is most commonly regulated by controlling the transcription of their genes (230) and by modulation of their stability (231, 232). Regulation of c-fos is a classic of example how AP-1 proteins can be regulated by different signaling mechanisms. The c-fos promoter is the target of several signal transduction pathways, which regulate c-fos levels in response to external stimuli. Stimulation of cells with different stimuli induces c-fos very rapidly (233). Several cis elements are known to mediate c-fos promoter induction: first, proximal to the c-fos TATA box is a CRE which binds to CREB (CRE-binding protein), inducing c-fos via

cAMP- and Ca2+- dependent signaling pathways in response to neurotransmitters and polypeptide hormones (234). A Second cis element is the sis-inducible enhancer (SIE) which is recognized by the STAT (signal transducer and activator of transcription) group of transcription factors. These stimuli activate the mitogen activated protein kinase (MAPK) cascades that enhance AP-1 function through the phosphorylation of distinct substrates. In addition, fos promoters can be induced by activated extracellular-signal-regulated kinase (ERK) in response to serum and various growth factors (235). Based on these studies it can be concluded that the c-fos promoter is a target of various cell signaling cascades induced by cell growth and proliferation promoting agents. For typical cancer disease models it is well established that various growth and proliferation promoting signaling cascades are constitutively active. In line with viewpoint, it is know that expression of fos is differentially regulated in various cancers. Therefore the c-fos promoter is an ideal candidate for the designing of a tumor specific gene expression promoter because tumor cells, as compared to normal cells, will have the appropriate cellular environment for its induction.

Cyclooxygenase-2 (Cox-2) Promoter

Cyclooxygenases are the key enzyme in arachadonic metabolism and catalyze the conversion of arachadonic acid to prostaglandin H2, the precursors for prostaglandins and thromboxanes (236). Two isoforms of Cox exists, Cox-1 and Cox-2. Cox-1 is constitutively expressed in many tissues (236), however Cox-2 is induced in many inflammatory reactions (237). Numerous studies have suggested that Cox-2 is regulated at the transcriptional level. Many growth factors, cytokines and inflammatory agents all appear to enhance the expression of Cox-2 by interacting with various regulatory

sequences in the promoter region of this gene (238). Evidence for involvement of Coxs in cancer was suggested by unusually high amounts of prostaglandins in various animal and human tumor tissues, including human colon cancer (239, 240). Recent studies have presented compelling evidence that Cox-2 is induced and at elevated levels in human colorectal cancers, azoxymethane-induced mouse tumors and in the polyps of mouse FAP (familial adenomatous polyposis) models (241-244). In addition, under physiological conditions Cox-2 is virtually undetectable in most of the tissues (245, 246). This evidence suggest that Cox-2 is differentially expressed in tumors as compared to majority of normal tissue. Differential expression of Cox-2 could be explained by studies which propose that Cox-2 promoter is differentially regulated in tumors. Further, it has been suggested that the Cox-2 promoter is regulated by the wnt signaling pathway which is aberrantly activated in a number of epithelial tumors (247). In addition, recently, a direct link between transcriptional regulation, at the promoter level, of Cox-2 by the wnt signaling pathway has been demonstrated (26).

In the past the Cox-2 promoter has been used and characterized as a tumor specific gene expression promoter for expression of therapeutic genes in different types of tumors (248-252). Therefore it is suggestive that the Cox-2 promoter has a potential utility for a tumor specific promoter and it could be successfully engineered by combining with various DNA elements to achieve an augmented level of specificity and activity.

CEA Promoter

The carcinoembryonic antigen (CEA) has a long history as a potential therapeutic tool for targeting metastatic colon carcinoma. CEA is a tumor-associated marker that is

expressed by most colorectal tumors (253, 254). CEA expression is fairly homogenous with metastatic tumors (254), and its promoter and enhancer sequences have been very well characterized. The CEA promoter has been mapped and the promoter elements critical for selective expression of transgenes in recombinant adenoviruses are located between nt. -229 to nt. + 111 (the translational start site) (253). The CEA promoter linked to a therapeutic gene and incorporated into an adenovirus has been used extensively in multiple studies and shown to be active in only CEA producing cells (255). Related to our proposed studies one report used a CEA/HSV-TK adenovirus, which was shown to have no hepatoxicity in nude mice, yet was effective at reducing the tumor volume of a human tumor xenograft following GCV administration (256). Although the use of CEA to target gene therapy has resulted in specificity, this has come at the cost of a 10-300 fold loss in activity compared with nonspecific viral promoters such as CMV (cytomegalovirus) or RSV (Rous sarcoma virus) (255, 257, 258). CEA is found specifically up regulated in colon tumors and at the same time more than 80% of colon tumors show constitutive activation of Wnt signaling pathway. Therefore, it is reasonable to speculate that a recombinant Tcf-4 / CEA promoter will have enhanced transcriptional activity as compared to CEA promoter alone

In addition to colorectal carcinoma, wnt signaling pathway is also shown to be activated in prostate cancer and is believed to play an important role in the development of prostate cancer (205, 207). Therefore, it is logical to think that Tcf-4 / β-catenin enhancer element can be potentially combined with promoters which are differentially up-regulated in prostate carcinoma.

PSMA Promoter

Prostate Specific Membrane Antigen (PSMA) is a membrane glycoprotein with folate hydrolase activity, predominantly expressed in prostate epithelial cells. Studies at the mRNA and protein levels support that PSMA is differentially regulated in benign and malignant prostate abnormalities as well as during tumor progression. In benign prostate hyperplasia (BPH) PSMA decreases whereas in prostate cancer (CaP) PSMA levels are increased reaching their highest values in hormone deprived and hormone refractory states. The mechanism of regulation of the gene in the various prostate abnormalities has yet to be identified. PSMA is most strongly expressed in the prostate with lower levels in the brain, salivary gland and small intestine (259, 260). Immunohistochemistry has consistently detected strong PSMA expression in prostate epithelia, with weaker expression in colon, small intestine, and kidney tubules (260-265). In addition week expression of PSMA has also been observed in cardiac muscle and sweat glands (261). PSMA expression in tumor tissue increases as the prostrate tumor increases in size (260). Furthermore, expression levels of PSMA, unlike PSA, has been reported to increase under conditions of androgen deprivation (259, 260, 264). The collective evidence suggests the potential use of the promoter of the PSMA gene in gene therapy of prostate disease. However, PSMA promoter itself is a week promoter and need to be coupled with other positive regulatory DNA elements to achieve satisfactory levels of therapeutic gene expression. We have combined the Tcf-4/β-catenin enhancer element with the minimal PSMA promoter to attain higher levels of gene expression. The significance of this approach lies in the fact that approximately 20% of the prostate show an aberrant

activation of wnt signaling pathway. Therefore, combining PSMA promoter and Tcf-4/β-catenin enhancer element is a rational approach for designing prostate specific gene expression promoters for the purpose of gene therapy.

PSA Promoter

Prostate specific antigen is a protein expressed exclusively by benign, hyperplastic and malignant prostatic epithelium (266). An increase in the serum levels of PSA are considered indicative of the benign hyperplasia or malignant carcinoma of the prostate (267) and this has allowed PSA to be used as a diagnostic marker for prostate disease. Expression of PSA is reported to be up regulated by androgens and is restricted exclusively to prostate (268). The restricted expression of PSA is attributed to the immediate 5' promoter region of the PSA gene and is shown to be sufficient to target expression to prostate tissue (269). Subsequent studies demonstrated that PSA promoter activity is also regulated by an upstream PSA enhancer element (270). A thorough analysis of PSA promoter revealed that coupling of minimal PSA promoter with its enhancer increases the specificity and level of gene expression (271). This suggest that for designing of prostate specific gene expression vectors, using PSA promoters, coupling of positive regulatory elements is very critical for achieving satisfactory levels of therapeutic gene expression. With the idea that wnt signaling pathway is frequently found activated in prostate carcinoma, it is reasonable to conceptualize that combing Tcf-4/β-catenin enhancer element with PSA enhancer will contribute in achieving higher levels of specificity and gene expression in prostate carcinoma.

Transcriptional Targeting Using Tcf-4/β-Catenin Enhancer

Selective targeting and killing of tumor cells is one of the major goals of all cancer therapies. Conventional chemotherapy and radiotherapy induce dose limiting normal cell toxicities, which reduce their clinical effectiveness. Cancer gene therapy has the potential to avoid or minimize normal cell toxicities. This could be achieved by employing suitable strategies to target the therapeutic gene directly to the tumor cells. The majority of the cancer gene therapy clinical trials have focused on the delivery genes directly to the tumor site by intratumoral injections using both viral and non-viral delivery agents, thereby largely avoiding normal tissues. However, direct intratumoral injections fail to achieve higher levels of tumor targeting because there are instances in which it is difficult to reach the tumor and it cannot be applied to disseminated metastatic tumors which are yet to be detected. A viable alternative to intratumoral injection is systemic delivery of a suitably packaged transgene. This would allow targeting of both the primary tumor and metastatic deposits, which must be controlled if therapy is to be successful. While increasing the therapeutic ratio, systemic delivery also runs the risk of exposing the normal cells to the harmful effects of the therapy. Cancer gene therapy offers several technological advances which may be helpful in addressing this issue. A number of gene therapy strategies are now being developed to target both viral and nonviral delivery agents to tumor cells. These include exploitation of natural viral tropisms; genetically modifying the virus to ablate native receptor interactions and incorporating a novel ligand into one of the viral coat proteins; using tissue specific ligands or monoclonal antibodies incorporated on to the surface of liposomes to direct them to target cells.

In cancer gene therapy expression of the therapeutic gene in target cells is an absolute requirement to minimize normal cell toxicity. Targeted delivery of the therapeutic vector to the tumor cells is one way to achieve this goal; however it is still desirable to have another level of control to ensure exclusive expression of the therapeutic gene in target cells. Transcriptional targeting of the therapeutic gene is a promising way to selectively target tumor cells and it involves designing of recombinant DNA element (promoters) which would actively express the therapeutic gene in tumor cells. The basic idea behind these tumor specific gene expression promoters is that they usually contain cis-acting elements which are activated in diseased states such as cancer. A decade of research has well established that genetic mutations result in aberrant activation of growth pathways which contribute towards the development of cancer. These growth signaling cascades, in general, target promoters of several oncogenes resulting in their constitutive activation. The products of these oncogenes are known to contribute towards the development of cancer. Cyclooxygenase-2, c-myc, c-fos, cyclinD etc are classic examples of such oncogenes which are found to be constitutively activated, at transcription level, in variety of cancers. Therefore, promoters of such oncogenes are ideal candidates for being used in designing of tumor specific gene expression promoters. It is expected that, owing to the differential activation of signaling pathways in a cancer cell environment, these oncogenic promoters will only be activated in cancer cells and not in normal cells. Thus, such promoters are extremely important DNA sequences for the purpose of constructing tumor specific gene expression construct. However, it is also well known that usually such oncogenic promoters are poor or weak activators of transcription. This can be an issue of concern because failure to express sufficient

amounts of the therapeutic gene may result in failure of the therapy. Besides, there is always a possibility that such oncogenic promoters can cause a leaky expression of the therapeutic gene in normal cells which can result in normal cell toxicity. In general, activity and specificity are the two major concerns while designing a tumor specific gene expression promoter. An ideal promoter should be able to achieve therapeutically significant levels of therapeutic gene expression specifically in cancer cells and should not show any biologically significant activity in normal cells. As described earlier, oncogenic promoters are good candidates but do not entirely fulfill the necessary requirements from the perspective of gene therapy. Therefore it is very crucial to engineer such oncogenic promoters before they can be used in cancer gene therapy.

One of the ways to accomplish this is to incorporate unique enhancer DNA sequences in such oncogenic promoters. These enhancer elements are specialized DNA sequences naturally found to be part of several gene promoters and are responsible for the transcriptional activation or repression of promoters in response to a biological signal. The ability of an enhancer element to transcriptionally activate or repress a promoter is facilitated by its ability to bind to a variety of proteins. These proteins may range from chromatin modifying agents to transcription factors and are usually components of signal transduction pathways. They have the ability to actively associate or dissociate from enhancer elements in response to a signal. Activation or repression of promoter is a very complex process and it is the result of a cumulative effect of the nature of proteins bound to enhancer/promoter DNA sequence at any given point of time. Therefore a biological signal received at the cell surface may trigger a cascade of events that may result in binding of proteins known to be activators of DNA transcription leading to transcriptional

activation of the promoter. On the other hand it may also repress transcription if the signals lead to dissociation of pro-transcription proteins or binding of factors which have a cumulative repressive effect on transcription. As mentioned earlier in this text that aberrant activation of growth promoting signal transduction pathways results in differential activation of genes in cancer. The promoters of these differentially transctivated genes usually have specific DNA elements (for example enhancer elements) binding to an array of proteins in response to a signal transduced by the activated signaling pathway. Such enhancer elements can be innovatively used in cancer gene therapy to control the expression of therapeutic genes.

Mutational inactivation of APC and/or beta-catenin is frequently found in epithelial tumors resulting in transcriptional activation of Tcf-4/β-catenin gene responsive genes. Therefore, it is rational to assume that incorporating Tcf-4/β-catenin enhancer in therapeutic gene expression vectors would result in tumor selective gene expression. In past several attempts have been made to exploit the tumor selective activation of the Tcf-4/β-catenin pathway for the purpose of designing tumor selective gene expression vectors. Typically, these include combing of Tcf-4/β-catenin enhancer with a promoter to drive the expression of a therapeutic gene. In a study it was shown that introduction of the cell death gene *fadd* under the control of a HSV-TK promoter containing wild-type Tcf/Lef-binding sites resulted in preferential killing of colon cancer cells with hyperactive b-catenin/Tcf activity (272). A synthetic promoter, incorporating Tcf-4/beta-catenin binding sites has been designed and shown to successfully express *E. coli* Nitroreductase gene in colon cancer xenografts (273). The suppression of colon cancer cell growth was demonstrated in nude mice when xenografts were targeted with an

adenoviral vector carrying HSV-TK gene driven by a recombinant CMV/Tcf-4/β-catenin promoter (274). These studies have been well designed and successfully demonstrate the proof-of-principle. However, none of the studies propose the idea of combining oncogenic promoter with Tcf-4/bet-catenin enhancer elements for designing tumor specific gene expression promoters. This is a very crucial aspect because various oncogenic promoters are found differentially activated in tumors and incorporating such DNA elements in therapeutic vectors would help in achieving a stringent control over the therapeutic gene expression. It is expected that such recombinant promoters will have a very low activity in normal cells and this would help in further minimizing the normal cell toxicity. Furthermore, none of the studies have conclusively shown the activity of recombinant promoters, with Tcf-4/beta-catenin binding sites, in normal colon cells which is a very important aspect in the strategic targeting of Tcf-4/β-catenin signaling pathway for gene therapy. It is expected that recombinant Tcf-4/β-catenin enhancer / promoter combination designed in this study would be very useful in construction of gene expression vectors for strategic delivery of therapeutic of choice to cancer cells. Another hallmark of the proposed enhancer / promoter design strategy is that it can be adapted to suit the clinical therapeutic requirements governed by the patient and the type of tumor. This can be achieved by combining Tcf-4/β-catenin enhancer with the chosen oncogenic promoter and therapeutic gene. Choosing an oncogenic promoter partner for Tcf-4/β-catenin enhancer for treating a patient can be aided by gene array analysis of a tumor biopsy taken from the patient. Furthermore, the ability to modulate the levels of therapeutic gene expression via genetic and chemical means is another novel aspect of the proposed enhancer/promoter system. As described on page number 34, the activity of

Tcf-4/β-catenin enhancer elements can be modulated by chemical means and these modulators could be strategically used in clinic for regulating the activity of the proposed enhancer/promoter system.

CHAPTER II

SPECIFIC AIMS

The goal of this research is to construct and identify enhancer/promoter combinations for tumor specific gene expression. Identified combinations will be tested, in vitro, for their ability to drive the expression of a therapeutic gene and kill cells. Further, delivery issues of the therapeutic gene construct will be addressed by incorporating these elements into a recombinant adenovirus. The efficacy of the recombinant adenovirus to deliver and selectively express the therapeutic gene in target cells will be evaluated by in vitro assays. Finally, various chemical and genetic modulators will be screened in an attempt to identify newer methods of regulating the activity of the recombinant enhancer/promoters.

Aim 1: Construction and screening of optimal recombinant Tcf-enhancer/Promoter combination

Experiments described in this aim are designed to accomplish a single goal: identify the optimal Tcf-4 enhancer/promoter combination(s) which have the ability to drive gene expression specifically in tumor cell as compared to normal cells. To accomplish these goals we plan to conduct luciferase reporter assays. Various Tcf-4/promoter luciferase reporter constructs would be generated by cloning optimized number of repeats of Tcf-4 enhancer element in combination with the chosen oncogenic promoter in a luciferase reporter construct. To ascertain the efficacy of these enhancer/promoter combinations, reporter constructs would be used to screen several colon and breast tumor cell lines. Since, it is known that in majority of colon and in fairly

high number of breast tumors, inactivating mutations in the APC/beta-Catenin gene results in stabilization of β-Catenin which leads to transactivation of β-catenin/tcf-4 enhancer regulated genes. Therefore, the selected colon and breast tumor lines are likely to be good model system for screening for optimal Tcf-4 enhancer/promoter combinations. Further, the results of these experiments will also provide additional information: comparative ability of the all enhancer/promoter combination to drive gene expression in different cell lines because it is expected that a certain combination might be optimal in certain cell lines and not in others. All the reporter constructs will also be tested for their ability to drive gene expression in a small panel of primary human normal (NCM) cell lines obtained from INCELL. These cell lines have been derived from normal human colon and are expected to have low or insignificant amounts of transcriptionally active beta-catenin. Therefore, the cloned Tcf-4 enhancer/promoter combinations are expected to be transcriptionally inactive and a lower level of reporter luciferase activity is expected in these normal cell lines.

Aim 2: Evaluation of the ability of chosen Tcf-enhancer/Promoter combination to selectively express a therapeutic gene in tumor cells and estimation of cell killing

These experiments are designed to accomplish two goals: 1) Evaluate the ability of the chosen enhancer promoter combination (from Aim # 1) to drive the expression of a therapeutic gene selectively in tumor cells and 2) estimate the efficacy of tumor selective gene expression in tumor cell killing. Reporter luciferase data from Aim#1 would help us to identify enhancer promoter combinations which are selectively active in tumor cells; next we would use the identified enhancer/promoter elements to drive the expression of a therapeutic gene. We plan to use HSV-TK as the therapeutic gene, which would be

cloned down stream of the chosen Tcf-4 enhancer/promoter element in a eukaryotic expression vector. This recombinant therapeutic gene expression construct would be used to transient transfect normal and tumor cell lines allowing us to evaluate the ability of the enhancer/promoter for tumor selective HSV-TK gene expression. Because of the tumor selective activity of the enhancer/promoter we expect to see a selective expression of HSV-TK in tumor cells and not in normal cells. In addition, to gene expression analysis, we would a conduct a functional assay to confirm the activity of the expressed HSV-TK. Transfected cells will be treated with labeled [3H] nucleosides followed by the extraction and assaying of nucleotides. Ability to phosphorylate nucleosides would reflect the functionality of the expressed HSV-TK and the amount of extracted nucleotides would be an index of the levels of HSV-TK expression in cells. In the next step we will investigate the ability of the pTcf-4/promoter-HSV-TK gene construct to selectively kill tumor cells by conducting GCV cytotoxic cell viability assays. For determination of GCV cytotoxicity, tumor cell lines will be transiently transfected with Tcf-4/promoter-HSV-TK construct followed by treatment with different doses of GCV. As a control normal cell will be transfected similarly followed upon by GCV treatment. GCV treated cells would be allowed to grow for 36-48 hours followed by counting of viable cells. We expect to see increased tumor cell killing as compared to the normal cells, which be could be explained on the basis of level of expression of HSV-TK in tumor cells as compared to normal cells.

Aim 3: Optimization of delivery of the therapeutic gene/promoter-enhancer combination to the tumor

The main objective of the experiments in this section is to design optimal delivery method which could deliver the recombinant therapeutic gene construct to the target cells. Gene delivery by naked DNA transfection is severely limited by the ability of the target cells to get transfected. Some cells are more susceptible for naked DNA uptake as compared to others. However, transfection efficiencies for a specific target cell can be improved by employing various transfection methods. Nevertheless, transfection procedures fail to achieve significant levels of gene delivery of the therapeutic gene to target cell and this would severely impede the objectives of cancer gene therapy which requires, ideally, targeting every single tumor cell. We plan to use recombinant adenoviral vectors to address the issue of delivering our recombinant therapeutic gene construct to the target cells.

Aim 4: Identification of strategies for modulating Tcf-4/Beta-catenin responsive promoters

For several therapeutic implications, it may be necessary to regulate the expression of the therapeutic gene, for e.g. stimulation of the gene expression is possibly needed, for increasing therapeutic gene expression, in cells which are marginally responsive. Conversely, the possibility of having a strategic repressor option in the context of viral-based gene therapies could be important. One of the several ways to control the expression of the therapeutic gene in target tissue is to regulate the activity of the enhancer/promoter which is driving its expression. Experiments in this specific aim are, primarily, geared towards identifying strategies to regulate the activity of the

recombinant Tcf-4 enhancer/promoter by chemical and genetic means. Short chain fatty acids like butyrate and trichostatin A have been reported to increase Tcf-4/β-catenin mediated gene expression. Butyrate is a histone de-acetylase inhibitor which can act differentially either promoting or repressing Tcf-4/β-catenin mediated gene expression. Therefore compounds like butyrate and trichostatin A could be potentially used for increasing therapeutic gene expression by stimulating the Tcf-4 enhancer/promoter. On the contrary, compounds like doxorubicin and 9-cis-retinoic acid have been reported to transcriptionally repress expression of Tcf-4/β-catenin responsive genes. Further, drugs like aspirin and indomethacin have recently been reported to reduce Tcf-4/β-catenin mediated signaling by increased stabilization of phosphorylated β-catenin the levels. Hence, there are several promising compounds which can be screened for potential chemical regulators of recombinant Tcf-4 enhancer/ promoter combinations. The luciferase reporter DNA constructs designed in specific aim#1 contain tcf-4 enhancer elements coupled with different oncogenic promoters used to drive the expression of a reporter luciferase gene. These constructs will be primarily used for screening of various compounds for modulatory acitivity on recombinant Tcf-4/promoter combination. The central idea is to measure the luciferase activity, following transfection of cells with reporter DNA, in presence or absence of the candidate drug. Levels of luciferase activity would assist in classifying the candidate compound into stimulators or repressors.

CHAPTER III

MATERIALS AND METHODS

Plasmids

Luciferase reporter plasmids pTop-flash and pFop-flash were gifted by Dr. Van de Wetering, Netherlands. Reporter plasmids pGL2-CEA was obtained as a gift from Dr. Kathy Molnar-Kimber, university of Pennsylvania, PA. pGL3-PSA and pGL3-PSMA were gifted by Dr. Tonia Vlahou, Eastern Virginia Medical School, VA.

Cloning Procedures

Restriction Digests

All restriction enzymes used in these studies unless otherwise noted, were bought from Promega. For most digests, 3-5 units of enzyme were used per μg of DNA digested in a company recommended restriction enzyme buffer and the reaction was incubated for approximately 2 hours at the appropriate temperature.

Ligation Reactions

All ligation reactions were performed using 20 μg of T4 DNA ligase (NEB) and buffer provided by the manufacturer. All ligation reactions had 100-300 ng of vector DNA and three to five molar excess of insert when necessary. The ligation reaction was incubated at 16° C for at least 16 hours.

Phosphatase Reactions

These reaction was carried to remove 5' phosphate on restriction digested DNA to prevent self ligation of the vector DNA in ligation reactions. Approximately 20 units of

calf intestinal phosphatase (NEB) were added to the reaction mixture containing manufacturer recommended buffer and restriction digested vector DNA. The reaction mixture was incubated at 37° C for 30 minutes, 20 units of phosphatase were added, and the reaction was incubated for an additional 30 minutes. The vector DNA was subsequently purified on a 1% agarose gel.

Competent Bacteria

E. coli DH5α competent cells used in these studies were prepared in the laboratory and JM109 competent cells were purchased (Promega). Single colony of *E. coli* DH5α cells was revived overnight in LB at 37°C at 200 rpm shaking. 1 ml of this culture was subcultured in 100 ml LB and grown till A_{600} reached 0.3. The culture was chilled on ice for 10 min and centrifuged at 4500×g for 15 min at 4°C. The cells were gently suspended in 15 ml of ice cold sterile solution of 100 mM $CaCl_2$ containing 15% glycerol, and incubated on ice for 30 min. Cells were harvested again at 4500×g for 15 min and gently suspended in 5 ml of 100 mM $CaCl_2$ solution containing 15% glycerol. Competent cells were aliquoted in small volumes (100-200 µl) in prechilled tubes and stored at -70°C.

Transformation of Bacteria

Competent cells, stored at -70°C, were slowly thawed on ice. The DNA (ligation mix (10 µl) or 100 ng of plasmid DNA) was added to 100 µl of competent cells. After gentle mixing, cells were incubated on ice for 1 h. Cells were given a heat shock at 37°C for 5 min or 42°C for 90 sec and then chilled on ice for 2 min. The cells were then allowed to grow in 1 ml of LB for 1 h at 37°C. Dilutions of cells were plated on LB-agar

plates containing specific antibiotics and incubated at 37°C overnight. The recombinants were screened for the presence of plasmid by mini-preparation of plasmid DNA and restriction endonuclease digestions.

Miniprep Analysis of Plasmid Constructs

For screening of *E. coli* cells harboring the desired plasmid small scale preparation of plasmids were made using Qiagen miniprep kit. Briefly, cells were picked up from a single colony and grown for 12 h in a small volume of LB containing appropriate antibiotic. Three ml of culture was centrifuged in microcentrifuge tubes at 8000×g for 2 min and the supernatant was discarded. The plasmid DNA was then purified using Qiagen miniprep spin columns following the protocol provided by the manufacturer in the product literature. The purified plasmids were appropriately restriction digested and analyzed on agarose gels.

Large Scale Plasmid Purification

For rapid large-scale preparation of plasmid DNA using the Qiagen midi-preparation kit, manufacturer's instructions were followed. Briefly, 50 ml of LB medium with appropriate antibiotics was inoculated with an ovrnight culture of *E. coli* cells harboring the desired plasmid. Cells were harvested at 4000×g and suspended in 5 ml of resuspension buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 μg/ml RNase A). 5 ml of lysis buffer (0.2 M NaOH, 1% SDS) was then added to the suspension, mixed thoroughly and incubated for 5 min at RT. To this, 5 ml of neutralization buffer (3 M potassium acetate, pH 5.5) was added and mixed and then incubated for 10 min on ice. The mixture was spun at 12,000×g for 30 min and clear supernatant was collected in a fresh tube. The supernatant was then loaded on Qiagen Tip-100 equilibrated in Buffer

QBT (0.75 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol) and bound DNA was washed with buffer QC (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol). Pure DNA was eluted in buffer QF (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5, 15% isopropanol) and precipitated with 0.7 volumes of isopropanol. DNA pellet was washed with 70% ethanol and dried and dissolved in TE ((10 mM Tris-Cl, pH 8.0, 1 mM EDTA).

Isolation of DNA Fragments from Agarose Gels

To isolate DNA fragments from a reaction mixture containing other products, the reactions were stopped with stop dye (50% glycerol, 100mM EDTA, 0.1% bromophenol blue and loaded onto a 1% agarose gel. Following electrophoresis, the desired band was excised with sterile scalpel blade and put in a microcentrifuge tube. The DNA was purified with the help of Qiaquick gel extraction kit (Qiagen). Briefly, the gel pieces were dissolved in solubilization buffer for 15 min at 55°C. Solubilized agarose with DNA was added to the spin column provided in the kit and was spun at 12,000xg for 1 min. The flow through was discarded and column was washed with a buffer containing 70% ethanol. Bound DNA was eluted at 55°C for 5 min in 10 mM Tris, pH 8.0.

PCR amplification of DNA fragments

PCR master mix (Promega) was used for all the PCR reaction described in these studies and the reactions were performed following the manufacturer's instruction. 100 ng of template DNA and 100 ng of each primer were used in 50 µl of reaction mixture. The following program was used in all amplication reactions on a thermal cycler (MJ research). Step 1: 94° C for 2 minutes; Step 2: 92° C for 1 minute; Step 3: 55° C for 1 minute; Step 4: 72° C for 1 minute; Step 5: cycle to step 2, 32 times; Step 6: 72° C for 5 minutes; Step 7: maintain at 4°C.

Human Normal and Tumor Cell Lines

Normal cell lines (NCM-460, NCM-425, CSC-1) were maintained in INCELL's specialty media M310 at 37 ° C and 5% CO2. Colon tumor cell lines (SW480, HCT8, SW620, HCT116, HT29) were maintained in RPM1 1640 (Cell Grow) supplemented with 10% fetal bovine serum (Cell Grow), 1% penicillin-Streptomycin (Cell grow), at 37 ° C and 5% CO_2.

Luciferase Vectors and Assays

Luciferase Reporter Vectors

Tcf-4/β-catenin enhancer and pGL3-basic/Tcf: pTop-Flash luciferase reporter plasmid was used as the source for Tcf-4/β-catenin enhancer fragment. pTop-Flash was restriction enzyme digested with Sal-I and the reaction mixture was resolved on a 15 % polyacryamide gel. Resolving gel consisted of 15% (w/v) acrylamide:bisacrylamide (29:1), 1X TBE, and 0.1% APS. Gel was polymerized with 1 µl/ml of TEMED. Tcf-4/β-catenin enhancer fragment was excised out of the gel and eluted in a elution buffer (0.5 M ammonium acetate, 1mM EDTA, pH 8.0). pGL3-basic (promega) reporter luciferase vector was restriction enzyme digested with Sal-I and purified on agarose gel. Sal-I digested 86 bp. insert of Tcf-4/β-catenin enhancer fragment was ligated with Sal-I digested pGL3-basic vector to generate pGL3-basic/Tcf.

pGL3-CEA/Tcf

pGL3-CEA vector was digested with Sal-I followed by purification on a 1% agarose gel. Purified SalI digested pGL3-CEA was treated with calf intestinal phosphatase and purified on a 1% agarose gel. Sal-I digested Tcf-4/β-catenin enhancer

fragment (86 bp.) was ligated to the restriction digested, CIAP treated pGL3-CEA to generate pGL3-CEA-Tcf.

pGL3-Cox2 and pGL3-Cox2/Tcf

A 720 bp fragment of Cox-2 promoter was PCR amplified from human genomic DNA. SW480 colon cancer cells were used as the source of genomic DNA which was isolated following the instructions provided with the Qiagen mammalian cell DNA extraction kit. Desired Cox-2 promoter fragment was amplified with XhoI (Cox-2/XhoI-CCGCTCGAGCGGGGGTACGAAAAGGCGGAA) and Bgl-II (Cox-2/Bgl-II-GAAGATCTTCCGCCAGGTACTCACCTGT) restriction enzyme end primers in a PCR reaction mix (Promega) with 100 ng of genomic DNA template. The amplified DNA fragment was agarose gel purified restriction enzyme digested in a reaction mixture containing Xho-I and Bgl-II. Following restriction enzyme treatment, 270 bp DNA fragment was resolved on an agarose gel and purified using Qiagen qiaquick gel extraction kit. The vector DNA was prepared by treating pGL3-basic plasmid DNA with Xho-I and Bgl-II. The restriction digested vector DNA was agarose gel purified using Qiagen Qiaquick gel extraction kit and ligated, with PCR amplified, and restriction enzyme treated 270 bp Cox-2 promoter DNA to generate pGL3-Cox2.

Sal-I restriction enzyme digested Tcf-4/β-catenin enhancer DNA was cloned in the Sal-I restriction enzyme site of pGL3-Cox2 to generate pGL3-Cox2/Tcf.

pGL3-PSA/Tcf

Sal-I restriction enzyme digested Tcf-4/β-catenin enhancer DNA was cloned in the Sal-I site of pGL3-PSA luciferase reporter vector to generate pGL3-PSA/Tcf.

pGL3-PSMA/Tcf

PSMA promoter (640 bp.) insert was excised out of pGL3-PSMA promoter using Kpn-I and Hind-III restriction enzymes. The 640 bp. promoter DNA fragment was resolved and purified on an agarose gel. For vector DNA, pGL3-Tcf plasmid was restriction digested with KpnI and Hind-III, followed by purification on an agarose gel. Kpn-I and Hind-III digested insert and vector DNA were ligated to generate pGL3-PSMA/Tcf.

Reporter Luciferase assays

To evaluate the ability of the recombinant enhancer/promoter combinations to express the reporter gene in cancer and normal cells, dual luciferase assays were performed. The assay relies on an internal control (pRL-TK) vector which carries a gene for Renilla luciferase expressed from a ubiquitously active eukaryotic promoter (HSV-TK). Renilla luciferase generates a signal which is distinct from the luciferase expressed by the test reporter vector and this signal is used to normalize for transfection efficiencies of different cell lines. Optimal transfection conditions for each cell lines were empirically determined. 2 X 10^5 cells/well were seeded in 6 well plates. The cells were transfected overnight with 10µl lipofectamine (Life Tech.) reagent, 2 µg luciferase reporter plasmid and 200 ng of pRL-TK in OPTIMEM media (Life Tech.). On following day, regular maintenance media was replaced. After 48 hours, the cells were lysed and assayed according to the protocol supplied with the Dual Luciferase Assay Kit (Promega). The relative light units were recorded with a Turner luminometer.

HSV-TK Constructs and Gene Expression Analysis

Construction of pCDNA-TK and pc-fos/Tcf-4-HSV-TK

pCDNA3.1(+)-5.4 kb (Invitrogen), was used as the eukaryotic expression vector. The native CMV promoter was removed by BglII and BamHI (promega) restriction digest, followed by gel purification of the vector backbone excluding the CMV promoter. Wild type HSV-TK gene (1.1 Kb) was excised from pLTK-ED (275) using 5' BglII and 3' BamHI restriction enzymes and cloned into BglII and BamHI sites of CMV promoter less pCDNA3.1(+) to generate pCDNA-TK. To generate pc-fos/Tcf-4-HSV-TK, Tcf-4-c-fos DNA fragment (186 bp.) was PCR amplified with 5' Bgl-II (GTAAGATCTGTTCTAGAGTCGACCTGCAGCCCAAG) and 3' Bam-HI (GTAGGATCCATGGGAGATCCTCTAGAGAGACACTG) primers and using PCR master mix reagent (promega). The PCR product (186 bp.) was resolved on a 1% agarose gel, purified and restriction enzyme digested (with Bam-HI and Bgl-II). Restriction digested and agarose gel purified enhancer/promoter DNA fragment was cloned into the BglII site of pCDNA-TK upstream of HSV-TK to generate pc-fos/Tcf-4-HSV-TK.

Construction of pPSMA/Tcf-4-HSV-TK

The luciferase gene of pGL3-PSMA/Tcf plasmid DNA was removed by restriction enzyme digestion (Hind-III, Xba-I) and resolving the products on a 1% agarose gel. The vector backbone without the luciferase gene was excised from the gel and purified. For insert, the HSV-TK gene (1.1 kb.) was PCR amplified with primers with Hind-III (CCCAAGCTTGGGATGGCTTCGTACCCCGGCCATC) and Xba-I (GCTCTAGAGCTCAGTTAGCCTCCCCCATCTG) restriction enzyme end primers. pLTK-ED (ref) was used as template DNA. Following agarose gel purification, HSV-TK

gene was restriction digested (Hind-III, Xba-I) and ligated with the prepared vector to generate pPSMA/Tcf-4-HSV-TK.

Transfection and Gene Expression Analysis

The ability of the HSV-TK expression vectors for tumor selective expression of HSV-TK was evaluated by transiently transfecting vectors in normal and tumor cells followed by Western analysis. $2x10^5$ cells were seeded in 6 well plates for 24 hours followed by overnight transfection with 2μg/well of DNA, 10μl/well of Lipofectamine transfection reagent and OPTIMEM media (Invitrogen). The day after transfection, regular growth media was replaced. After 48 hours of transfection, protein extracts were prepared by boiling in reducing sample loading buffer. Protein samples were subjected to SDS-PAGE and western blot analysis.

SDS-PAGE Analysis

SDS-PAGE analysis was carried out as described by Laemmli (1970). Resolving gel consisted of 15% (w/v) acrylamide:bisacrylamide (29:1), 0.4 M Tris-Cl, pH 8.8, 0.1% SDS, and 0.1% APS. Gel was polymerized with 1 μl/ml of TEMED. 5% stacking gel was made similarly in 0.125 M Tris-Cl, pH 6.8. Samples were boiled in the reducing sample buffer (4M urea, 20mM DTT, 100mM Tris pH 8.0, 4% SDS, 0.1% bromophenol blue) prior to loading onto the gel. The gel was electrophoresed in running buffer (25 mM Tris-Cl, pH 8.3, 250 mM glycine and 0.1% SDS) at 15 V/cm for 2 h. The gel was then stained with Coomassie brilliant blue G-250 (0.05% w/v) for 1 h and destained in methanol: glacial acetic acid: water (4:1:5).

Immunoblotting

The samples analyzed by SDS-PAGE were electrotransferred onto nitrocellulose membrane at 30 mA for 10 h in transfer buffer (20 mM Tris, 150 mM glycine, pH 8.0 and 20% methanol). Non-specific sites were blocked by 5% non-fat milk in TBST (0.01 M Tris pH 8.0, 0.14 M Sodium Chloride and 0.1 % Tween-20) for 1 h on a rocker. The membrane was then incubated with primary rabbit polyclonal IgG against HSV-TK in 1X TBST (1:1000) followed by incubation with HRP conjugated goat anti-rabbit antibody (1:5000). The blot was washed thoroughly with TBST between successive incubations. Fluorimetric detection of bands was attained using ECL western blotting kit (Amersham Pharmacia Biotech, UK). Blots overlaid with detection reagents were exposed to X-ray film (Kodak) and were developed using an automated X-ray film developer (Kodak).

Metabolic Labeling with [3H] GCV

For metabolic labeling procedure (171). Parental and HSV-TK expressing vector transfected cells ($2x10^5$) were labeled in triplicate with 1μCi of [3H]GCV for 8-10 hours, and then nucleotides were extracted from pelleted cells in 0.2ml of 70% methanol at 4°C for 15 min. An aliquot of each methanol-soluble supernatant was analyzed for radioactivity by scintillation counting using a bench top scintillation counter (Beckman). The residual cell pellets were analyzed for radioactivity, by scintillation counting, for nucleotides present in DNA.

GCV Cytotoxicity: Cell Viability Assays

$1x10^5$ cells/well, tumor and normal cells were seeded in 6 well plates. Next day cells were transfected as previously described in these studies. Similarly, control cells were mock transfected with the same amount of non-specific DNA / lipocfectamine.

Following 18-20 hours of transfection, regular growth media was replaced with 0, 1 and 10μm GCV. After 48 hours of growth, cells were washed once with PBS to dislodge dead cells, adherent cells were collected by trypsinization and trypan blue stained live cells were counted using a haemocytometer/microscope.

GCV sensitivity: Clonal dilution Assays

For determination of GCV sensitivity, cells were seeded in 24 well plates ($2x10^5$/well) in 1 ml media. On the following day, cells were transfected with appropriate plasmid DNA constructs and control cells were mock transfected similarly with same amount of lipid and non specific control plasmid DNA. The next day, after transfection, 0, 0.1, 1 or 10μl GCV was be added to each cell line in triplicate. After 24 hours of GCV treatment, the media was removed, cells were rinsed in fresh media, trypsinized, and then media was added to a final volume of 1 ml/well. Each well of cells was then sequentially diluted from 1:10 to 1:10,000 in 1 ml fresh media on a separate 24 well plate. After approximately 7 days, surviving cell colonies were fixed in 100% methanol, stained with 0.1% methylene blue and were counted.

Adenoviral Vectors and Gene Expression Analysis

Construction of the Recombinant Adenoviral Vectors

The adenoviral vectors used in the study are E1/E3 deleted replication incompetent viruses and are constructed by using pAdeasy technology and a pAd-easy recombinant adenoviral vector construction kit (Qbiogene). Instruction and reagents provided by the manufacturer were used to construct two recombinant adenoviral vectors; Ad-Tcf-fos-TK and Ad-CMV-TK. Recombinant adenovirus were constructed in three

steps, in the first step two vector pShuttle-Tcf-fos-TK and pShuttle-CMV-TK, were generated.

pShuttle-Tcf-fos-TK

pc-fos/Tcf-4-HSV-TK construct was restriction enzyme digested (Bgl-II, BamH-I), products were resolved on a 1% agarose gel and an insert of 1.3 kb fragment (Tcf-c-fos-TK) was purified. pShuttle vector (provided in the kit) was prepared by Bgl-II restriction digestion followed by phosphatase treatment and agarose gel purification. Prepared Tcf-c-fos-TK DNA fragment was liagated in the Bgl-II restriction site of the prepared vector to generate pShuttle-Tcf-fos-TK.

pShuttle-CMV-TK

pLTK-ED (ref) plamisd DNA was restriction digested (Bgl-II, BamH-I), products were resolved on a agarose gel and an insert DNA of 1.1 kb. of HSV-TK gene was purified. Vector was prepared by treating pShuttle-CMV (provided in the kit) with Bgl-II restriction enzyme and agarose gel purifying the restriction digested DNA. HSV-TK insert with Bgl-II and BamH-I restriction ends was ligated with purified Bgl-II treated pShuttle-CMV to generate pShuttle-CMV-TK.

In the second step, using a recombination positive bacterial strain (provided in the kit), pShuttle-Tcf-fos-TK and pShuttle-CMV-TK were recombined with pAdeasy-1 (33.4 Kb) plasmid to generate the recombinant adenoviral DNA. These recombinant constructs were transfected in HEK 293 cells to produce adenoviral particles. The recombinant adenoviruses were amplified and purified as suggested by the manufacturer. The viral stocks were titered using an adenovirus rapid titration kit (Clontech) and following instructions provided by the manufacturer.

Adenoviral Infections and gene expression analysis for HSV-TK

Infecting cells with adenoviruses simply involves placing viruses in contact with cells. For the first 3-4 hours of infection, to increase the infection efficiency, the volume of the media used was minimized to allow close contact between the cells and recombinant adenoviruses. Infection was carried at 37° C and 5% CO_2. Regular media was then added to cover the cells and were allowed to grow normally. For HSV-TK expression analysis $2x10^5$ cells /well were infected with recombinant adenoviruses in 6 well plates. After 48 hours of infection, protein extracts were prepared by boiling samples in reducing sample buffer and were analyzed by SDS-PAGE / Western blot analysis.

Screening of Drugs with Modulatory Properties

To evaluate the ability of the candidate drug to modulate the activity of recombinant Tcf-4 enhancer/promoter dual luciferase assays were. 2 X 10^5 cells/well were seeded in 6 well plates. The cells were transfected overnight with 10μl lipofectamine (Life Tech.) reagent, 2 μg luciferase reporter plasmid and 200 ng of pRL-TK in OPTIMEM media (Life Tech.). The drug, to be tested, was added to the cells in different concentrations at the time of transfection. On the following day, regular maintenance media was replaced. The pRL-TK construct was used as an internal control for transfection efficiency. After 48 hours, the cells were lysed and assayed according to the protocol supplied with the dual Luciferase Assay Kit (Promega). The relative light units were recorded with a Turner luminometer.

CHAPTER IV

RESULTS

Introduction

The ability to distinguish a normal cell from a tumor cell is one of the central requirements in a cancer gene therapy protocol. This can be accomplished by restricting the entry of the therapeutic vector into the cells and/or at the level of transcription by directing the expression of the therapeutic gene from a tumor specific promoter (276, 277). Transcriptional control strategies mainly involve designing therapeutic vectors by incorporating promoter/enhancer elements that show little or no activity in normal cells under non-pathological conditions, but are turned on or up regulated in certain types of tumors. For example, erb-2 and muc-1 promoters, which are frequently found up regulated in adenocarcinomas due to the altered signaling pathways, have been successfully exploited in experimental therapeutic models (278, 279). However, some tumor specific promoters are inefficient activators of transcription, which severely limits their applicability. One of the approaches for improving the transcriptional strength or specificity is to couple positive regulatory elements of enhancer domains with the basal minimal, differentially regulated, promoters of oncogenes. This strategy has been successfully used for carcino embryonic antigen (CEA) and prostate specific antigen (PSA) promoter (271, 280). We have used a similar rationale to design promoters for gene therapy vectors based on the wnt signaling pathway. It is well established that wnt signaling pathway is aberrantly activated in a variety of adenomas of epithelial origin (205, 281-285). This results in stabilization of β-catenin levels leading to constitutive

activation of gene promoters that contain Tcf-4/β-catenin enhancer element. Therefore it is logical that the Tcf-4/β-catenin enhancer element could be incorporated in the design of tumor specific promoters to achieve tumor specificity of therapeutic gene expression.

The purpose of the experiments described in this chapter is to establish that the Tcf-4/β-catenin enhancer element can be combined with minimal promoters of known oncogenes to achieve specificity of gene expression in tumor cells as compared to normal cells. A Tcf-4/β-catenin enhancer element was combined with various minimal oncogenic promoters in reporter luciferase constructs. These constructs were used to screen a panel of tumor and normal cell lines to evaluate the ability of the Tcf-4/β-catenin enhancer / promoter combination to direct gene expression specifically in the tumor cells. A chosen Tcf-4/β-catenin enhancer / promoter combination was cloned upstream of an HSV-TK gene in a therapeutic eukaryotic expression vector to test the ability of the recombinant promoter to tumor specific gene expression. To address the issue of delivery of the therapeutic vector to the cells, an adenoviral vector was generated and evaluated for its ability to deliver and express the therapeutic gene in cells. Finally, the possibility of regulating the activity of recombinant Tcf-4/β-catenin enhancer / promoter with chemical means was also explored.

Aim 1: Construction and screening of recombinant Tcf-enhancer/Promoter combinations

Construction and cell line testing of Tcf-4-enhancer/c-fos recombinant promoter

The Tcf-4/β-catenin enhancer element was coupled with different basal minimal promoters in a luciferase reporter plasmid to generate various recombinant

enhancer/promoter combinations as described in materials and methods. These combinations were then screened in a panel of normal and tumor cell lines to identify the most active enhancer/promoter combination based on selectivity of expression of the reporter gene in tumor cells as compared to normal cells. The luciferase reporter plasmids pLuc-fos/Tcf (pTOP-FLASH) and pLuc-fos/fct (pFOP-FLASH) were kindly provided by Dr. Mark Van der Wetering. The pLuc-fos/Tcf construct consists of four tandem repeats of the Tcf-4 binding sequence (CCTTTGATCT, 82 bp total), 5' to a murine c-fos promoter (186 bp) driving expression of the luciferase gene. The pLuc-fos/fct is a negative control plasmid that contains altered Tcf enhancer repeat sequences (CCTTTGGCCT) not recognized by Tcf-4 (18). Therefore pLuc-fos/fct served as an excellent negative control reporter plasmid and demonstrated that binding of Tcf-4 factor to the enhancer element is crucial for the transcription of the reporter gene. For transfections, 2 X 10^5 cells were seeded (in triplicate) and incubated overnight with 2 μg pTCF/fos-Luc, 1 μg renilla luciferase (pRL-TK), and 8-14 μl lipofectin reagent in Opti-MEM media. A Dual-Luciferase Reporter kit and a Turner TD-20e luminometer were used to determine luciferase activity, with values expressed as the ratio (relative light units, RLU) of firefly luciferase activity to Renilla luciferase activity 48 hrs. post transfection. In addition, to examine the effect of tandem repeats of Tcf-4 enhancer on the activity of c-fos promoter, a Tcf-4 enhancer deleted vector containing only c-fos promoter driving the expression of luciferase gene was made. The Tcf-4 enhancer-deleted vector, pTOPLESS, was generated by digesting pTOPFLASH with Sal I to remove the 82 bp TCF enhancer sequences. Following gel purification, the pTOPLESS plasmid was generated by religation of the Sal I site. The constructed plasmids were used to evaluate

the ability of the Tcf enhancer sequences to express the luciferase reporter in normal and cancer cell lines. In dual luciferase assays, as described in the methods section, different cell lines were transfected with either pLuc-fos/Tcf or pTOPLESS or with just the blank luciferase reporter vector (pGL3). Table-1 shows the comparative luciferase activity in a panel of selected cell lines. The results show that the combination of Tcf-4/β-catenin enhancer with the c-fos promoter results in increased luciferase activities as compared to the luciferase activities of the c-fos promoter (pTOPLESS) or the blank vector (pGL3) alone. Shown in the panel are colon cancer cell lines (HCT 8, SW480, SW620, colo 320) and normal colon cell line (NCM 460). All of the colon cancer cell lines tested are known to have APC or β-catenin gene mutations (286) resulting in aberrant constitutive activation of the wnt signaling pathway. Therefore a higher Tcf-4 enhancer activity is expected in these cell lines which is reflected in the increased amount of luciferase gene product (higher luciferase activity) produced by pLuc-fos/Tcf. NCM460 is a normal cell line derived from non diseased colon (272) and is used as a negative control in the presented experiments. Use of NCM 460 as a negative control is based on the rationale that being a normal cell line it does not have APC or β-catenin gene mutations (no wnt signaling) and is expected to have very low Tcf-4/ β-catenin enhancer activity, resembling a normal cell situation. Figure 1 is an alternative way of showing the proof of principle. The activity of pLuc-fos/Tcf and pLuc-fos/fct were compared in normal and cancer cell lines by conducting dual luciferase assays. Either pLuc-fos/Tcf or pLuc-fos/fct was co-transfected with pRL-TK (internal control) in selected cell lines. 48 hours post transfection cell lysates were prepared and relative luciferase ratio (RLU) readings

Table 1. Fold activation by Tcf-4/β-catenin enhancer element. pLuc-fos/Tcf, pTOPLESS (c-fos promoter alone, no Tcf-4 enhancer), or pGL3 (blank vector) luciferase reporter plasmids were co-transfected with pRL-TK in selected cell lines. Whole cell lysates were prepared 48 hours later and assayed for luciferase activity using a dual luciferase reporter assay kit. Shown are the fold increases in the luciferase activities by Tcf-4/β-catenin enhancer element in selected cell lines.

Cell Line	**pTOPLESS**	**pGL3**
HCT8	5	150
SW480	34	500
SW620	24	420
Colo320	40	550
NCN460	1	4

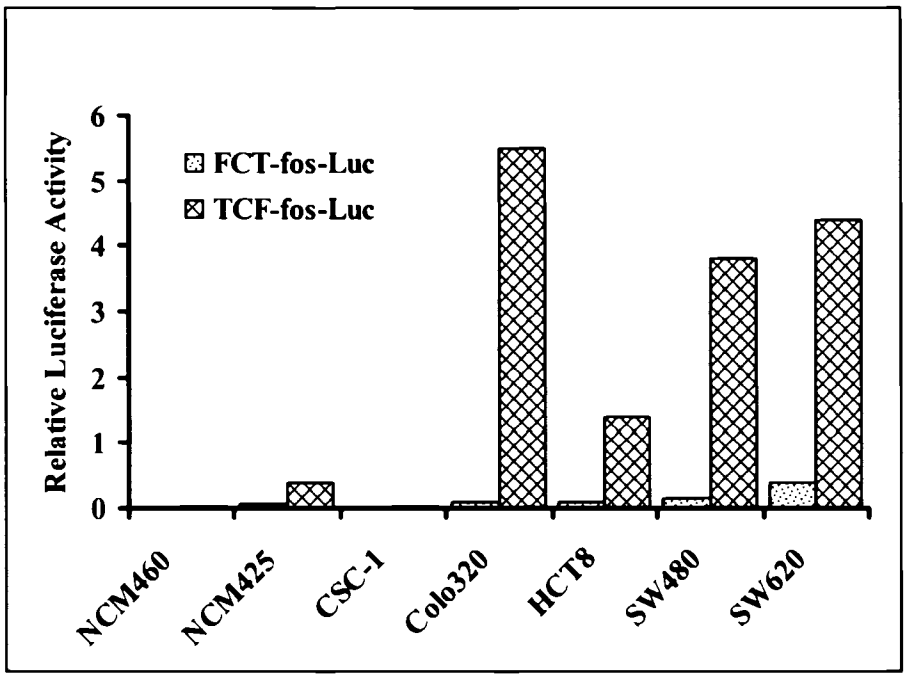


Figure 1. fos/Tcf and fos/fct recombinant promoter activities in normal and colon tumor cells. Normal and colon cancer cell lines were co-transfected using pRL-TK with either pLuc-fos/Tcf or pLuc-fos/fct reporter plasmids.Wholecell lysates were prepared 48 hours post transfection and assayed for luciferase activities. Shown are the recombinant promoter activities in terms of relative luciferase units derived by dividing the firefly luciferase activity value by the renilla luciferase value.

were recorded as previously described in the text. pLuc-fos/Tcf has much higher activity in colon cancer cell lines (colo 320, HCT8, SW480, SW620) as compared to pLuc-fos/fct. On the other hand pLuc-fos/Tcf did not show any appreciable activity in chosen normal cell lines (NCM460, NCM425, CSC1).

Construction and cell line testing of Tcf-4-enhancer/CEA recombinant promoter

The carcinoembryonic (CEA) antigen is a tumor associated antigen and is expressed by a majority of colorectal tumors. The feasibility of the use of the CEA promoter in the design of a tumor specific gene expression promoter was tested. As described in the methods section, the TCF-4 enhancer element was cloned into the Sal-I restriction site of pGL3-CEA to generate pGL3-CEA/Tcf. Dual luciferase assays were performed to evaluate the activity of these CEA constructs. A pane of selected cell lines (table-2) were transfected with either pGL3-CEA or pGL3-CEA/Tcf, in dual luciferase assays. Luciferase activities were monitored after 48 hours of transfection as described earlier. In order to compare the activities of the CEA constructs to c-fos/Tcf promoter, the same panel of cell lines were also transfected with either pLuc-fos/Tcf or pTOPLESS, in dual luciferase assays (table-2). The activity of the recombinant CEA promoter was also tested in two breast cancer cell lines; MCF-7 and MDA 435. Breast cancer cell lines were chosen because deregulation of the wnt signaling pathway has also been reported in breast carcinoma (284). In luciferase reporter assays, the combination of the Tcf-4 enhancer element with the CEA promoter did not yield any significant increase in luciferase activities as compared to the CEA promoter alone. As shown in table-2, by fold induction, the CEA/Tcf combination did not result in any significant increase in luciferase activities, in the tested cancer cell lines (MCF-7, MDA-435, SW620), over

Table 2. Effect of Tcf-4 enhancer element on the activity of c-fos and CEA promoters. Normal and colon cancer cell lines were co-transfected using pRL-TK with either pLuc-fos/Tcf or pLuc-fos/fct reporter plasmids. Whole cell lysates were prepared 48 hours post transfection and assayed for luciferase activities. The promoter activities in terms of relative luciferase units were derived by dividing the firefly luciferase activity value by the renilla luciferase value.

Cell Line/Promoter	No TCF RLU Ratio	Plus TCF RLU ratio	Fold Increase
CSC-1-Fos	0.009	0.04	4.4
CSC-1-CEA	0.01	0.01	1.0
CSC-1-LTR	6.0	-	-
NCM460-Fos	0.003	0.04	13.3
NCM460-CEA	0.005	0.01	2.0
NCM460-LTR	8.7	-	-
NCM425-Fos	0.06	0.39	6.5
NCM425-CEA	0.02	0.13	6.5
NCM425-LTR	16.7	-	-
MCF7-Fos	0.31	1.1	3.5
MCF7-CEA	0.05	0.08	1.6
MCF7-LTR	0.78		-
MDA435-Fos	0.03	0.71	23.6
MDA435-CEA	0.04	0.05	1.25
MDA435-LTR	8.5		-
MDA436-Fos	0.07	0.68	9.71
MDA231-Fos	0.04	0.96	24
SW620-Fos	0.32	4.2	13.2
SW620-CEA	0.27	1.4	5.1
SW620-LTR	7.4	-	-

CEA promoter alone. On the other hand, as demonstrated in the previous experiment, the combination of the Tcf-4 enhancer with the c-fos promoter resulted in a significant increase of luciferase activity in the SW620 and MDA 435 cancer cell lines, when compared to c-fos promoter alone. Unexpectedly, in the panel of selected normal cell lines (CSC-1, NCM460, NCM425), the combination of c-fos/Tcf also resulted in appreciable increase in luciferase activities when compared to c-fos alone. The significance of these unexpected findings is included in the discussion section of the presented study. Furthermore, in order to demonstrate that the selected cell lines were capable of plasmid DNA uptake in transfection assays and are transcriptionally active, dual luciferase assays were performed using pGL3-LTR reporter luciferase assay plasmid. The pGL3-LTR plasmid containing the wild-type U3 LTR of the Moloney murine leukemia virus was a gift from Dr. Robert Saylors, University of Arkansas for Medical Sciences. It was expected that LTR would be typically activated in eukaryotic cells and would express the luciferase gene. As a positive control, dual luciferase assays were performed by transfecting pGL3-LTR in the same panel of cell lines (table-2). As shown in table-2, in all the normal cell lines (CSC-1, NCM460 and NCM 425) and cancer cell lines (MCF7, MDA435 and SW620) pGL3-LTR showed significant luciferase activity. These results are significant especially in reference to the normal cell lines because a lower Tcf-4 enhancer activity could be argued as a result of poor transfection efficiency and inability to express the reporter DNA. Therefore in the light of these results it is suggested that the chosen cell lines are transfectable, transcriptionally active and are appropriate controls for analyzing Tcf-4 activity in normal cells.

Construction and cell line testing of combination of Tcf-4-enhancer with Cox-2, PSMA and PSA promoter

The possibility of using cyclooxygenase-2 (cox-2), prostate specific membrane antigen (PSMA) and prostate specific antigen (PSA) promoter, in combination with the Tcf-4 enhancer, for designing of tumor/tissue specific gene expression promoter was also examined. Cox-2, is an enzyme, that is induced in many inflammatory reactions (237). Many growth factors, cytokines and inflammatory agents all appear to enhance the expression of Cox-2 by interacting with various regulatory sequences in the promoter region of this gene (238). The Cox-2 promoter was selected as a candidate promoter for gene therapy because of the deregulation of cox-2 expression levels commonly found in tumors of epithelial origin such as colon cancer (287). Studies have shown that the minimal cox-2 promoter can be used for designing tumor specific gene expression promoters (248). In an effort to improve the specificity and activity of a minimal cox-2 promoter, it was combined with the Tcf-4 enhancer element. A 720-bp minimal cox-2 promoter was amplified from genomic DNA isolated from SW480 cells and cloned in pGL3-basic vector to generate pGL3-Cox-2. Subsequently, the Tcf-4 enhancer was cloned into the enhancer cloning site of pGL3-Cox-2 to generate pGL3-Cox-2/Tcf.

Prostate Specific Membrane Antigen (PSMA) is a membrane glycoprotein with folate hydrolase activity, predominantly expressed in prostate epithelial cells. Studies at the mRNA and protein levels demonstrate that PSMA is differentially regulated in benign and malignant prostate abnormalities as well as during tumor progression. Thus, the promoter of the PSMA gene may be of use in gene therapy of prostate cancer. However, available data indicates that the PSMA promoter, by itself, is a weak activator of

transcription and may not necessarily be able to achieve therapeutically significant levels of gene expression. Therefore, in an effort to achieve higher tumor specific activity of the PSMA promoter, it was combined with Tcf-4 enhancer element. A 640-bp minimal PSMA (cloned in pGL3-PSMA) promoter has been previously defined and was cloned in pGL3-Tcf to generate pGL3-PSMA/Tcf as described in materials and methods.

Prostate specific antigen is a protein expressed by benign, hyperplastic and malignant prostatic epithelium (266) and it is well established as a prostate cancer specific biomarker. Expression of PSA is regulated by androgens and is restricted to the prostate (268). PSA expression is attributed to the selective activation of the PSA promoter in prostate. Therefore, the PSA promoter is a very attractive candidate for the design of a tumor/tissue specific gene expression promoter for the purpose of gene therapy. Indeed the PSA promoter has been shown to be a promising candidate for constructing tumor/tissue specific gene expression promoters (271). However, it is associated with weak transcriptional activity and in the past, the PSA promoter has been coupled with enhancers in an effort to improve its activity. With the back ground knowledge that wnt/β-catenin signaling pathway is activated in a significant number of prostate cancer cases, the idea of combining Tcf-4 enhancer element with a minimal PSA promoter to construct a tumor/issue specific gene expression promoter was explored. pGL3-PSA reporter vector, containing a minimal 410 bp PSA promoter, was a gift from Dr. Antonia Vlahou. pGL3-PSA/Tcf was generated by cloning the Tcf-4 enhancer element into pGL3-PSA.

The promoter/enhancer activity of the constructed luciferase reporter plasmids, pGL3-Cox/Tcf, pGL3-PSMA/Tcf and pGL3-PSA/Tcf, was assessed by screening a panel

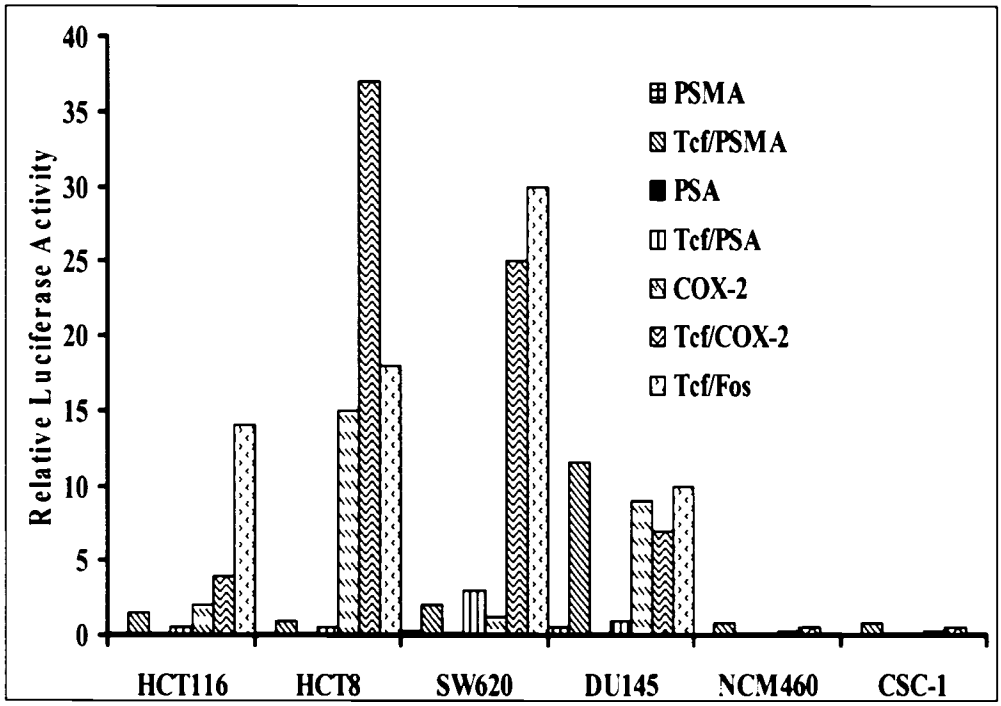


Figure 2. Cell line screening of the activities of PSMA, PSA, COX-2 promoters. Dual luciferase assays were performed in each cell line using plasmids containing the test promoter with or without the Tcf-4/β-catenin enhancer element. Relative luciferase unit ratios are shown for each of the tested promoter. Also shown, for comparison, are the RLU ratios obtained in dual luciferase assays performed using plasmid containing the c-fos/Tcf recombinant promoter (pLuc-fos/Tcf. The data shown is a representative of multiple experiments.

of selected cancer and normal cell lines (figure-2) in luciferase assays. pGL3-Cox2, pGL3-PSMA and pGL3-PSA reporter constructs were used as controls. The selected cell lines were transfected and processed for luciferase activities as described in the materials and methods section. The activities of the various promoters and promoter/enhancer combinations were assessed by the fold increase in the luciferase activities. In HCT8 (colon cancer cell line) coupling of the Tcf-4 enhancer element with the Cox-2 promoter resulted in a dramatic increase of luciferase activities (see figure-2) in colon cancer cell lines HCT8 (15 fold) and SW620 (23 fold). However, combination of Tcf-4 with Cox-2 caused only marginal increase in luciferase activities in the colon cancer cell line HCT116 and at the same time it resulted in a marginal decrease of luciferase activity in the prostate cancer cell line DU145. The combination of the Tcf-4 enhancer with the PSMA or PSA promoters resulted in a marked increase (PSMA/Tcf-12 fold, PSA/Tcf-9 fold) in luciferase activities, over the control promoters, in the prostate cancer cell line DU145 (figure-2). In addition, shown in figure-2, are the luciferase activities of the indicated plasmids in two normal cell lines NCM460 and CSC1, suggesting that the combination of the Tcf-4 enhancer with either the PSMA, PSA or Cox-2 promoters did not result in any appreciable increase of luciferase activity in normal cells.

As evident from the results of the reporter assay based cell line screening of the Cox-2/Tcf-4 recombinant promoter, the Cox-2 promoter was found to be a promising candidate and could be used for designing a tumor specific gene expression promoter. Therefore the activity of the recombinant Cox-2/Tcf-4 promoter was compared with c-fos/Tcf-4 and CEA/Tcf-4 recombinant promoters in reporter luciferase assays in a panel of selected cell lines (figure-3). As indicated from the RLU ratios in figure-3, the activity

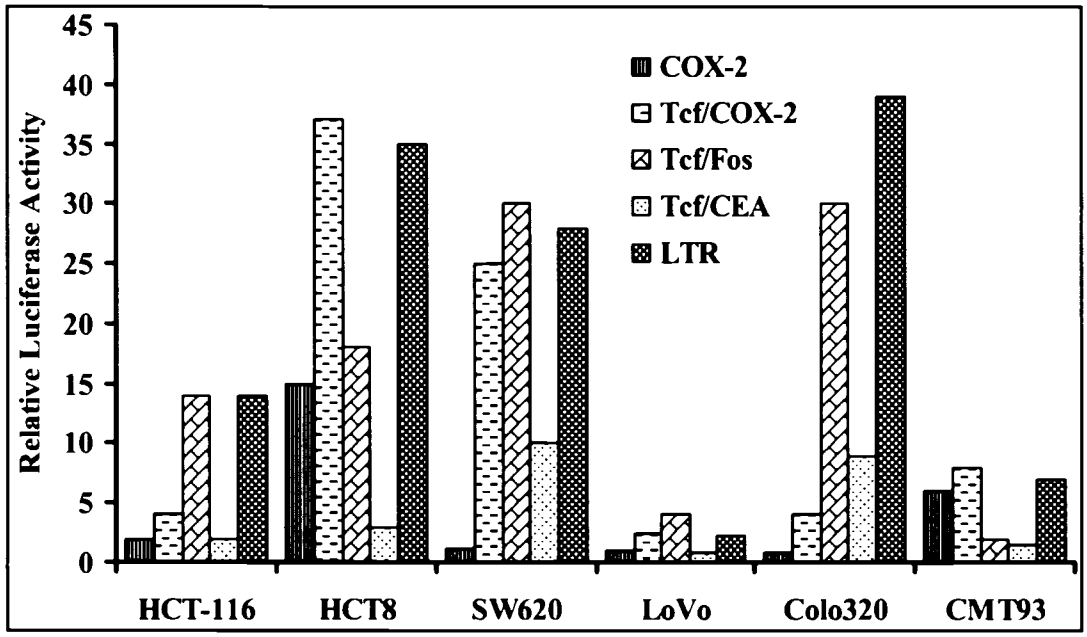


Figure 3. Cell line screening of the activities of COX-2, c-fos and CEA promoters. Dual luciferase assays were performed in each cell line using plasmids containing the test promoter with or without the Tcf-4/β-catenin enhancer element. Relative luciferase unit ratios are shown for each of the tested promoter. Also shown, for comparison, are the RLU ratios obtained in dual luciferase assays performed using plasmids containing the viral LTR promoter (pGL3-LTR) and c-fos/Tcf recombinant promoter (pLuc-fos/Tcf).

of Cox-2/Tcf-4 promoter was comparable to or higher than the c-fos/Tcf-4 promoter in the colon cancer cell lines(HCT8, LoVo, CMT93 and SW620). On the other hand, cox-2/Tcf-4 promoter activity was found to be lower than the c-fos/Tcf-4 promoter in colon cancer cell line HCT116 and Colo320. With the exception of CMT93, Cox-2/Tcf recombinant promoter activities were found to be higher than the activities of CEA/Tcf-4 in the selected cell lines. Also, shown in figure-3 are the luciferase activities of viral LTR promoter (from pGL3-LTR) in different cell lines. The LTR activity was used for the purpose of determining comparative luciferase activities and to serve as a positive control.

In the prostate cancer cell line DU145, the combination of the Tcf-4 enhancer element with the PSMA promoter showed an appreciable increase in promoter activity over that of the PSMA promoter alone. In an effort to further characterize the PSMA/Tcf-4 recombinant promoter, luciferase assays were performed using the pGL3-PSMA/Tcf luciferase reporter construct in a panel of prostate cancer (Tramp, PC3 and DU145) and normal (NCM460 and normal prostate) cell lines in luciferase assays (figure-4). The PSMA/Tcf-4 recombinant promoter showed an appreciable increase in activity over the PSMA promoter alone, in the PC3 prostate cancer cell line but did not show any significant activity in the normal prostate cell line and normal colon cell line (NCM460). The possibility of using a mouse model for prostate cancer (TRAMP) was also explored by including TRAMP cells in the panel of cell lines assessed for PSMA/Tcf-4 activity. However, the TRAMP cell did not show any appreciable PSMA/Tcf-4 recombinant promoter activity as assessed from the RLU ratios (figure-4).

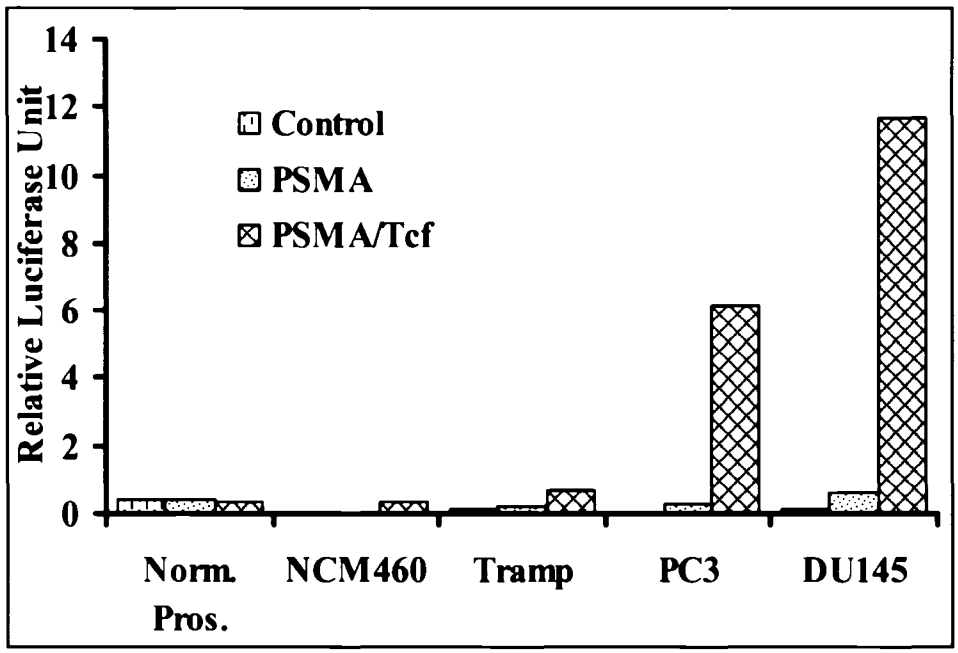


Figure 4. Cell line screening of the activities of the PSMA promoter. Selected cell lines were co-transfected with pRL-TK and reporter plasmids containing the PSMA promoter with or without the Tcf-4 enhancer Tcf-4/β-catenin enhancer element. Transfected cells were processed 48 hours later using a dual luciferase assay kit and relative luciferase units were determined. RLU ratios from each transfection are shown in the figure.

Evaluation of NCM460 as a negative control

NCM460 is cell line derived from normal colon mucosa and has been extensively used in the presented study as a negative control. The rationale behind using NCM460 as a negative control was that being derived from non-cancerous tissue, mutations in genes responsible for regulating β-catenin levels are unlikely to be found and it is expected that a constitutively active β-catenin will not be found in the nucleus. It has been suggested that in a normal cell β-catenin plays a role in cell to cell junctions and is found as part of multi-protein complex at the inner surface of cell membrane, which includes proteins like E-cadherin (288). Therefore, to evaluate the cellular localization of β-catenin in this cell line immunoflorescence studies were performed by staining for β-catenin as described in materials and methods. As expected in a normal cell, beta-catenin was found to be located on the inner side of the cell membrane and no nuclear staining was observed (figure-5). To assess whether introduction of a constitutively active β-catenin (13) can allow for Tcf-4/β-catenin enhancer mediated transcriptional activity in the NCM460 cells, luciferase reporter assays were performed in the presence of plasmid expressing constitutively active β-catenin. NCM460 cell were co-transfected with pLuc-fos/Tcf and β-catenin expressing plasmid and after 48 hours cells were processed for luciferase activity. A 100 fold increase in luciferase activity of pLuc-fos/Tcf was observed in presence of constitutively active beta-catenin indicating these cells were capable of Tcf-4/β-catenin transcriptional activity.

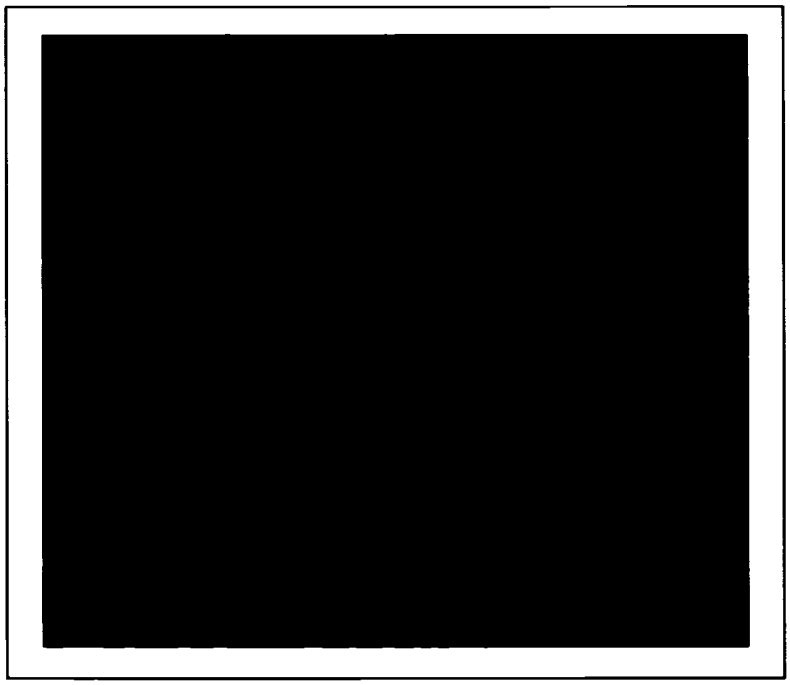


Figure 5. Validation of NCM-460 colon cells as a negative control. To visualize the localization of β-catenin immunoflorescence staining for β-catenin was performed in NCM460 cells. Cells were stained with a primary anti β-catenin antibody followed by incubation with a secondary FITC labeled anti mouse IgG antibody.

Aim #2. Evaluation of the ability of Tcf-enhancer/Promoter combinations to selectively express a therapeutic gene in tumor cells

Results presented under aim-1 strongly suggested that specific combinations of the Tcf-4 enhancer with minimal promoters could result in tumor/tissue specific gene expression. Such promoter/enhancer combinations are potential candidates for use in directing therapeutic gene expression. However, these findings are based on luciferase reporter assays suggesting the potential of these recombinant promoters in cancer gene therapy. To further evaluate the tumor specific gene expression efficacy of these recombinant promoters, therapeutic gene expression vectors were generated incorporating the therapeutic gene encoding the Herpes Simplex Virus Thymidine Kinase expressed from Tcf-4-enhancer/promoter combinations. For experiments presented in this section the following recombinant plasmid DNA vectors were constructed as described in material and methods.

a) pc-fos/Tcf-4-HSV-TK: Consists of the HSV-TK gene expressed from a recombinant Tcf-4 enhancer/c-fos promoter combination.

b) pSc-fos/Tcf-4-HSV-TK: Derived from pc-fos/Tcf-4-HSV-TK in which a significant portion of the c-fos promoter was deleted.

c) pPSMA/Tcf-4-HSV-TK: Consists of the HSV-TK gene expressed from a recombinant Tcf-4 enhancer/PSMA promoter combination.

d) pCDNA-HSV-TK: Negative control plasmid consisting of only the HSV-TK gene without any promoter.

Characterization of HSV-TK expression constructs

HSV-TK expression analysis

The pc-fos/Tcf-4-HSV-TK was constructed to test the ability of Tcf-4/β-catenin enhancer coupled with the c-fos promoter to drive the expression of HSV-TK selectively in tumor cells. The new plasmid was used to transiently transfect colon cancer cell lines (HCT-8, SW480 and SW620) and normal colon cells (NMC-460). After transfection, 48 hours later, whole cell lysates were made from each cell line and were subjected to SDS-PAGE, Western blot analysis using anti-HSV-TK antibodies. As shown in figure-6, transient transfection of pc-fos/Tcf-4-HSV-

TK resulted in significant amounts of HSV-TK in the tested cancer cells lines (HCT-8, SW620, and SW480). On the contrary, under similar conditions, HSV-TK expression was not detected in the normal cell (NCM460). Also shown in figure-6 are the results from similar transient transfection assays using the promoter less negative control plasmid pCDNA-HSV-TK where no detectable expression was observed in any of the tested cell lines. In addition, the ability of c-fos/Tcf-4 recombinant promoter to express HSV-TK in breast cancer cell lines was tested. As mentioned earlier in the text, this was done because evidence of aberrant activation of wnt signaling pathway in breast cancer has been documented. Two breast cancer cell lines were chosen; MDA435 and MCF7. As presented in figure 7, transient transfection of pc-fos/Tcf-4-HSV-TK resulted in significant expression of HSV-TK in MCF7 and MDA435. As a control, pCDNA-HSV-TK was transiently transfected in both breast cancer cell lines and assayed for expression of HSV-TK. As seen in figure 7, no expression, from pCDNA-HSV-TK, was detected in MCF7 cells; however trace amounts of HSV-TK were detected in the MDA435. For

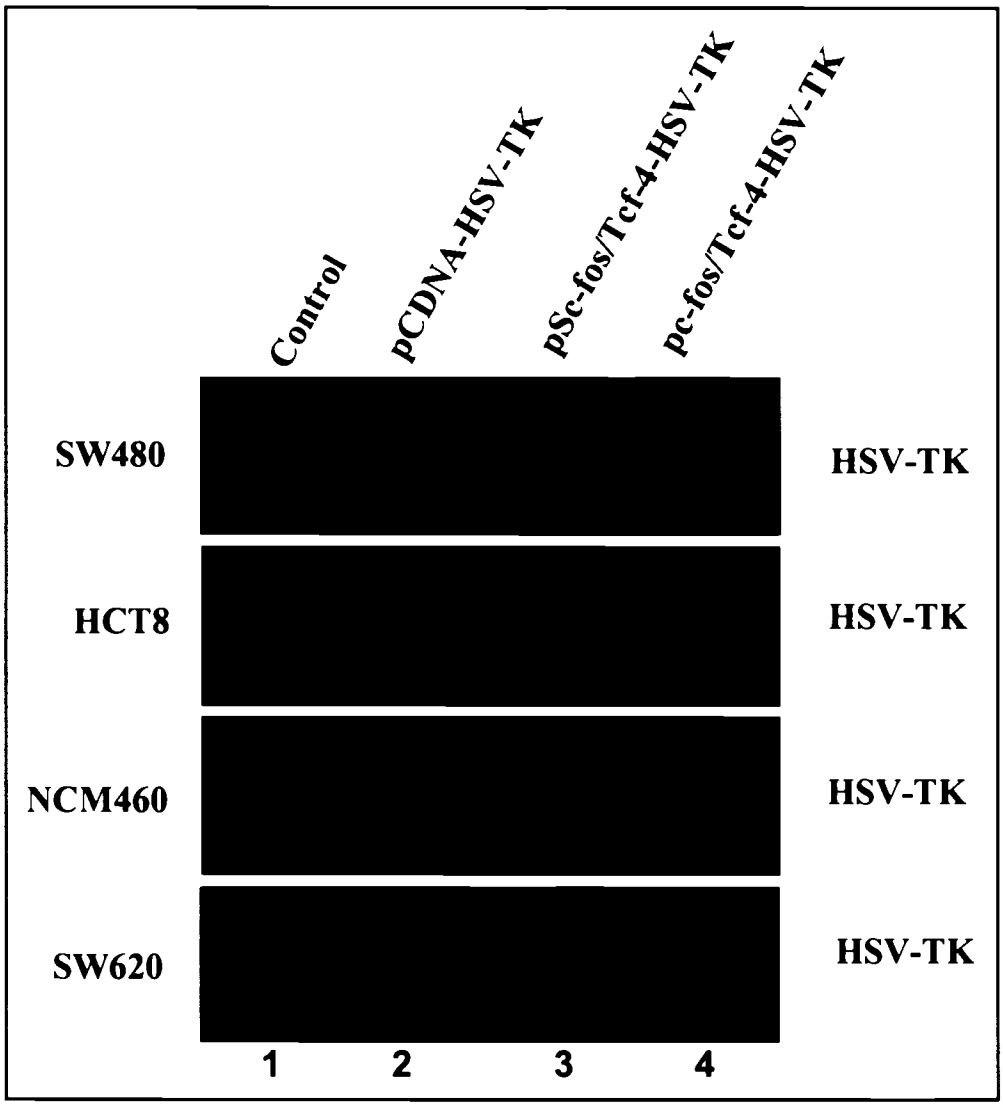


Figure 6. HSV-TK Western blots to assess the gene expression ability of c-fos/Tcf recombinant promoter in normal and tumor colon lines. Selected cell lines were transiently transfected with plasmids encoding HSV-TK gene driven by: cfos/Tcf (lane 4), truncated c-fos/Tcf (lane 3) or without any promoter (lane 2). Whole cell lysates were prepared after 48 hours of transfectionand were subjected to Western blot analysis using an anti HSV-TK antibody. Lane 1 is an untransfected negative control.

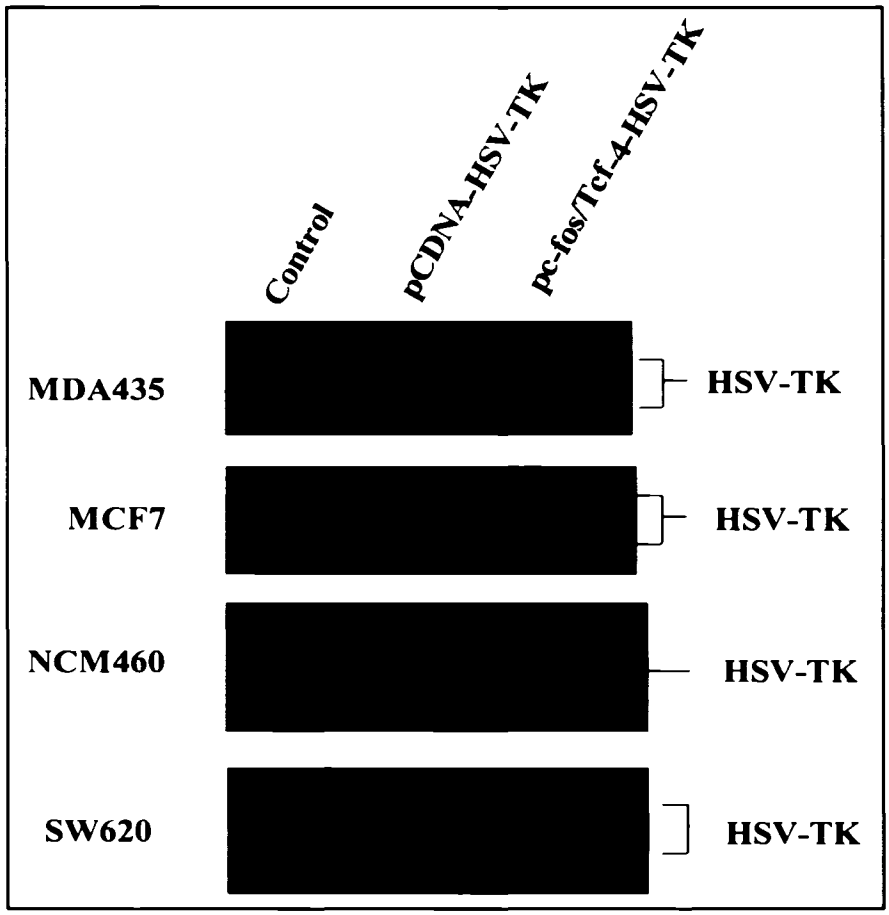


Figure 7. HSV-TK Western blot to assess the gene expression ability of c-fos/Tcf recombinant promoter in breast tumor cell lines. Selected cell lines were transiently transfected with plasmids encoding HSV-TK gene driven by: cfos/Tcf (lane 3) or without any promoter (lane 2). Whole cell lysates were prepared after 48 hours of transfectionand were subjected to Western blot analysis using an anti HSV-TK antibody. Lane 1 is a untransfected negative control. Bands corresponding to HSV-TK are labeled.

comparison of HSV-TK expression, results from transient transfection of HSV-TK expression constructs in NCM460 and SW620 cell lines are also shown in figure 7. These results demonstrate that c-fos/Tcf-4 recombinant promoter can selectively express the therapeutic gene, HSV-TK, in examined tumor cell lines. However, selective expression of HSV-TK does not necessarily mean that a functional HSV-TK gene product is expressed which would facilitate tumor cell killing upon treatment with ganciclovir. To determine whether the expressed HSV-TK was functional GCV metabolic labeling and sensitivity assays were performed.

GCV Metabolic Labeling and Sensitivity in pc-fos/Tcf-4-HSV-TK transfected cells

To determine whether the expressed HSV-TK was functional, identical transfection protocols were performed except that at 24 hours post-transfection, 1 μM [^{3}H] GCV was added for 12 hours. Cells were isolated, counted and then extracted with 70% methanol. The soluble supernatant and insoluble DNA pellet were quantitated for levels of [^{3}H] GCV incorporation by scintillation counting. To separate GCV from phosphorylated GCV metabolites in the methanol soluble fractions, thin layer chromatography, using PEI-cellulose plates developed in 0.8 M LiCl, was performed. As shown in Figure 8, only cells transfected with the pc-fos/Tcf-4-HSV-TK construct had significant levels of soluble [^{3}H] GCV-phosphometabolites. Again, only minimal metabolism of [^{3}H] GCV was found for the NCM460 control cells. Analysis of [^{3}H] GCV levels in the methanol insoluble pellet, indicative of GCV incorporation into cellular DNA, was also performed. As presented in Figure 9, the levels of [^{3}H] GCV incorporated into the DNA were highest in the pc-fos/Tcf-4-HSV-TK transfected tumor cells, but low in the NCM460 cells. Similar, GCV metabolic labeling, experiments were

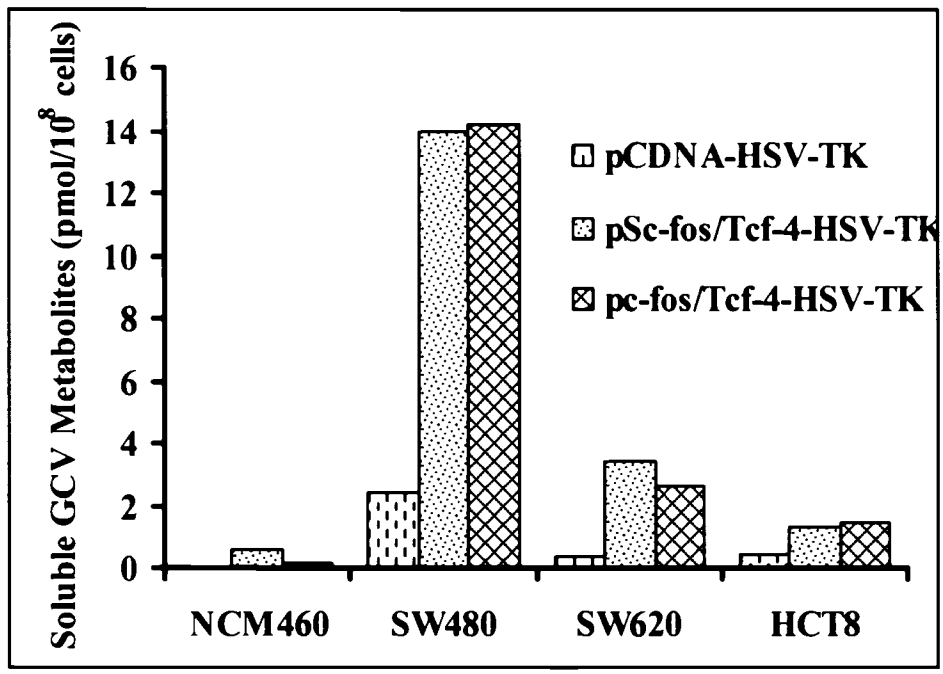


Figure 8. Assessment of functionality of HSV-TK expressed by c-fos/Tcf recombinant promoter in colon cell lines. Expressed HSV-TKwas evaluated for its ability to phosphorylate GCV. Selected cell lines were transiently transfected with plasmids encoding HSV-TK gene driven by: cfos/Tcf (pc-fos/Tcf-4-HSV-TK), truncated c-fos/Tcf (pSc-fos/Tcf-4-HSV-TK) or without any promoter (pCDNA-HSV-TK). Following 36 hours after transfection radio labeled GCV was added to the cell. 6-8 hours later, GCV metabolites were extracted and counted for radioactivity. Shown are the estimated amounts of soluble GCV metabolites for each cell line.

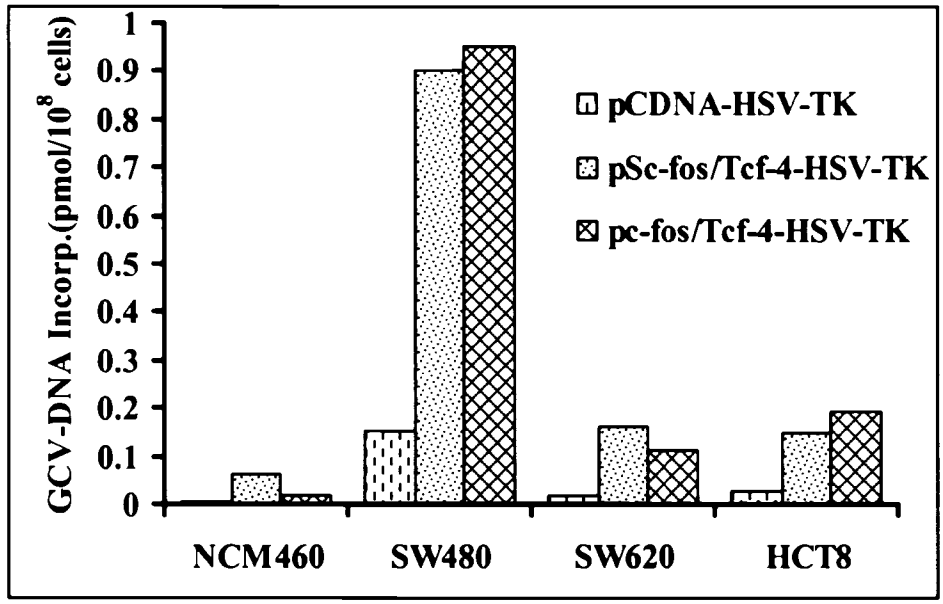


Figure 9. Assessment of functionality of HSV-TK expressed by c-fos/Tcf recombinant promoter in colon cell lines. DNA incorporation of radio labeled GCV was used as an index to asses the functionality of expressed HSV-TK. Selected cell lines were transiently transfected with plasmids encoding HSV-TK gene driven by: cfos/Tcf (pc-fos/Tcf-4-HSV-TK), truncated c-fos/Tcf (pSc-fos/Tcf-4-HSV-TK) or without any promoter (pCDNA-HSV-TK). Following 36 hours after transfection radio labeled GCV was added to the cell. 6-8 hours later, insoluble GCV metabolites were separated from soluble metabolites followed by scintillation counting. Shown are estimated amounts of incorporated GCV metabolites for each cell line.

performed in breast cancer cells line; MCF7 and MDA435, and a higher metabolism of [^{3}H] GCV was detected in pc-fos/Tcf-4-HSV-TK transfected cells (figure 10). To examine whether GCV metabolism resulted in increased tumor cell killing, SW480, MDA435 and NCM460 cells were transfected with pCDNA-HSV-TK or pc-fos/Tcf-4-HSV-TK. After 24 hours, half of the samples received 10 μM GCV for an additional for 48 hrs. Viable cell numbers were determined using trypan blue dye exclusion and automated cell counting. As shown in Figure 11, only in the pc-fos/Tcf-4-HSV-TK transfected SW480 (figure 11a) and MDA-435 (figure 11c) cells did GCV addition have any significant toxic effect, while no effect was observed in the similarly treated normal NCM460 (figure 11b) and CSC-1(figure 11d) cells. As a control, the promoterless pCDNA-HSV-TK vector was transfected in SW480 (figure 11a), MDA435 (figure 11c), NCM460 (figure 11b) and CSC-1 cells (figure 11d) in identical transfection experiments. Results presented in figure 11 show that pCDNA-HSV-TK construct did not result in any significant toxicity in the chosen cell lines. The cumulative results presented in Figures 8-11 indicate functional and tumor cell specific expression of HSV-TK delivered via the pc-fos/Tcf-4-HSV-TK plasmids. The results, so far, strongly support the proof of principle that a minimal oncogenic promoter can be successfully combined with Tcf-4/β-catenin enhancer to achieve tumor specific gene expression. With these encouraging results further experiments were performed using constructs in which the PSMA promoter was coupled with Tcf-4/β-catenin enhancer (pPSMA/Tcf-4-HSV-TK) to express HSV-TK.

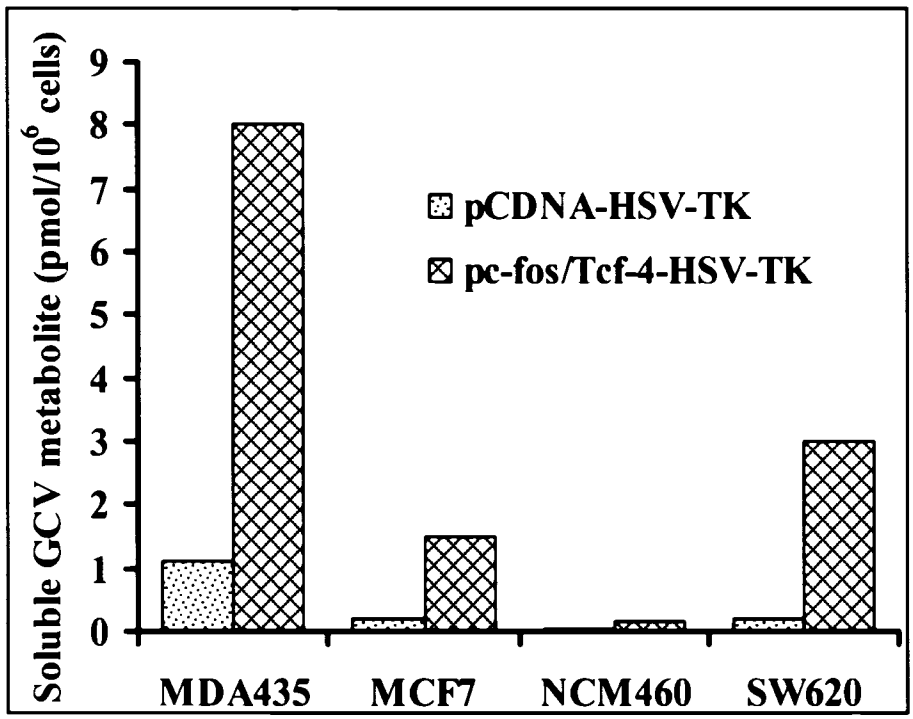
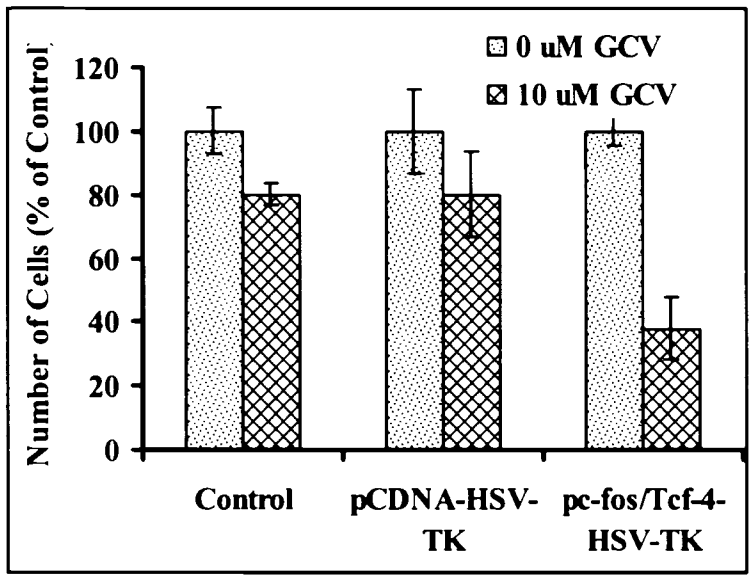


Figure 10. Assessment of functionality of HSV-TK expressed by c-fos/Tcf recombinant promoter. Expressed HSV-TK was evaluated for its ability to phosphorylate GCV in MDA435 and MCF7 breast cancer cell lines. Selected cell lines were transiently transfected with plasmids in which the HSV-TK gene was driven by cfos/Tcf (pc-fos/Tcf-4-HSV-TK). pCDNA-HSV-TK is used as . Following 36 hours after transfection radio labeled GCV was added to the cell. 6-8 hours later, GCV metabolites were extracted and counted for radioactivity. Shown are the estimated amounts of soluble GCV metabolites for each cell line.Also shown, for comparison, soluble GCV amounts from the normal cell line NCM460 and the colon cancer cell line SW620.

11 (a)



11 (b)

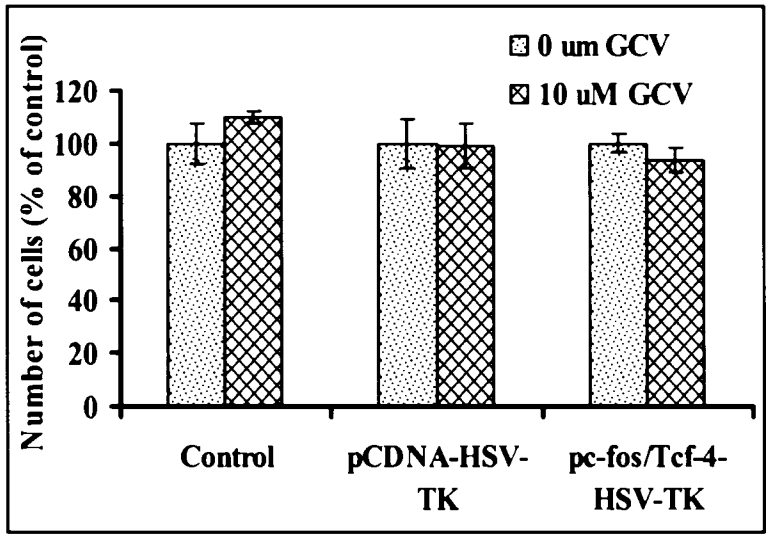


Figure 11 (a-b-c-d). GCV sensitivity in pc-fos/Tcf-4-HSV-TK transfected cells. Selected cell lines were transiently transfected with either pc-fos/Tcf-4-HSV-TK or pCDNA-HSV-TK (control) and treated with GCV 24 hours after transfection. Number of live cells was assessed 72 hours later. Cell numbers, determined as percentage of control, are shown for SW480 (a), NCM460 (b), MDA435 (c) and CSC1 (d). Figure 11 (c) is shown on page 105 and 11 (d) on page 106.

11 (c)

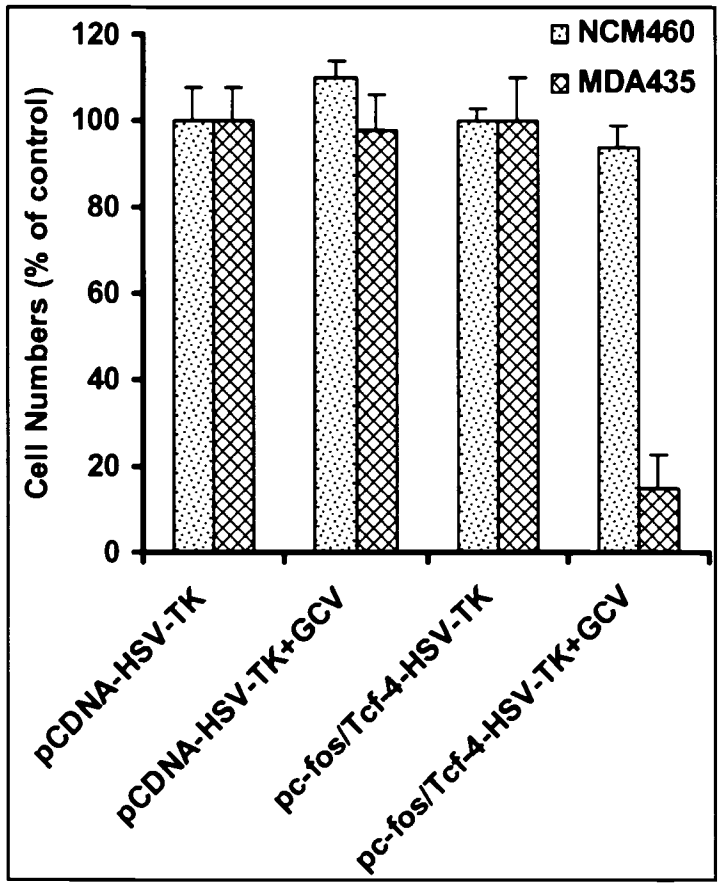


Figure 11 Continued.

11(d)

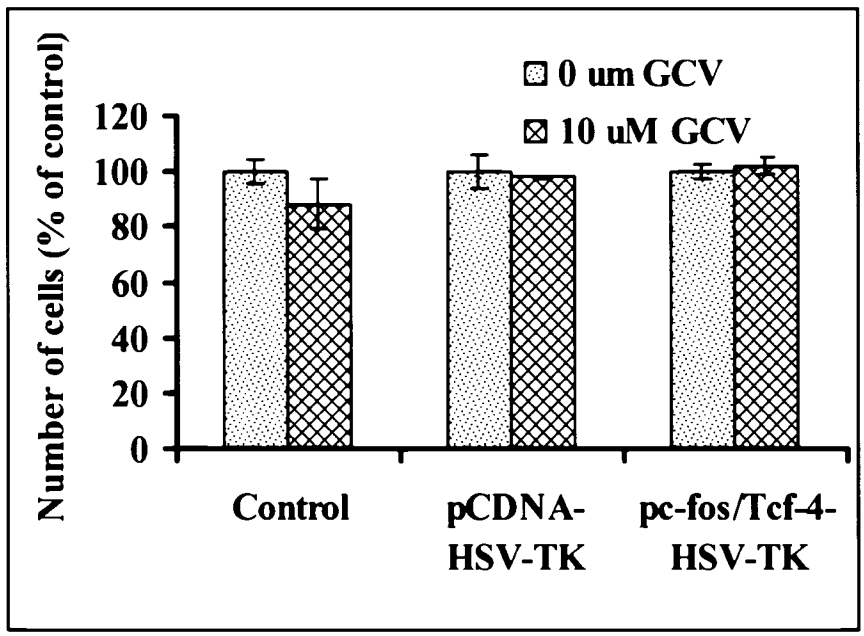


Figure 11 Continued.

GCV Sensitivity in pPSMA/Tcf-4-HSV-TK transfected cells

In a significant number of prostate tumor cases aberrant activation of wnt signaling pathway is documented (205). In addition, in prostate carcinoma PSMA is frequently found to be overexpressed, which is attributed to the increase in transcription activity of the PSMA promoter (289). Therefore the ability of PSMA/Tcf-4 recombinant promoter to direct tumor specific gene expression in prostate tumor cells was explored. Two prostate cancer cell lines DU145 and PC3 were transiently transfected with pPSMA/Tcf-4-HSV-TK and pCDNA-HSV-TK. After 24 hours, half of the samples received 10 μM GCV for an additional for 48 hrs. Viable cell numbers were determined using trypan blue dye exclusion and automated cell counting. As shown in figure 12, pPSMA/Tcf-4-HSV-TK transfection resulted in significant cellular toxicity upon addition of GCV in PC3 cells (figure 12b). On the other hand, under identical conditions pPSMA/Tcf-4-HSV-TK transfection did not cause any noticeable GCV toxicity in normal NCM460 cells (figure 12a). Simultaneously, there was no GCV mediated cellular toxicity observed in NCM460 and PC3 cells when transfected with control promoter less vector pCDNA-HSV-TK. However, at 10 μm GCV concentration, in DU145 prostate cancer cells the combination of GCV with transfection of pPSMA/Tcf-4-HSV-TK did not cause any increased toxicity as compared with the control pCDNA-HSV-TK transcfection (figure 12c). This inconsistency in the results could be due to the differential metabolism of GCV by DU145 cells. Therefore, the experiment described in figure 12c was repeated and this time transiently transfected cells were treated with an increased dose of 25 μm GCV. The data presented in figure 12d clearly exihibit that DU145 prostate cancer cells show significant cell killing when transiently transfected by

12 (a)

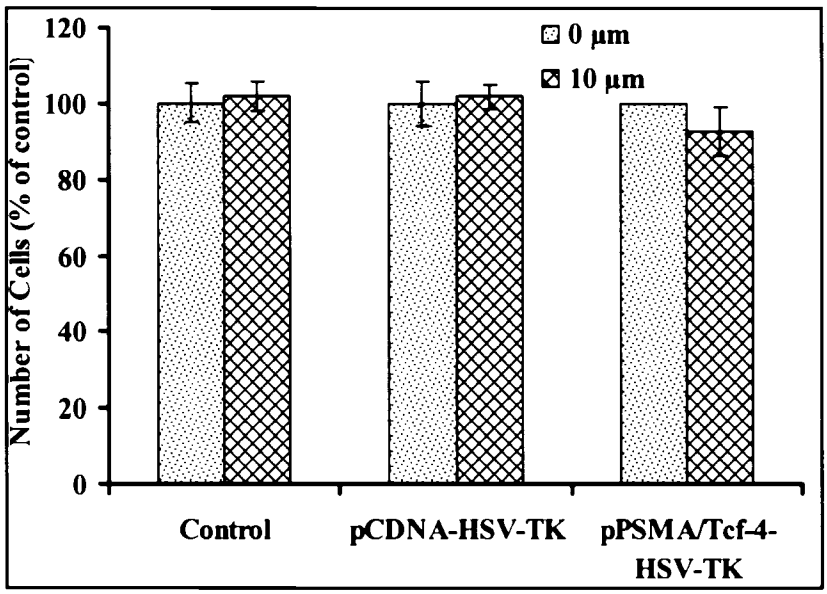
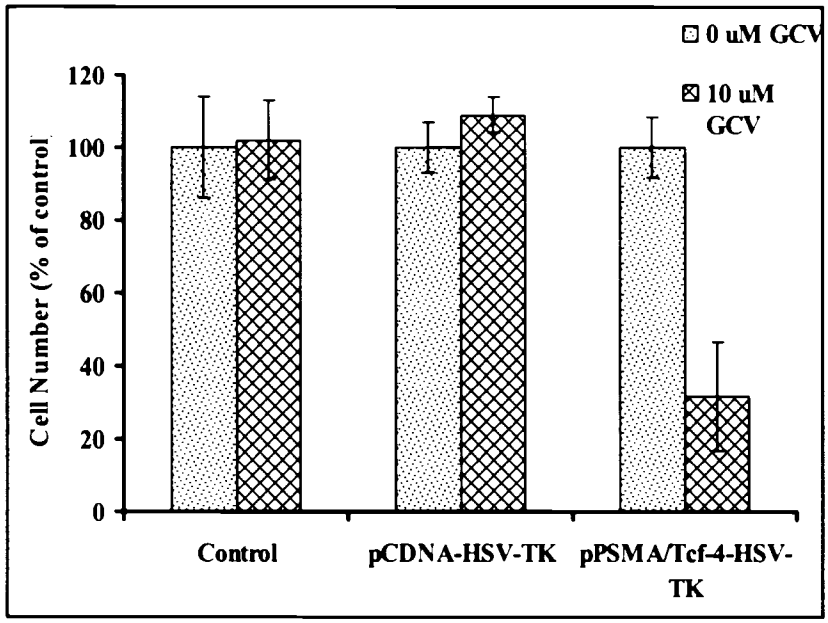


Figure 12(a-b-c-d). GCV sensitivity in pPSMA/Tcf-4-HSV-TK transfected prostate cancer cells. Selected cell lines were transiently transfected with either pPSMA/Tcf-4-HSV-TK or pCDNA-HSV-TK (control) and treated with GCV 24 hours after transfection. Number of live cells was assessed 72 hours later. Cell numbers, determined as percentage of control, are shown for NCM460 (a), PC3 (b) (page 109) and DU145 (c-d) (page109-110). As a control, GCV sensitivity caused by transient pPSMA/Tcf-4-HSV-TK transfections in normal NCM460 was also determined (figure a). pc-fos/Tcf-4-HSV-TK mediated GCV sensitivity in DU145 cells was compared to GCV sensitivity caused by pPSMA/Tcf-4-HSV-TK and is shown in figure d. For b, c and d, see pages 109-110.

12 (b)



12 (c)

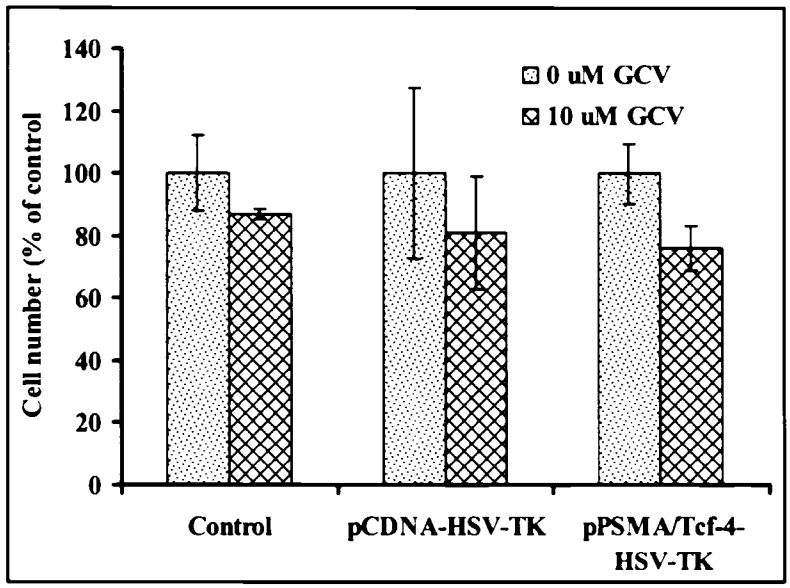


Figure 12 Continued.

12 (d)

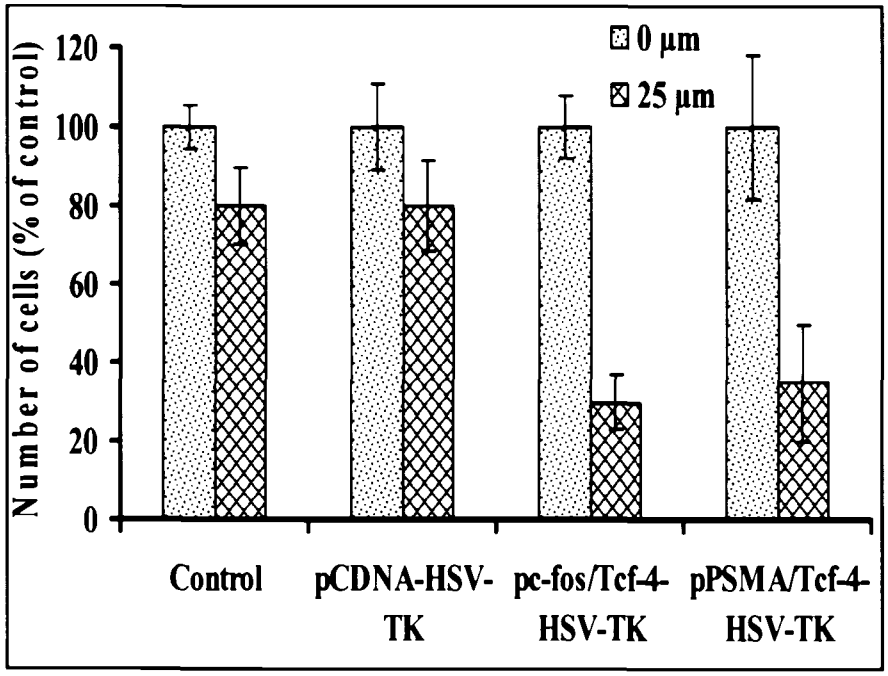


Figure 12 Continued.

pPSMA/Tcf-4-HSV-TK and followed by 25 μm GCV treatment. For comparison, in figure 12D, pc-fos/Tcf-4-HSV-TK/GCV mediated DU145 cell killing is also shown. Further, having successfully tested the ability of pPSMA/Tcf-4-HSV-TK vector to kill human prostate cancer cells, the PSMA/Tcf-4 vector was assessed for its toxicity in a mouse prostate cancer cell line (Tramp). The primary objective behind testing the HSV-TK expression construct in Tramp cells was to exploit the already established Tramp mouse model for certain in vivo gene therapy experiments. Tramps cells were transiently transfected with pPSMA/Tcf-4-HSV-TK, pCDNA-HSV-TK or with blank vector. After 24 hours, half of the samples received 10 μM GCV for an additional 48 hrs followed by 10 μM of GCV treatment 24 hours later. Viable cell numbers were determined using trypan blue dye exclusion and automated cell counting. As shown in figure 13 treatment of Tramp cell with pPSMA/Tcf-4-HSV-TK/GCV caused approximately 25% cell death as compared to the control pCDNA-HSV-TK/GCV treated cells. The marginal toxicity of pPSMA/Tcf-4-HSV-TK/GCV system in Tramp cells could be attributed to lower transfection efficiency of TRAMP cells and /or to the lower Tcf-4 transcriptional activity in these cells.

Cumulatively, the results presented in figure 12 suggest that the pPSMA/Tcf-4-HSV-TK construct can successfully express a functional HSV-TK gene in specific prostate cancer cells. In addition, the pPSMA/Tcf-4-HSV-TK/GCV system can result in significant cell death in prostate cancer cells and shows little or insignificant amount of toxicity in normal NCM460 cells.

In summary, the presented results so far are highly suggestive that a recombinant oncogenic promoter/Tcf-4 enhancer combination can successfully express a functional

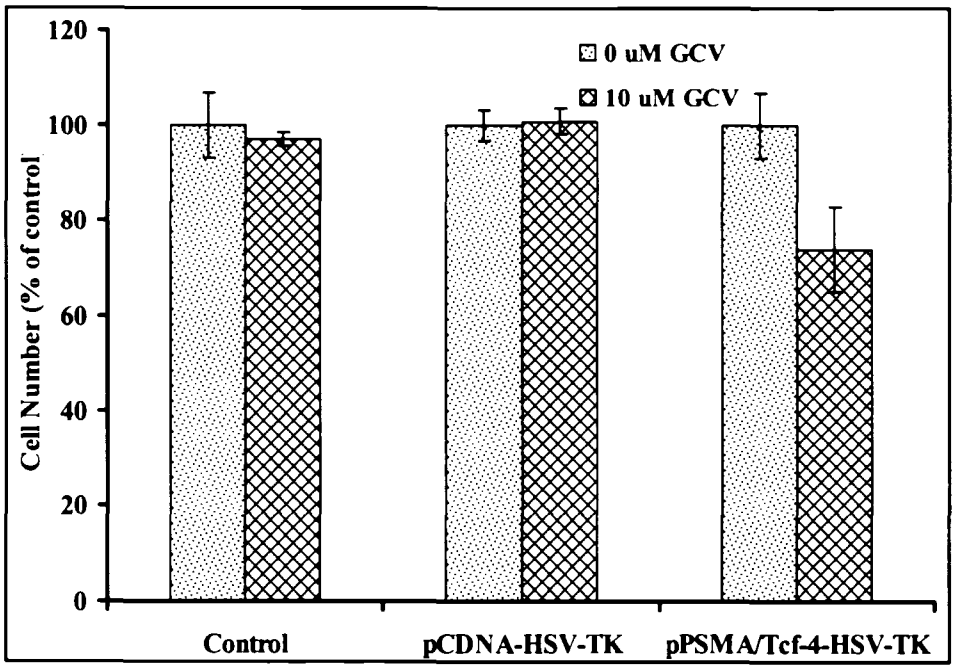


Figure 13. GCV sensitivity in pPSMA/Tcf-4-HSV-TK transfected Tramp cells. Tramp cells were transiently transfected with either pPSMA/Tcf-4-HSV-TK or pCDNA-HSV-TK (control) and treated with GCV 24 hours after transfection. Numbers of live cells were assessed 72 hours later. Cell numbers, determined as percentage of control, are shown.

therapeutic gene specifically in tumor cells. In transient transfection experiments, in combination with GCV the recombinant promoter system shows the ability to selectively kill tumor cells. However, therapeutic gene facilitated tumor cell killing is severely compromised by the delivery of the therapeutic gene expression vector inside the tumor cells. In other words, effective tumor cell killing cannot be achieved unless the therapeutic vector is not successfully delivered inside the target tumor cell. Experiments presented in aim No. 2 have used lipid based transient transfection methods to deliver the therapeutic vector inside the target cells. Such delivery methods are effective in demonstrating the proof of principle under *in vitro* conditions. However, such transfection methods do not successfully deliver the DNA vector, in most cases, to majority of target cells. Given that one of the primary objectives in cancer gene therapy is to target as many as tumor cells, we next looked to optimize the delivery of the therapeutic vector to the tumor cells.

Aim #3. Optimization of delivery of the therapeutic gene/promoter-enhancer combination to the tumor

To improve the delivery of the therapeutic gene/promoter-enhancer combination to tumor tissue/cells, recombinant adenoviruses were constructed. As mentioned in the introduction chapter, there are several advantages of using adenoviruses for delivering the therapeutic gene expression constructs. These advantages include: the ability to produce high titer (10^{12}-10^{13} virus particles per ml) of recombinant viruses; postmitotic cells can be effectively infected including a wide variety of cell types and the ability to accommodate up to -8 kb of foreign DNA, including expression cassettes or other

regulatory sequences. Further, the current generation of engineered adenoviruses are considered relatively safe for the purpose of gene therapy as they do not integrate into the genome and are rendered replication incompetent by deletion of certain essential viral genes.

Using a pAdeasy recombinant adenoviruses construction kit (Qbiogene) the following adenoviruses incorporating the specified DNA elements were generated as described in materials and methods section.

Ad-c-fos/Tcf-4-HSV-TK

The c-fos/Tcf-4 promoter-enhancer combination and the HSV-TK gene, was excised out of the pc-fos/Tcf-4-HSV-TK plasmid vector. The excised DNA fragment was incorporated into a promoterless pShuttle vector which was subsequently used for generating recombinant Ad-c-fos/Tcf-4-HSV-TK adenoviral particles. The hall mark of Ad-c-fos/Tcf-4-HSV-TK vector is that it was designed to express HSV-TK gene from a recombinant c-fos/Tcf-4 promoter.

Ad-CMV-HSV-TK

The HSV-TK gene was incorporated into a pShuttle-CMV vector which was subsequently used to generate Ad-CMV-HSV-TK viral particles. Ad-CMV-HSV-TK was used as positive control adenoviral vector because the presence of a CMV promoter allowed it to express HSV-TK ubiquitously in tested cell lines.

The adenoviral particles were amplified and quantitated as described in materials and methods section. To ensure that the generated adenoviral particles were expressing HSV-TK, colon cancer cells (SW480, SW620 and NCM460) were infected with Ad-c-fos/Tcf-4-HSV-TK and Ad-CMV-HSV-TK and 48 hours later whole cell lysates were

made from infected and uninfected cells. Prepared lysates were subjected to SDS-PAGE and Western blot analysis. Blots were probed for HSV-TK with a HSV-TK antibody. Figure 14 shows the result from Western blot analysis done on whole cell lysates from HSV-TK expressing adenoviral vectors. As shown in figure 14, Ad-CMV-HSV-TK expressed HSV-TK in all the chosen cell lines including the normal NCM460 colon cells. In contrast, Ad-c-fos/Tcf-4-HSV-TK mediated HSV-TK expression was observed only in SW480 and SW620 colon cancer cell lines, and no detectable expression of HSV-TK was seen in NCM460 cells. It can be concluded from the results that Ad-CMV-HSV-TK has the ability to express HSV-TK in both normal and cancer cell lines. This is because of the presence of strong CMV promoter which is expected to be active in any given eukaryotic cell environment (290). On the other hand, Ad-c-foc/Tcf-4-HSV-TK vector expresses the therapeutic gene only in cancer cell lines and not in the NCM60 normal cells.

Having confirmed that the HSV-TK expressing adenoviral vectors can successfully express the therapeutic gene in chosen cells, the adenoviral vector infected cells were tested for their GCV sensitivity. Such sensitivity experiments are crucial for insights into the functional aspects of the adenovirally delivered and expressed HSV-TK gene. Effective tumor cell killing is entirely dependent upon the ability of the expressed HSV-TK gene to convert the prodrug GCV into toxic metabolites. For the GCV sensitivity assay, SW480, SW620 and NCM cells were infected with Ad-CMV-HSV-TK and Ad-c-foc/Tcf-4-HSV-TK adenoviral particles and 24 hours later 0.1 μm and 10.0 μm GCV was added to the infected cells. Adenovirally infected cells without the addition of GCV were assessed as controls. The cells were allowed to grow for the next 48 hours. Viable cell numbers were determined using trypan blue dye exclusion and automated cell

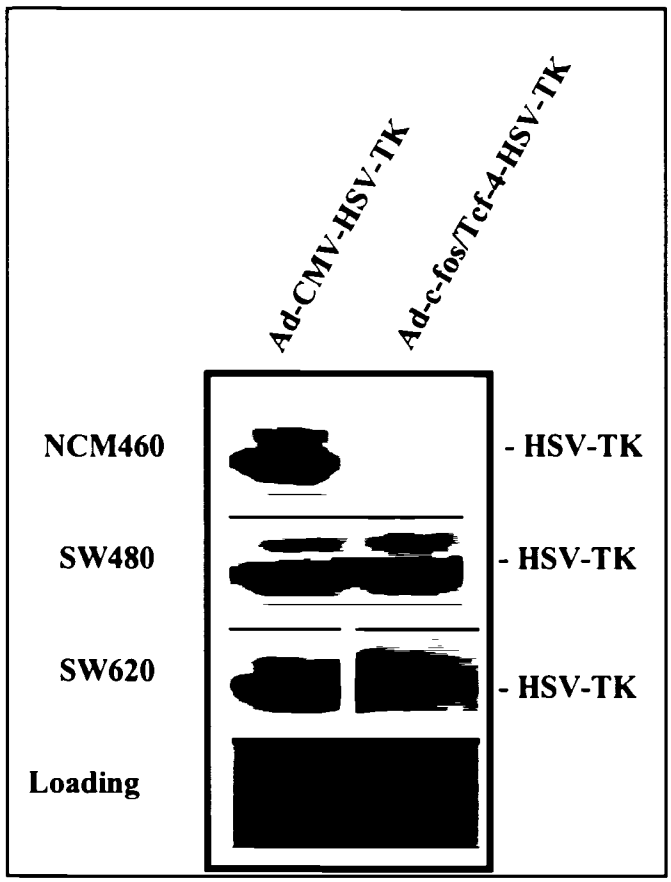


Figure 14. Recombinant adenoviral vector infections: HSV-TK Western Blot. Normal (NCM460) and tumor colon cells (SW480 and SW620) were infected with either Ad-CMV-HSV-TK or Ad-c-fos/Tcf-4-HSV-TK. Each cell line was infected with same MOI for both the recombinant adenoviral vectors. Whole cell lysates were prepared 48-72 hours later and were subjected to SDS-PAGE/Western blot analysis using an anti HSV-TK antibody. Bands Corresponding to HSV-TK are labeled.

counting. All of the Ad-CMV-HSV-TK infected cell lines showed a significant GCV dose dependent cellular toxicity. However, of the cell lines infected with Ad-c-fos/Tcf-4-HSV-TK, only SW620 and SW480 colon cancer cells showed significant GCV dose dependent cellular toxicity, whereas normal NCM460 cells showed a minimal GCV sensitivity (figure 15). Based on the results presented in figure 15 it can be concluded that the adenoviral vectors, Ad-CMV-HSV-TK and Ad-c-fos/Tcf-4-HSV-TK are capable of delivering and expressing a functional HSV-TK in specific cancer cell lines and exihibit significant cell death upon addition of GCV. In addition, Ad-c-foc/Tcf-4-HSV-TK infection does not result in extensive GCV mediated cellular toxicity in the normal NCM460 cells.

So far in this study, it has been shown that Tcf-4 enhancer element can be successfully combined with a minimal oncogenic promoter to generate a tumor specific gene expression promoter. In aim 1 we showed by reporter gene assays that's these promoters can direct tumor specific gene expression. Furthermore, we demonstrated the ability of the designed recombinant promoter to specifically express a therapeutic gene, under in vitro conditions, in tumor cells. In addition studies in specific aim 2 show that the HSV-TK therapeutic gene expressed in the tumor cells was functional and capable of directing tumor cell killing. Finally, in specific aim 3 we constructed recombinant adenoviruses engineered to deliver the therapeutic gene expression cassette to tumor cells. The data presented prove that the adenoviral viral vectors successfully express the chosen therapeutic gene (HSV-TK) and cause significant tumor cell death upon addition of the prodrug (GCV). Together, these studies demonstrate that we can generate an adenoviral vector capable of directing tumor cell specific killing.

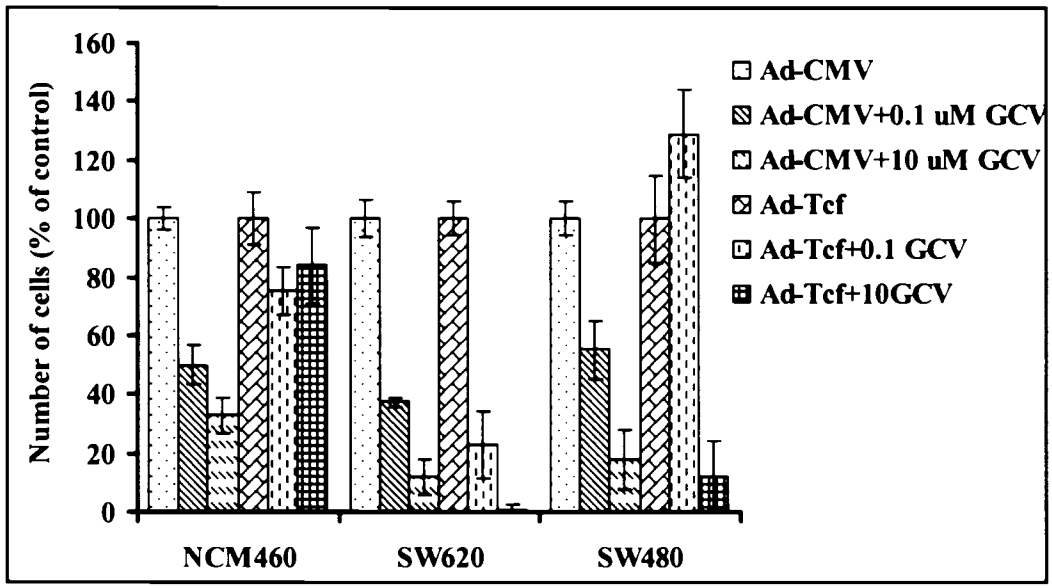


Figure 15. GCV sensitivity in infected with HSV-TK expressing adenoviral vectors. Normal (NCM460) and tumor colon cells (SW480 and SW620) were infected with either Ad-CMV-HSV-TK or Ad-c-fos/Tcf-4-HSV-TK. Each cell line was infected with same MOI for both the recombinant adenoviral vectors. 24 hours after infection cells were treated with GCV and 72 hours later viable cell number was determined. Numbers of live cells are shown as percentage of control. SW620 and SW480 are colon cancer cell lines where as NCM460 is a normal colon cell line.

Aim #4. Identification of strategies for modulating Tcf-4/β-catenin responsive promoters

Recombinant adenoviruses would help in improved targeting of the tumor cells because most of the tumor cells have up-regulated CAR receptors. To continue identifying novel strategies which would allow better targeting and control of therapeutic gene expression, the idea of regulating the activity of the recombinant Tcf-4 enhancer/promoter combination was also explored. The significance of this approach lies in the fact that regulating the promoter activity would allow control over the amount of therapeutic gene expressed inside the target cell which might be necessary under clinical situations. For instance, it might be required to turn off the expression of the therapeutic if accidentally expressed ectopically. On the other hand, a boost in expression of the therapeutic gene might provide for increased tumor cell death. In an effort to identify genetic and chemical modulators for regulating the activity of recombinant Tcf-4 enhancer/promoters, several candidate compounds were screened. Short chain fatty acids, like butyrate, have been previously shown to influence the normal homeostasis of colon epithelial cells (224). In addition, butyrate has also been shown to influence the wnt/β-catenin signaling pathway (223). In order to test the ability of butyrate to modulate the activity of our recombinant promoters, reporter luciferase assays in the presence or absence of sodium butyrate were performed. Either pLuc-fos/Tcf or pLuc-fos/fct (Tcf enhancer sites are scrambled, a negative control reporter vector) was co-transfected with pRL-TK (internal control) in selected cell lines. 24 hours post-transfection 1.4 mM sodium butyrate was added to the cells. 48 hours post transfection cell lysates were prepared and relative luciferase ratio (RLU) readings were recorded as previously

described in the text. The data generated from this experiment is presented in figure 16. The relative luciferase ratios as shown in figure 16 did not presented a clear picture. Sodium butyrate treatment, in general, did not significantly appear to affect the relative luciferase units (RLU-firefly luciferase reporter gene activity divided by the internal control renilla luciferase activity) in cases when cell lines were transfected with the negative control plasmid, pLuc-fos/fct. Butyrate seemed to have a variable effect on expression of the luciferase gene in cell lines transfected with pLuc-fos/Tcf. For example, in pLuc-fos/Tcf transfected HCT8 cells, butyrate significantly increases luciferase expression, as assessed from increased relative light units. In contrast, under similar conditions, Lovo colon cancer cell line showed only a marginal increase in luciferase expression. Further, the presence of butyrate in pLuc-fos/Tcf transfected Colo320, SW480 and SW620, decreased luciferase expression. However, it should be noted that, by nature, butyrate is also known as a histone deacetylase inhibitor and it's generally known to increase gene expression by opening up the chromatin structure (291). Therefore relative luciferase units shown in the figure 16 should be interpreted in the light of the fact butyrate is also expected to increase transcription of the Renilla luciferase gene (pRL-TK, internal control) besides the test firefly luciferase reporter (pLuc-fos/Tcf). Indeed a look at the firefly luciferase numbers alone revealed that butyrate did enhanced the expression of luciferase from pLuc-fos/Tcf in colon cancer cell lines (Lovo, Colo320, HCT8, SW480 and SW620) (figure 17). Importantly, butyrate did not seem to affect the luciferase gene expression from pLuc-fos/Tcf or pLuc-fos/fct when transfected in NCM460 (normal cells). These results are very preliminary but suggest that specific compounds may be identified that can affect Tcf-4/β-catenin mediated transactivation of

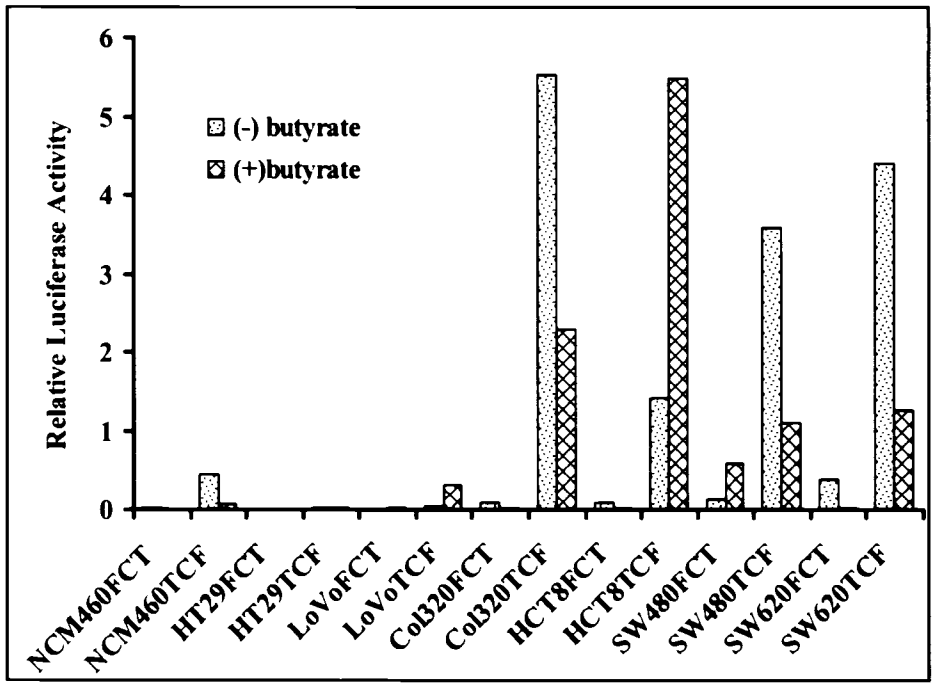


Figure 16. Effect of 1.4 mM Butyrate (RLU Firefly:Renilla) on c-fos promoter activity with and with out Tcf-4 enhancer element. Dual Luciferase assays were performed to asses the effect of butyrate on recombinat c-fos/Tcf-4 promoter activity. Selected cell lines were co-transfected with pRL-TK and with either pLuc-fos/Tcf or pLuc-fos/fct. 24 hours later cell were treated with 1.4 mM sodium butyrate and 48 hours later, cells were processed for dual luciferase activities. Shown are c-fos/Tcf-4 and c-fos/fct promoter activities, in different cell lines, in terms of relative luciferase units.

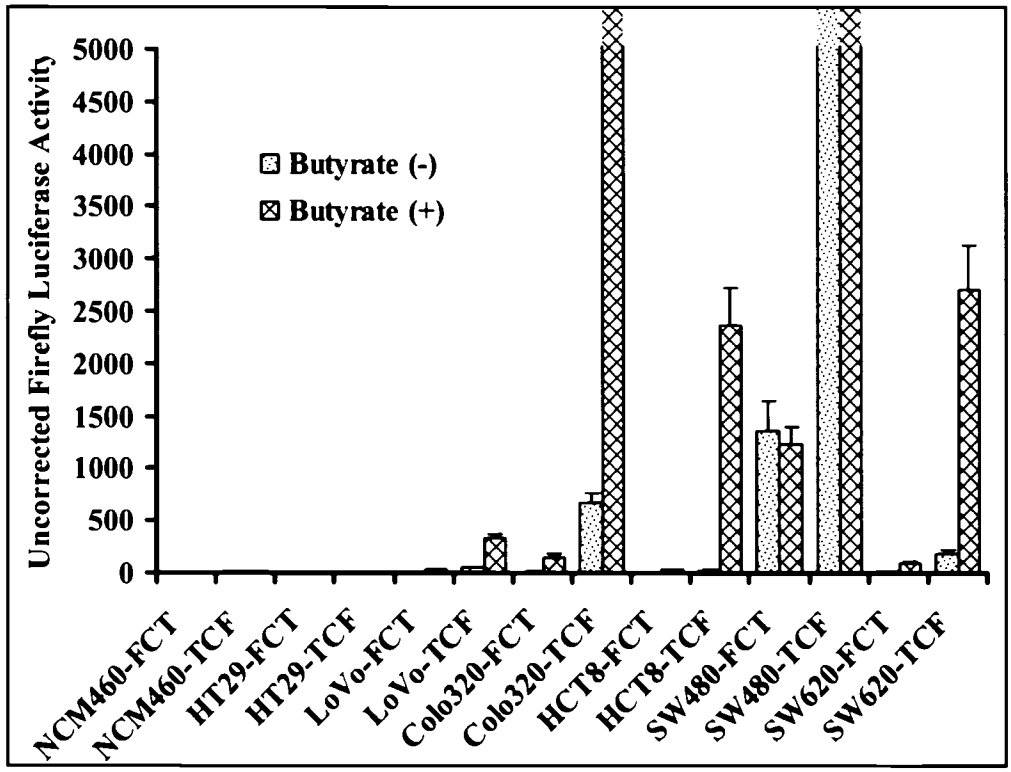


Figure 17. Effect of 1.4 mM Butyrate on c-fos promoter activity with and with out Tcf-4 enhancer element. The data is derived from experiment performed in figure 16. Effect of butyrate on the activity of c-fos/Tcf-4 and c-fos/fct promoter, in selected cell lines, is shown as uncorrected Firefly Luc values in absence and presence of butyrate.

gene expression. From the data it can be concluded that under the conditions tested butyrate increases the levels of luciferase gene expression and this effect may not be specific to modulating the transcriptional activity of the recombinant TCf-4 enhancer/promoter combination. In addition to butyrate, other chemicals which were previously shown to be associated with colon cancer prevention/treatment such as non-steroidal anti-inflammatory drugs; Indomethacin and cycloxygenase-2 inhibitors; Celecoxib (292), were also tested. Colo320 and SW620 cells were transiently transfected with pc-fos/Tcf-4-HSV-TK and 24 hours later treated with appropriate concentrations of sodium butyrate, Indomethacin and Celecoxib (figure 18, lane 2-4). Whole cell lysates were prepared 48 hours post transfection and were subjected to SDS-PAGE and western blot analysis. The blot was probed with antibodies raised against HSV-TK. Butyrate caused significant increase in HSV-TK expression levels as compared to untreated control in Colo320 cells and did not seem to have any effect in SW620 cells (figure 18 lane 2). In contrast, treatment with Indomethacin and Celecoxib appeared to result in lowering of HSV-TK gene expression (figure 18, lanes 3, 4). The results shown in figure 17 are not very conclusive; however they do suggest that transcriptional activity of the recombinant Tcf-4enhancer/promoter combination can be modulated by chemical means. Additional experiments, including screening of more candidate compounds, are needed to confirm these findings.

The presence of a constitutively active β-catenin inside a cell is one of the primary requirements for the transcriptional activation of the proposed recombinant Tcf-enhancer/promoter elements. Therefore it can reasonably argued that increasing the expression of the constitutively active β-catenin may result in enhancement of the

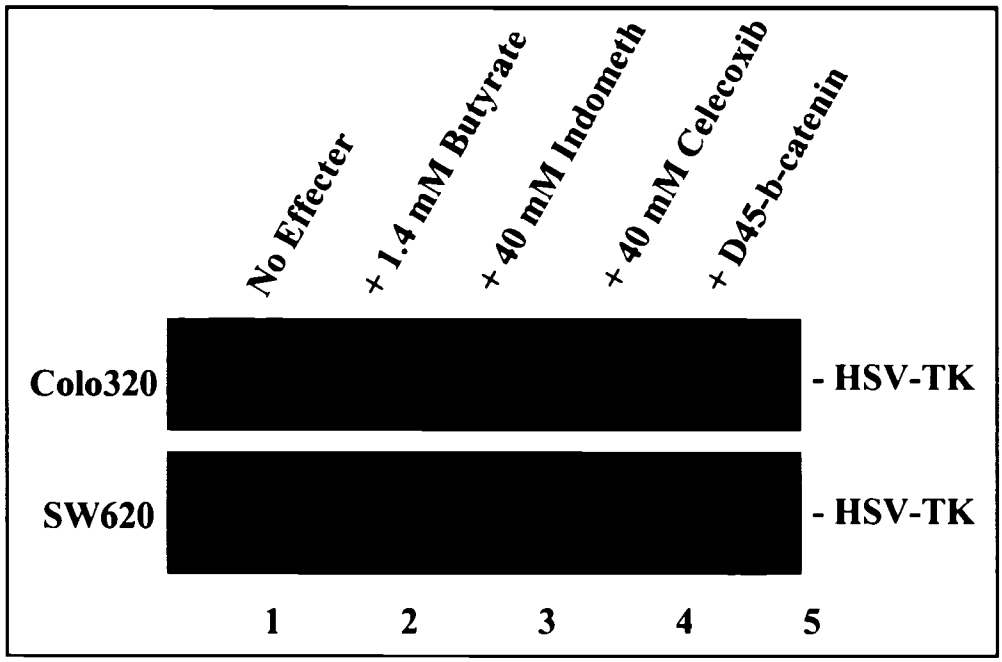


Figure 18. Effect of various modulating agents on expression of HSV-TK driven by c-fos/Tcf-4 promoter. HSV-TK Western blot. Colon cancer cells (colo320, SW480) were transiently transfected with pc-fos/Tcf-4-HSV-TK (lanes 1-4) and treated with butyrate (lane 2), indomethacin (lane 3) and celecoxib (lane 4). To asses the effect of overexpression of constitutively active β-catenin on c-fos/Tcf-4 promoter driven HSV-TK expression, pc-fos/Tcf-4-HSV-TK was co-transfected with a plasmid expressing for mutated form of β-catenin (lane 5). Whole cell lysates were prepared 48 hours post transfection and were subjected to SDS-PAGE/Western blot analysis. Bands corresponding to HSV-TK are shown.

recombinant Tcf-enhancer/promoter element driven therapeutic gene expression. This idea was explored by co-transfecting Colo320 and SW620 cells with pc-fos/Tcf-4-HSV-TK and with a plasmid encoding for a constitutively active β-catenin (Δ-45 β-catenin). Whole cell lysates were prepared 48 hours post transcription and subjected to Western blot analysis using an antibody against HSV-TK. Indeed, Δ-45 β-catenin co-transfection resulted in increased gene expression of HSV-TK in SW620 and Colo320 (figure 17, lane 5). However, this enhancement of c-fos/Tcf-4 driven HSV-TK expression mediated by Δ-45 β-catenin may be a general response of Δ-45 β-catenin protein as a known transcription factor and may not be specific for c-fos/tcf-4 promoter. Additional experiments are required to explore the nature of increase of gene expression caused by Δ-45 β-catenin. However these data indicate that the activity of the recombinant tcf-4 enhancer/promoter elements can be modulated by chemical and genetic means.

CHAPTER V

DISCUSSION AND CONCLUSION

One of the most challenging aspects in cancer therapy is how to exclusively target cancer cells and spare the normal cells? Conventional therapies, owing to their lack of specificity in targeting tumor cells, frequently cause serious side effects. Because of this narrow therapeutic window, most metastatic and local malignancies become refractory to treatment. In contrast, gene therapy for cancer has the ability to distinguish between normal and tumor cells. This very aspect of gene therapy gives it an edge over conventional cancer therapies as it has the ability to minimize normal cell toxicity by selectively attacking the tumor cells. However, this aspect of gene therapy is very challenging and needs innovative strategies for specific targeting to the site of the tumor. One of the ways this can be achieved is by limiting the expression of the therapeutic gene to the tumor cells and sparing the normal cells from the toxic effect of the therapeutic gene. This strategic approach of limiting the expression of the therapeutic gene to the tumor cells is known as transcriptional targeting and has been described in detail in the Introduction. In general, successful expression of any gene is dependent upon activation of specialized DNA sequences known as promoters. Promoters provide the binding sites for transcription factor/proteins which are required for the successful initiation and completion of gene transcription. Activation of a promoter is a function of the availability of the transcription factor/proteins, inside the target cell, which bind to specific sites in the promoter DNA to form a transcription activation complex. In continuation, a universally active promoter implies that the promoter DNA sequence is active in a variety

of cells owing to its ability to bind constituitively expressed transcription factor/proteins that form a transcription initiation complex.

Typically in cancer gene therapy, a toxin gene is coupled with a promoter incorporated in a gene expression cassette which is delivered to the target tumor cells. Therefore, activity of the promoter in the target tissue will govern the expression of the therapeutic gene. A universally active promoter is not a worthy candidate for such therapy protocols because its activity in normal cells would result in expression of the toxin therapeutic gene leading to widespread normal cell toxicities. Hence, an ideal situation would be to have a gene expression promoter which is exclusively activated in tumor cells. These promoter DNA elements are known as tumor specific gene expression promoters. As explained in the Introduction, minimal promoters of various oncogenes, such as Cyclooxygenase-2, c-myc, c-fos, cyclinD etc, when combined with an enhancer DNA sequence are ideal candidates for being used in designing of tumor specific gene expression promoters. Enhancer elements are naturally found to be part of several genes differentially regulated in various types of cancers. As mentioned earlier in this text, aberrant activation of growth promoting signal transduction pathways results in differential activation of genes in cancer. The promoters of these differentially transactivated genes usually have specific DNA elements (for example enhancer elements) binding to an array of proteins in response to a signal transduced by the activated signaling pathway. Such enhancer elements can be innovatively used in cancer gene therapy to control the expression of therapeutic genes.

Aberrant activation of the Wnt-signaling pathway has been associated with an increasing number of neoplasms. Deregulation of this pathway disrupts normal proteosome mediated degradation of β-catenin, resulting in a nuclear localization. β-catenin shows nuclear localization in a majority of colorectal carcinomas and has been associated with a significant proportion of prostate cancers, melanomas, ovarian cancers, glioblastomas, and breast cancers (281-285, 293). Nuclear β-catenin pairs with members of the Lef/Tcf family of proteins and trans-activate several cancer promoting genes (19, 20, 22, 24). This occurs by binding of the constitutively active β-catenin/Tcf-4 complex to the Tcf-4 enhancer regions present in the promoter regions of these target genes. In other words the presence of a Tcf-4 enhancer contributes to the expression of these cancer promoting genes by its ability to bind to the transcriptionally active β-catenin/Tcf-4 complex resulting in transcriptional activation of their promoters. Given the fact that the canonical Wnt signaling pathway is constitutively activated in a large number of adenocarcinomas and not in normal cells, the Tcf-4 enhancer element could be a very valuable tool for design of tumor specific gene expression promoters.

Specific Aim 1

In this Specific Aim, the effectiveness of the Tcf-4 transcriptional enhancer element was evaluated for its ability to act as a tumor specific regulator of therapeutic gene expression in a panel of human tumor and normal colon cell lines. These experiments were geared towards establishing the proof of principle that the Tcf-4/β-catenin enhancer could be used for regulation of gene transcription in tumor cells. To achieve the objectives of this aim, extensive cell line screenings were done using dual

luciferase assays. The Tcf-4 enhancer element was cloned with a panel of chosen minimal promoters from various oncogenes; c-fos, CEA, Cox-2, PSMA and PSA. CEA (280), Cox-2 (252), PSMA (294) PSA (271, 295) promoters have been previously used for constructing tumor specific gene expression promoters. On one hand, these candidate gene therapy promoters have shown vast potential but, in general, they are very weak activators of gene transcription. This is especially true for PSMA, PSA and PSMA promoters, and in previous studies this issue has been addressed by coupling these promoters with specific enhancer elements to increase the transcriptional activity of the native promoters (271, 280, 294, 295).

c-fos promoter

A minimal c-fos promoter has never been used as such, for expressing a therapeutic gene for the purpose of gene therapy. Given the fact that promoters of several members of the AP-1 family of proteins are targets of various signaling pathways found to be activated in variety of cancers(296), c-fos could be a very strong candidate for use in cancer gene therapy. Our results from luciferase assays show that coupling of a Tcf-4 enhancer element with a minimal c-fos promoter results in a significant increase in the transcriptional activity as compared to the c-fos promoter alone (Table 1). As expected, owing to the activation of the Wnt signaling pathway in the chosen colon cancer cell lines (HCT8, SW480, SW620, and Colo320), a c-fos/Tcf recombinant promoter showed a higher activity in terms of the measured luciferase levels. At the same time, no significant levels of c-fos/Tcf transcriptional activity was observed in the normal NCM460 colon cells. This was because NCM460 cells are derived from normal colon epithelia and do not have transcriptionally active β-catenin present in the nucleus required to transactivate

the Tcf-4 enhancer elements (discussed later). In the next step, we compared the activities of c-fos/Tcf and c-fos/Fct (Tcf-4 enhancer with mutated Tcf-4 binding sites) in luciferase assays (figure 1). As expected, we observed a dramatic increase in the transcriptional activity of c-fos/Tcf over c-fos/Fct, demonstrating that a functional Tcf-4 enhancer sequence is essential for the recombinant promoter activity in a tumor cell environment having a constitutively active β-catenin/Tcf-4 complex. In addition, in normal cells (NCM460, NCM425) presence of Tcf-4 enhancer element did not result in any significant increase in promoter activities. Therefore it can be concluded that in luciferase assays, coupling of the Tcf-4 enhancer increases the transcriptional activity of a minimal c-fos promoter.

CEA promoter

Encouraged by these results, we next tested the possibility of using the CEA promoter in combination with the Tcf-4 enhancer element. The CEA promoter is found typically activated typically in gastrointestinal cancers and several previous studies have shown the potential of the CEA promoter for tumor specific therapeutic gene expression. However, CEA promoter is also a weak activator of gene transcription which could be an issue in cases where a high level of therapeutic gene expression is required. We tested the hypothesis that the activity of a CEA promoter could be improved by coupling it with a Tcf-4 enhancer element. We tested CEA/Tcf recombinant promoter activity in a panel of cell lines (table2) and noticed that it is active only in SW620 colon cancer cells. CEA/Tcf promoter activity was also assessed in breast cancer cells (MDA435 and MCF7) and no appreciable increase of promoter activity was observed as compared to the CEA promoter activity alone. At the same time, one of our collaborators (Dr. Kathy Molnar-Kimber) at

the University of Pennsylvania tested the activity of the CEA/Tcf recombinant promoter in Lovo and Colo320 colon cancer cells. A dramatic increase in CEA promoter activity was seen in the presence of the Tcf-4 enhancer element in Colo320 cells and a modest increase was noticed in Lovo cells. Therefore coupling of a Tcf-4 enhancer element with a CEA promoter may be advantageous under some situations. Although the cells in which CEA/Tcf recombinant promoter did not show significant activity have been shown to have deregulation of Wnt signaling pathway, it could be possible that these cells have very low CEA activity and coupling of Tcf-4 enhancer is not enough for high levels transcriptional activation. Results presented in table 2 also show that, except in MCF7 breast cancer cells, the c-fos/Tcf combination is significantly active in the test cancer cells lines, again emphasizing the point that the minimal promoter coupled with Tcf-4 enhancer element is also an important factor contributing to the overall activity of the recombinant promoter.

NCM460 as negative control cell line

The normal colon cells, NCM460, have been extensively used in this study. The NCM460 cell line is a very useful tool for evaluating the activities of the candidate promoter/enhancer combination in a normal cellular environment. This becomes very important because the goal of the study was to design novel promoter/enhancer combinations, active in tumor cells and not in normal cells, which could be used for driving therapeutic gene expression. The c-fos/Tcf recombinant promoter showed a 10-fold increase in activity in NCM460 cells (table 2) which was an unexpected result because being normal cells, NCM 460 do not have a constitutively active Wnt signaling pathway (272). However, it is worth mentioning that these results are derived from a dual

luciferase assay which is a screening tool and not an assay which would directly measure the levels of therapeutic gene expression in terms of cell killing or viability. In other words, a more functional assay would be a better way to evaluate the activity of the designed recombinant promoter/enhancer combinations. Another important parameter was to assess how transfectaable NCM460 cells were, as it could be argued that if these cells were not efficiently transfectable, then this would result in lower activities of the candidate recombinant promoters. We addressed this issue by transfecting NCM460 cells with a reporter construct in which the luciferase gene was driven by a constitutively active MMLV LTR promoter (table 2). Significant levels of luciferase gene expression was achieved from the LTR promoter indicating successful reporter plasmid DNA uptake, and also that these cells are not transcriptionally inactive.

Additionally, we showed by immunostaining for β-catenin that there is no nuclear β-catenin present in NCM460 cells and it was found to be present on inner side of cell membranes, indicating that wnt signaling pathway is not deregulated in these cells (figure 5). Further, we co-transfected NCM460 cells with pLuc-fos/Tcf (luciferase gene expressed by c-fos/Tcf recombinant promoter) and a plasmid DNA encoding constitutively active beta-catenin. As expected, a dramatic increase in the activity of c-fos/Tcf promoter was observed suggesting that NCM460 cells do not have constitutively active β-catenin which is available for the transactivation of recombinant promoters consisting of Tcf-4 enhancer elements. With all the given data it can be concluded that NCM460 cells are good negative controls for the experiments performed in Specific Aim1.

Cox-2, PSMA and PSA promoter

Cox-2 is found to be up-regulated in a variety of epithelial tumors and has been shown to be a target of the wnt signaling pathway (26, 247). In addition cox-2 promoter activity has been shown to be inactive in liver which can be very helpful in minimizing the liver toxicity in patients being treated with suicide gene therapy (252). Previous studies have indicated the potential of using the Cox-2 promoter as a tumor specific gene expression promoter (248). We hypothesized that coupling of the Tcf-4 enhancer element with a Cox-2 promoter would achieve higher transcriptional activities. Indeed, in dual luciferase assays using plasmid constructs in which the luciferase gene was driven by a Cox-2 promoter or Cox-2/Tcf promoter enhancer combination, we found that the Tcf-4 enhancer element significantly increased the activity of the Cox-2 promoter in the colon cancer cell lines HCT8 and SW620, where as it gives a modest boost to the Cox-2 promoter activity in the HCT116 colon cancer cell line (figure 3). This was expected given the fact that Cox-2 in known to be upregulated in gastrointestinal cancers (287), and moreover the tested cell lines are also known to have an activated wnt signaling pathway (286).

In our continuing efforts to identify more candidate promoter/Tcf-4 enhancer combinations, we also tested the activity of PSA and PSMA promoters in the presence and absence of a Tcf-4 enhancer element by performing dual luciferase assays. No significant PSA or PSMA activity, either in the presence or absence of Tcf-4 enhancer, was observed in the tested colon cancer cells (HCT116, HCT8, and SW620). However, coupling of the Tcf-4 enhancer did significantly increase the PSMA promoter activity in prostate cancer cells DU145 and PC3 (figure 4).

Conclusion: Specific Aim 1

Based on the results from experiments performed in specific aim 1 it can be concluded that coupling of a Tcf-4 enhancer element to a minimal promoter of an oncogene is a viable strategy for designing of tumor specific gene expression promoters. In this promoter/enhancer combination the role of the promoter partner is very significant; as evidenced by the following observations:

- Lovo and Colo320 colon cancer cells showed a high CEA/Tcf activity, in contrast they show comparably less Cox-2/Tcf-4 activity
- SW620, HCT116 and HCT8 colon cancer cells had a low CEA/Tcf recombinant promoter activity but high Cox-2/Tcf and c-fos/Tcf recombinant promoter activity.
- Prostate cancer cell lines DU145 and PC3 registered a high PSMA/Tcf recombinant promoter activity but low PSA/Tcf recombinant promoter activity.

Therefore the emerging theme is, one particular promoter/Tcf-4 recombinant promoter combination may be active in one specific target cell and may not show the same activity in a different cell line.

Specific Aim 2

In Aim 1 we established that Tcf-4 enhancer elements can be successfully combined with minimal promoters of various oncogenes for the purpose of engineering of a tumor specific gene expression promoter. In the next step, we evaluated the ability of the chosen c-fos/Tcf enhancer promoter combination to express the therapeutic gene HSV-TK specifically in targeted tumor cells. By transient transfection experiments we

showed successful expression of c-fos/Tcf driven HSV-TK expression in selected colon cancer cells (SW480, HCT8 and SW620) and breast cancer cells (MDA435 and MCF7) (figures 6&7). At the same time no c-fos/Tcf driven HSV-TK expression was detected in the normal NCM460 cells. These results are in perfect agreement with the results luciferase assays performed in aim 1 using c-fos/Tcf recombinant promoter. However, it is noteworthy here that although, in luciferase assays, we did noticed a 10 fold increase in promoter activity upon coupling of Tcf-4 enhancer element with c-fos promoter, we did not see any detectable levels of the therapeutic gene expression as measured by western blots. Therefore indicating that activity of the c-fos/Tcf recombinant promoter in normal cells is not significantly enough for the expression of HSV-TK. Further by metabolic labeling assays we demonstrated that the HSV-TK gene product expressed from c-fos/Tcf recombinant promoter is functional and is capable of phosphorylating the prodrug GCV into GCV phosphate (figures 8 and 9). The high level of HSV-TK metabolically labeled GCV detected in SW480 cells conform to the high level of HSV-TK expression seen by western blotting. However, HCT8 cells did seem to have comparable GCV metabolites, although they show levels of HSV-TK expression. This anomaly could be due to the difference in metabolism of GCV by HCT8 cells. Several cancer cells are know to develop drug resistance via effluxing the drug with the help of specialized pumps present in their cell membranes. This effectively reduces the bio-availability of the drug in the target tissue. In our GCV sensitive cell viability assays we showed that transient transfection of colon cancer (SW480) and breast cancer cells (MDA 435) followed by GCV treatment results in significant cell death. Whereas, in identical assays no significant GCV mediated cell killing was observed in the normal NCM460 cells. These

results are as expected, given that c-fos/Tcf selectively expresses the HSV-TK gene in tumor cells (here SW480 and MDA435) and not in normal cells (NCM 460), it is likely that tumor cells will have a significantly higher GCV toxicity as compared to the normal cells (figure 11).

The ability of PSMA/Tcf recombinant promoter combination was also tested to sensitize prostate cancer cells (DU145 and PC3) to GCV. In transient transfection assays followed by GCV treatment, using pPSMA/Tcf-4-HSV-TKconstruct, PC3 cells showed were found to be highly sensitive to GCV (figure 12). This was highly expected because in our luciferase assays we had already shown that PSMA/Tcf recombinant promoter is very active in PC3 cells. Although, in luciferase assays, we had seen a high PSMA/Tcf recombinant promoter activity in DU145 cells, we did not notice significant cell death at 10um GCV concentrations. Again this could be an issue of reduced bioavailability of GCV in DU145 cells owing to the activation of drug resistance mechanisms commonly found in cells (figure 12). Therefore, we repeated the GCV sensitivity assay in DU145 cells, using pPSMA/Tcf-4-HSV-TKconstruct, and increased the GCV treatment levels to 25um. A dramatic increase in DU145 cell killing was observed, indicating increased level of GCV sensitivity. This increase was highly specific to the expression of HSV-TK in DU145 cells because mock or pCDNA-HSV-TK (promoter less) transfected cells were not found to be sensitive to 25um GCV. In addition, transfection of pPSMA/Tcf-4-HSV-TKconstruct did not cause any significant level of GCV toxicity in the normal NCM460 cells indicating non-expression HSV-TK in these cells due to weak PSMA/Tcf recombinant promoter activity.

Conclusion: Specific Aim 2

From the data provided it can be concluded that c-fos/Tcf and PSMA/Tcf recombinant promoters can successfully express a functional copy of the chosen therapeutic gene, HSV-TK, in tumors cells.

Specific Aim 3

The ultimate aim in this step of the study was to engineer a delivery system in which the gene expression cassette could be packed and delivered to the target tumor cells. Adenoviral vectors are widely used for in vitro and in vivo gene transfer in several animal models and in clinical trials as well. They are know to efficiently transduce a wide range of cell types and have shown enormous potential for being used in cancer gene therapy for delivery of the therapeutic gene to the tumor cells (297). However, there are several issues related to the Biosafety of a adenoviruses for example adenoviruses are known to generate an acute inflammatory immune response which can cause widespread toxicities in the patient (298). However, newer generation adenoviral vectors have a better safety profile as they are engineered to carry less adenoviral proteins and unable to replicate inside the host (297). The main objective of this specific aim was to demonstrate that a therapeutic gene expression cassette, in which the therapeutic gene is under the control of a recombinant c-fos/Tcf promoter-enhancer combination, can be successfully delivered and expressed in a target cell. Ad-Fos/Tcf-TK was designed to express HSV-TK from a c-fos/Tcf recombinant promoter. Ad-CMV-TK was constructed as a control vector in which HSV-TK gene was under the control of a CMV promoter. The tested cell lines were infected with the recombinant adenoviruses and were assessed for HSV-TK

expression by Western blotting. Ad-c-fos/Tcf4-HSV-TK was found to successfully express HSV-TK in the tested colon cancer cells (SW620 and SW480). Owing to the high activity of C-fos/Tcf recombinant promoter and the ability of adenoviruses to infect tumor cells, this result was highly expected. However, no expression of HSV-TK was detected in normal (NCM460) cells. A logical explanation for this observation could be the inactivity of c-fos/Tcf recombinant promoter in these normal cells. However it can also be speculated that NCM460 cells cannot be infected by adenoviruses resulting in their failure to deliver the HSV-TK therapeutic gene construct to the normal cells. To address this issue NCM460 cells were infected with Ad-CMV-TK, followed by a western blot analysis for the HSV-TK gene expression. As expected, significant levels of HSV-TK protein were detected in NCM460 cells suggesting a successful delivery of the HSV-TK therapeutic gene by Ad-CMV-TK. Further, we established adenovirus mediated delivery of HSV-TK causes GCV sensitivity in target cells. Following infection with the recombinant adenoviruses, cells were treated with GCV and 48 hours later live cells were counted. Ad-c-fos/Tcf4-HSV-TK (figure 15)was found to cause significant cell death in colon cancer cell lines (SW480 and SW620) and was not observed to cause any significant GCV sensitivity in the normal cells (NCM460). These results are in accordance with the HSV-TK western blot shown in figure 6 in which, owing to the inactivity of the recombinant promoter, Ad-c-fos/Tcf4-HSV-TK was not found to be active in the normal cells. Therefore, it can be speculated that lack of Ad-c-fos/Tcf4-HSV-TK mediated HSV-TK expression in the normal NCM460 cells resulted in insignificant cell death. This speculation was further confirmed by the observation that, owing to the universally active CMV promoter, Ad-CMV-TK caused significant GCV

toxicity in the normal cells (NCM460) as well as the colon tumor cells (SW480 and SW620).

Conclusion Specific Aim 3

The following points can be concluded from the experimental evidence provided in specific aim 3.

- The recombinant therapeutic gene expression cassette can be successfully packaged inside an adenoviral delivery vector.
- Adenoviruses can successfully deliver and express the therapeutic gene expression cassette to the colon cancer cells (SW480 and SW620).
- It is probable that the Adenoviral vectors would be able to deliver and express the therapeutic gene expression cassette in other types of cancers especially the ones in which wnt signaling pathway is found to be aberrantly activated.

Specific Aim 4

Finally, having successfully delivered the therapeutic gene expression cassette to the tumor cells, the next step was to discern strategies by which the therapeutic gene expression could be regulated. This regulatory aspect of therapeutic gene expression could be very critical under certain clinical situations which may require increasing or decreasing the level of therapeutic expression. Chemical modulators like sodium butyrate have been previously shown to increase the expression of Tcf-4 responsive genes (223). Therefore the efficacy of sodium butyrate to increase the recombinant promoter activity was explored by conducting luciferase assays. Sodium butyrate was found to increase the activity of our recombinant Tcf-4/c-fos promoter combination as well the HSV-TK

promoter which drives the expression of the internal control 'Renilla Luciferase' gene. This is evident from the results presented in figure 16 Which show that after adding sodium butyrate the relative luciferase value actually goes down, this is because sodium butyrate (general histone deacetylase inhibitor) non specifically increases the rate of transcription of both the HSV-TK promoter (expresses the Renilla luciferase gene), as well as the transcriptional activity of the tested Tcf-4/c-fos promoter combination (expressing the luciferase gene). Although non-specifically, sodium butyrate did increase the activity of our tested Tcf-4/c-fos promoter combination (figure 17), therefore it may be useful for cancer gene therapy under situations where increased therapeutic gene expression is needed for enhancing tumor cell killing. Further, we screened NSAIDs (Non-steroidal anti-inflammatory drug-NSAID) like indomethacin and Celecoxib (Cox-2 inhibitor) for their ability to modulate the transcriptional activity of our recombinant enhancer/promoter system. Indomethacin has been suggested to have chemo preventive properties against colon cancer and is believe to act by upregulating tumor suppressive gene PTEN, affecting the cell survival and inhibition of apoptosis by negatively regulating the AKT/PI3K pathway (292). In addition indomethacin has been directly shown to down-regulate wnt signaling by stabilizing the phosphorylated forms of β-catenin (299). Similarly Cox-2 inhibitors have been increasingly used as a chemo preventive agent for colon cancer and have been shown to inhibit wnt signaling pathway by affecting the levels of nuclear β-catenin (287, 300). In our preliminary experiments we found marginal reduction in the activity of the c-foc/Tcf-4 recombinant promoter. This was evidenced by the reduced HSV-TK levels in western blots performed on SW620 and Colo320 colon cancer cell lines transfected pc-fos/Tcf-4-HSV-TK and treated with

Indomethacina and Celecoxib (figure 18). Given the fact that NSAIDs have been shown to inhibit wnt signaling pathway, these results are highly encouraging and are indicative that the activity of our recombinant enhancer/promoter system can be controlled by chemical means. Taken together, it can be stated that there is a good possibility of regulating the activity of our recombinant enhancer/promoter system, in clinical settings, by chemical means. However extensive screening and analysis of these chemical modulators is needed to further characterize their modulatory properties.

In our on going efforts to find mechanisms to regulate the transcriptional activity of our recombinant enhancer/promoter system, we hypothesized that over expressing a constitutively active form of β-catenin in the target cell should enhance the transcriptional activity of the recombinant enhancer/promoter. Indeed, our results show increased HSV-TK expression levels when a plasmid expressing for constitutively active β-catenin was co-transfected with pc-fos/Tcf-4-HSV-TK in SW620 and Colo320 colon cancer cell lines. The most logical explanation for this result is that β-catenin over expression increased the activity of the pc-fos/Tcf recombinant promoter which is driving the expression of the HSV-TK gene. This observation is very interesting and may have significant clinical implications. For example, an adenoviral delivery vector can be designed with a dual expression cassette containing the therapeutic gene and gene for constitutively active β-catenin under the control of our recombinant Tcf-4 enhancer/promoter system. This system would express higher amounts of the therapeutic gene because of the constitutively active β-catenin. Since the expression of β-catenin will also be controlled by the Tcf-4 enhancer/promoter, β-catenin levels will further stimulate the transcriptional activity of the recombinant promoter greatly increasing the levels of

therapeutic and β-catenin gene expression levels. This dual expression strategy is particularly more beneficial in cancers which have low levels of nuclear β-catenin to activate the recombinant Tcf-4 enhancer/promoter system.

Conclusion Specific Aim 4

- Sodium butyrate increases the activity of the c-fos/Tcf-4 recombinant promoter. Although this effect of sodium butyrate is non-specific, it can still be used as a positive regulator of the Tcf-4 enhancer/promoter system.
- NSAIDs, which are known to affect the wnt signaling pathway by stabilizing the levels of phosphorylated β-catenin, can be used as negative regulators of the Tcf-4 enhancer/promoter system.
- Constitutively active β-catenin gene expression enhances the level of HSV-TK gene expression, most likely by increasing the transcriptional activity of the pc-fos/Tcf recombinant promoter.

The Big Picture

Because mutations in the APC gene and β-catenin gene are frequently associated with progression of colon carcinoma and most other types of epithelial carcinomas, the effectiveness of a Tcf-4/ β-catenin transcriptional enhancer element was evaluated for its ability to act as a tumor specific regulator of therapeutic gene expression in a panel of human tumor and normal colon cell lines. Using the HSV-TK gene in combination with GCV, we demonstrated that the Tcf-4/ β-catenin enhancer and c-fos promoter can efficiently direct expression of HSV-TK resulting in phosphorylation of GCV and cell killing.

1) Our approach with the Tcf-4/ β-catenin enhancer is based on tumor specific conditions with transcriptional activity dependent on transcription factor complexes found only in the tumor phenotype. In several studies, coupling of the Tcf enhancer elements with constitutive viral promoters (TK, SV40, Ad E1B,E2) in adenoviral vectors have proven to be effective vehicles for tumor specific gene expression in cell culture and in animal models (272, 273, 301). We hypothesized that linking the Tcf enhancer elements with a mammalian promoter associated with a tumor phenotype or involved in the wnt signaling pathway could be even more effective. An increasing number of genes with Tcf-4/ β-catenin 5' regulatory sequences have been identified, including c-myc, cyclin D, c-jun, and MMP-7 (19-22, 24). Promoter sequences from these genes, and others yet to be identified, could be manipulated for combination testing with the Tcf-4/ -catenin enhancer.

2) This approach can be very useful in customizing cancer gene therapy treatment based on the gene expression of the tumor biopsy taken from the patient. Depending on which oncogenes are found to be up-regulated in the tumor, a custom gene expression cassette can be tailor made for the patient by coupling Tcf-4 enhancer element with the chosen oncogenic promoter.

3) The potential of this strategy can be further explored by combining the Tcf-4 enhancer elements with newly discovered/designed tumor/tissue specific gene expression promoters. There are several potential candidate promoters, for example, epithelial cell adhesion molecule promoter, stress inducible grp78 promoter and the tyrosine hydroxylase promoter (302-304) .

4) Owing to the leaky therapeutic gene expression in normal cells, cancer gene therapy may cause normal cell toxicities. Usually liver cell toxicity is one of the prime concerns in cancer gene therapy and our approach can address this issue by selecting special promoters in combination with Tcf-4 enhancer element. For example, Cox-2 promoter has been shown to have minimal activity in liver cells, and as already shown in the presented data Cox-2 promoter, can be successfully combined with Tcf-4 enhancer element for tumor specific gene expression.

5) We have demonstrated that these innovatively designed therapeutic gene expression cassettes can be successfully delivered using adenoviral vectors. In a clinical situation, this would allow stringent control of the delivery and expression of the therapeutic gene expression cassette. In addition, systemically administered adenoviral vectors can reach widely disseminated tumors which may go undetected by conventional therapies. However there are certain safety issues associated with the use of adenoviral vectors as delivery vehicle, these concerns can be addressed to reasonable levels by using the newer generation adenoviral vectors (298).

6) Another unique aspect of Tcf-4/ β-catenin regulation is the modulation of responsive genes by chemical modifiers like butyrate, phorbol esters, doxorubicin and retinoids. Short chain fatty acids like butyrate and trichostatin A have been reported to increase Tcf-4/ β-catenin mediated gene expression (223, 305). Addition of the phorbol ester PMA has also been reported to increase gene expression (305). Conversely, addition of NSAIDs has been suggested to inhibit Tcf-4/β-catenin mediated transcription by stabilizing the levels of phosphorylated β-catenin. In addition, there are other compounds which have the potential for being used as negative regulators of Tcf-4/β-catenin

mediated transcription. For example, the anti-cancer drug, Adriamycin (226) and retinoids (227) have been shown to decrease Tcf-4/ -catenin mediated gene expression. The effects of these compounds on HSV-TK expression in the Tcf-TK-transfected cells is currently under investigation. Because of the demonstrated variability of transcriptional regulation of Tcf-4/ β-catenin expression in different tumor cell lines (as in Figure 1), addition of an inducible stimulating agent could enhance expression in marginally responsive cells. Conversely, the possibility of having a repressor option in the context of patient safety for viral-based gene therapies could be important. Thus, the drug inducibility (or repression) options conferred with the Tcf-4/ β-catenin enhancer will be further characterized for potential clinical use of this element in therapeutic gene expression.

7) Innovative cancer therapy protocols can be designed by combining our approach with conventional cancer therapies. For example, a Tcf-4 enhancer element could be combined with a radioinducible promoter like Egr (306) for expressing the desired therapeutic gene. This multi-modal therapeutic approach may result in improved therapeutic outcomes.

8) Our recombinant Tcf-4 enhancer/promoter system can be utilized to control the replication of conditionally replicative oncolytic adenoviruses. This can be achieved by placing the expressing of key genes needed for the replication of oncolytic adenovirus under the control of our recombinant enhancer/promoter system. In an appropriate tumor cell environment, the Tcf-4 enhancer/promoter would be highly active allowing increased replication of the oncolytic adenovirus. On the other hand, in a normal cell environment, the recombinant promoter will not be active and will prevent normal cell toxicity by not allowing the oncolytic adenovirus to replicate.

9) Our gene therapy approach is solely a tumor specific gene expression promoter based approach. Therefore, it has the flexibility of being used with any therapeutic gene of choice allowing 1) killing of the tumor cell 2) substituting the mutated gene responsible for neoplastic transformation or 3) immunomodulating the host immune response to target and eliminate the tumor.

Future Directions

The results presented in the study successfully demonstrate the 'proof of principle' that Tcf-4/β-catenin enhancer can be used for the design of tumor/tissue specific gene expression promoters for the purpose of cancer gene therapy. However, it should be noted that this conclusion is derived solely from in vitro experiments. The validity of these in vitro results needs to be tested in animals to demonstrate the robustness of the proposed enhancer promoter system. There are several key points which can be derived from the in vitro data presented, for example 1) aberrant activation of the wnt signaling pathway, due to mutational inactivation of the APC or β-catenin gene in the target tumor is an absolute requirement for the success of the proposed system, 2) the minimal promoter partner is very crucial in determining the over all activity of the recombinant promoters, 3) the promoters incorporating the Tcf-4 enhancer element are not active in the tested normal cells (we will elaborate this point later) and finally, 4) the therapeutic gene expression construct comprising of the recombinant promoters expressing the gene of choice can be successfully delivered by using an adenoviruses to the tumor cells under in vitro conditions. As mentioned earlier, each of these points will need to be further characterized in animal model systems.

There are several rodents models for colorectal cancer that are based upon mutational inactivation of the APC gene(307). These APC mutation based animal models would be ideal for testing the tumor specific gene expression ability of the proposed promoter/enhancer system under in vivo situations. The APC/min mouse (min mouse) is one of the most extensively used animal models for colon cancer studies. These animals carry germline heterozygous mutations in the APC gene resulting in a truncated APC gene product and develop more than 100 intestinal tumors mainly in the upper gastrointestinal tract (GI). The Min mouse has been widely used in colon cancer research although it does not forms tumors in the large intestine, its use can be justified from the fact that patients suffering from familial adenomatous polyposis are susceptible to a broad spectrum of extra-colonic manifestations, the most clinically relevant of which are the desmoid and upper GI tract tumors (307). Therefore, we believe that for our studies, this model would be highly useful in replicating our in vitro studies under in vivo situations.

However, to use the min mouse model for our studies, several key issues including the choice of therapeutic gene, delivery vector, and route of administration of the vector or drug will have to be worked out before we can observe the expected results. In addition, there is the issue of which minimal promoter to be pair with the Tcf-4 enhancer for testing in the animal model. This is a very crucial aspect and stems from the demonstrated fact that the activity of the minimal promoter is also an important part of the whole recombinant promoter. In other words, if a minimal promoter partner is chosen which has a fairly high amount of activity in normal cells/tissues besides being highly active in the target tumor cells; it may result in normal cell/tissue toxicity and lowers the

therapeutic index. One of the inherent positive points of using the Tcf-4 enhancer elements for designing such recombinant promoters is the mechanism by which they control gene expression. Tcf-4 elements in the absence of nuclear β-catenin, a situation expected in normal cells, act as transcription repressors. Several proteins like the histone deacetylases CtBP and Rpd3 are recruited to the TCf-4 elements (308) resulting in the transcriptional repression of the promoter. Therefore, this unique gene expression controlling feature of Tcf-4 elements can act as a safeguard mechanism by silencing the expression of the toxic therapeutic gene in normal cells. Hence, even if the minimal promoter partner has some activity in normal cells/tissues, we predict it is likely to show much reduced transcriptional activity when coupled with a Tcf-4 element.

By optimizing the above mentioned factors we expect to see a reduced tumor load in the min mouse upon treatment with our therapeutic expression vectors along with reduced normal cell toxicity. However, complete eradication of the tumors and minimizing normal cell toxicity may require several intervening steps. For example, rationally chosen therapeutic gene(s) could result in an enhanced bystander effect targeting increasing number of tumor cells or the gene therapy approach could be combined with other treatment modalities like chemotherapy for better therapeutic outcome. Cancer immunotherapy is one of the most promising ways to achieve substantial levels of a bystander effect. The proposed therapeutic gene expression system in this study can be easily adapted for such purposes. Several studies have shown the benefits of targeting the tumors with an immunogenic approach. Cytokines, like GM-CSF (granulocyte-macrophage colony-stimulating factor), when expressed in the tumor micro-environment have been shown to stimulate an intense inflammatory reaction

characterized by tumor infiltrating granulocytes, macrophages and dendritic cells. Furthermore, the presence of dendritic cells (the antigen presenting cells) suggests that GM-CSF creates an advantageous intracellular environment for tumor antigen presentation resulting in the activation of lymphocytes against the tumors (309). Other promising cytokines which can be used for cancer immunotherapy are IL-12 and IL-21 (310, 311). Genes for such cytokines along with tumor antigens can be incorporated into the Tcf-4 enhancer/promoter containing vectors for targeting colon tumors. Such cancer vaccines are expected to be highly effective in targeting widely disseminated tumors.

The possibilities of using our proposed gene expression system are potentially unlimited, in theory it can be used to express any desired gene in tumors especially of the colon. Therefore, in future studies, it will be quite interesting to see the efficacy of the Tcf-4 enhancer containing recombinant promoters to express genes other than HSV-TK. Efforts are needed to harvest the customizable aspect of our proposed gene expression system. Given the possibility of the spectrum of therapeutic genes which can be used to target tumor tissue, a thorough genetic screening of the patient would be extremely beneficial in choosing the appropriate therapeutic gene on an individual patient basis.

As far as the question of minimizing of normal cell toxicity, it may not be possible to attain absolutely no normal cell toxicity, but it can be minimized. The presented in vitro data clearly shows that the proposed promoter/enhancer system is not active in the normal NCM460 cells. These cells are derived from normal colon cells, are non cancerous and have been previously used as standard negative control cells in a published study (272). In addition we have demonstrated that, like normal cells, these cells do not have nuclear β-catenin and the Tcf-4 enhancer activity can be increased by

over expression of constitutively active β-catenin. However, it must be emphasized that although these cells may have characteristics of normal colon cells, they may not be a true representation of the entire spectrum of the epithelial cells lining the colon or GI tract. Two other normal colon cell lines, NCM425 and CSC-1 were also tested in our study and showed lower activities of the promoter/enhancer system. However, more studies are required to assess the ability of NCM425 and CSC-1 to serve as negative controls.

In relation to normal cell toxicity, another issue which may impact the usage of our proposed promoter/enhancer system is the presence of a small number of stem cells at the base of each colonic crypt along the length of intestine. These stem cells differentiate into several types of epithelial cells found in the lining of the GI tract and are responsible for replenishing the epithelial cells lost during normal wear and tear of the GI tract (4). It has been suggested that these stem cells require wnt signaling for the process of differentiation, and nuclear staining of β-catenin has been documented in these cells. At the same time these cells do not show any APC staining, reinforcing the fact that the presence of wild type APC protein is crucial for regulation of β-catenin levels. As the cells mature along the crypt-villi axis and move towards the periphery of villi (the center of the GI tract), they progressively show less nuclear β-catenin levels and increased APC expression. In the given scenario, in theory, our recombinant promoter/enhancer/gene construct could be toxic to these cells because of the activated wnt signaling pathway and may result in stem cell toxicity. However, how much stem cell toxicity our proposed therapy system would cause is a matter of speculation. This is because these stem cells have very low minimal levels of wnt signaling activation which may be significant only

from a differentiation and development perspective (312). How this background wnt signaling will activate our recombinant Tcf-4 enhancer/promoter elements, needs to be determined, but based on known considerations we do not expect it to cause widespread stem cell toxicity. This is based on the fact that too much wnt signaling usually causes a normal cell to undergo apoptosis. Therefore, levels of wnt signaling in these colon stem cells may not be as comparable to the colon cancer cells (in which mutational inactivation of APC/β-catenin causes aberrant activation of wnt signaling), and this might not significantly activate our proposed recombinant promoter system. In addition, normal stem cell toxicity can be further minimized by directly targeting the tumor with the therapeutic vector by using variety of means, for example by combining imaging techiniques like ultrasound biomicroscopy can be used to guide direct injection of the vectors into the tumors (313). Furthermore, the transgene expression can also be monitored by combining molecular imaging with radio-nuclide based gene therapy approaches and could assist in minimizing normal cell toxicity (314).

Another attractive aspect of the proposed system is the potential ability to regulate (induce or suppress gene expression) the levels of therapeutic gene expression under clinical situations. Although, the data presented here to support this aspect are very preliminary and need further characterization, the initial results are suggestive of the regulatory potential of the recombinant promoters containing the Tcf-4 enhancer element. Among the existing transcriptional gene regulatory systems, the Tet-regulatable system is the most widely exploited tool for controlling gene expression. For gene therapy purposes the Tet-regulatory system has been encoded with lentiviruses, adenoviruses, adeno-associated viruses and retroviruses. The Tet system has several advantages. For example,

tetracycline is non toxic to humans at the dosage levels required for gene activation (although it can be toxic to humans at high concentrations) and it does not exhibit any pleiotropic effect on cellular metabolic pathways. The down side of using this system to control gene expression in the clinic is the possibility of the Tet system proteins being immunogenic in human because of their bacterial origin (315). At the same time, the Tet system involves incorporation of extra DNA sequences encoding the Tet component proteins which may be critical for the gene delivery vector in terms of the carrying capacity. In contrast, for regulation of the therapeutic gene, the system proposed in this study requires no extra set of genes to be included in the therapeutic gene expression vector. In addition, no potentially immunogenic substances are involved. For example, short chain fatty acids (like sodium butyrate and retinoic acid) are naturally occurring substances. However, what adverse affects these substances might have on normal cells will have to be worked out, for example short chain fatty acids are also known to cause apoptosis in cells. Therefore, extensive studies are needed both in vitro and in vivo to demonstrate the feasibility of using these chemical regulators to control transcriptional activity of Tcf-4 enhancer containing promoters.

The proposed Tcf-4 enhancer based recombinant promoter system is a promising approach for expressing the therapeutic genes specifically in tumors especially of the colon. This system needs further characterization under in vivo situations. There are some hurdles, as described above, which may limit the use of these Tcf-4 based recombinant promoters in the clinic. However, with innovative design and application of comprehensive knowledge from the fields of molecular biology, immunology, virology and molecular medicine, these issues can be rationally addressed. The field of cancer

gene therapy is an area of active translational research and requires concerted efforts of scientists from several different areas. In fact, this is one of the limitations of successful implementation of cancer gene therapies in clinic. For the future more resources and dedicated efforts are required for the success of cancer gene therapy. In general, the field of gene therapy is very young and has given the mankind a ray of hope for the treatment of a broad spectrum of human diseases ranging from genetic disorders to heart diseases and cancer. Given the nature of the pain and suffering involved in these human diseases, gene therapy has generated high hopes and great hypes. There is huge burden on the scientific community to innovatively tap the potential of gene therapy and the same assure the public that the patient's welfare and health is their major goal.

REFERENCES

1. Pisani, P., Parkin, D. M., Bray, F. Ferlay, J. Estimates of the worldwide mortality from 25 cancers in 1990. Int J Cancer, 83:18-29, 1999.

2. Sparks, A. B., Morin, P. J., Vogelstein, B. Kinzler, K. W. Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res, 58:1130-1134, 1998.

3. Huang, J., Papadopoulos, N., McKinley, A. J., et al. APC mutations in colorectal tumors with mismatch repair deficiency. Proc Natl Acad Sci U S A, 93:9049-9054, 1996.

4. Fodde, R., Smits, R., Clevers, H. APC, signal transduction and genetic instability in colorectal cancer. Nat Rev Cancer, 1:55-67, 2001.

5. Groden, J., Thliveris, A., Samowitz, W., et al. Identification and characterization of the familial adenomatous polyposis coli gene. Cell, 66:589-600, 1991.

6. Rubinfeld, B., Souza, B., Albert, I., et al. Association of the APC gene product with beta-catenin. Science, 262:1731-1734, 1993.

7. Polakis, P. Wnt signaling and cancer. Genes Dev, 14:1837-1851, 2000.

8. Bhanot, P., Brink, M., Samos, C. H., et al. A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature, 382:225-230, 1996.

9. Kishida, S., Yamamoto, H., Hino, S., et al. DIX domains of Dvl and axin are necessary for protein interactions and their ability to regulate beta-catenin stability. Mol Cell Biol, 19:4414-4422, 1999.

10. Behrens, J., von Kries, J. P., Kuhl, M., et al. Functional interaction of beta-catenin with the transcription factor LEF-1. Nature, 382:638-642, 1996.

11. Ben-Ze'ev, A. Geiger, B. Differential molecular interactions of beta-catenin and plakoglobin in adhesion, signaling and cancer. Curr Opin Cell Biol, 10:629-639, 1998.

12. Munemitsu, S., Souza, B., Muller, O., et al. The APC gene product associates with microtubules in vivo and promotes their assembly in vitro. Cancer Res, 54:3676-3681, 1994.

13. Morin, P. J., Sparks, A. B., Korinek, V., et al. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science, 275:1787-1790, 1997.

14. Liu, W., Dong, X., Mai, M., et al. Mutations in AXIN2 cause colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signalling. Nat Genet, 26:146-147, 2000.

15. Clevers, H. Axin and hepatocellular carcinomas. Nat Genet, 24:206-208, 2000.

16. Jen, J., Powell, S. M., Papadopoulos, N., et al. Molecular determinants of dysplasia in colorectal lesions. Cancer Res, 54:5523-5526, 1994.

17. Powell, S. M., Zilz, N., Beazer-Barclay, Y., et al. APC mutations occur early during colorectal tumorigenesis. Nature, 359:235-237, 1992.

18. Smith, A. J., Stern, H. S., Penner, M., et al. Somatic APC and K-ras codon 12 mutations in aberrant crypt foci from human colons. Cancer Res, 54:5527-5530, 1994.

19. He, T. C., Sparks, A. B., Rago, C., et al. Identification of c-MYC as a target of the APC pathway. Science, 281:1509-1512, 1998.

20. Tetsu, O. McCormick, F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature, 398:422-426, 1999.

21. Shtutman, M., Zhurinsky, J., Simcha, I., et al. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A, 96:5522-5527, 1999.

22. Crawford, H. C., Fingleton, B. M., Rudolph-Owen, L. A., et al. The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. Oncogene, 18:2883-2891, 1999.

23. Wielenga, V. J., Smits, R., Korinek, V., et al. Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. Am J Pathol, 154:515-523, 1999.

24. Mann, B., Gelos, M., Siedow, A., et al. Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. Proc Natl Acad Sci U S A, 96:1603-1608, 1999.

25. Koh, T. J., Bulitta, C. J., Fleming, J. V., et al. Gastrin is a target of the beta-catenin/TCF-4 growth-signaling pathway in a model of intestinal polyposis. J Clin Invest, 106:533-539, 2000.

26. Araki, Y., Okamura, S., Hussain, S. P., et al. Regulation of cyclooxygenase-2 expression by the Wnt and ras pathways. Cancer Res, 63:728-734, 2003.

27. Korinek, V., Barker, N., Moerer, P., et al. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat Genet, 19:379-383, 1998.

28. Barker, N., Huls, G., Korinek, V.Clevers, H. Restricted high level expression of Tcf-4 protein in intestinal and mammary gland epithelium. Am J Pathol, 154:29-35, 1999.

29. Melhem, M. F., Meisler, A. I., Finley, G. G., et al. Distribution of cells expressing myc proteins in human colorectal epithelium, polyps, and malignant tumors. Cancer Res, 52:5853-5864, 1992.

30. Yuwono, M., Rossi, T. M., Fisher, J. E.Tjota, A. Oncogene expression in patients with familial polyposis coli/Gardner's syndrome. Int Arch Allergy Immunol, 111:89-95, 1996.

31. Roose, J., Huls, G., van Beest, M., et al. Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. Science, 285:1923-1926, 1999.

32. Kinzler, K. W. Vogelstein, B. Lessons from hereditary colorectal cancer. Cell, 87:159-170, 1996.

33. Friedberg, E. C. How nucleotide excision repair protects against cancer. Nat Rev Cancer, 1:22-33, 2001.

34. Ellis, N. A. German, J. Molecular genetics of Bloom's syndrome. Hum Mol Genet, 5 Spec No:1457-1463, 1996.

35. Jacob, S. Praz, F. DNA mismatch repair defects: role in colorectal carcinogenesis. Biochimie, 84:27-47, 2002.

36. McCormick, F. Signalling networks that cause cancer. Trends Cell Biol, 9:M53-56, 1999.

37. Fearon, E. R. Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell, 61:759-767, 1990.

38. Forrester, K., Almoguera, C., Han, K., Grizzle, W. E.Perucho, M. Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. Nature, 327:298-303, 1987.

39. Bos, J. L., Fearon, E. R., Hamilton, S. R., et al. Prevalence of ras gene mutations in human colorectal cancers. Nature, 327:293-297, 1987.

40. Vogelstein, B., Fearon, E. R., Hamilton, S. R., et al. Genetic alterations during colorectal-tumor development. N Engl J Med, 319:525-532, 1988.

41. Fearon, E. R., Hamilton, S. R.Vogelstein, B. Clonal analysis of human colorectal tumors. Science, 238:193-197, 1987.

42. Law, D. J., Olschwang, S., Monpezat, J. P., et al. Concerted nonsyntenic allelic loss in human colorectal carcinoma. Science, 241:961-965, 1988.

43. Vogelstein, B., Fearon, E. R., Kern, S. E., et al. Allelotype of colorectal carcinomas. Science, 244:207-211, 1989.

44. Rodrigues, N. R., Rowan, A., Smith, M. E., et al. p53 mutations in colorectal cancer. Proc Natl Acad Sci U S A, 87:7555-7559, 1990.

45. Hahn, S. A., Schutte, M., Hoque, A. T., et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science, 271:350-353, 1996.

46. Blobe, G. C., Schiemann, W. P.Lodish, H. F. Role of transforming growth factor beta in human disease. N Engl J Med, 342:1350-1358, 2000.

47. Thiagalingam, S., Lengauer, C., Leach, F. S., et al. Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. Nat Genet, 13:343-346, 1996.

48. Loeb, L. A. A mutator phenotype in cancer. Cancer Res, 61:3230-3239, 2001.

49. Rajagopalan, H., Nowak, M. A., Vogelstein, B.Lengauer, C. The significance of unstable chromosomes in colorectal cancer. Nat Rev Cancer, 3:695-701, 2003.

50. Lengauer, C., Kinzler, K. W.Vogelstein, B. Genetic instabilities in human cancers. Nature, 396:643-649, 1998.

51. Duesberg, P., Rausch, C., Rasnick, D.Hehlmann, R. Genetic instability of cancer cells is proportional to their degree of aneuploidy. Proc Natl Acad Sci U S A, 95:13692-13697, 1998.

52. Lengauer, C., Kinzler, K. W.Vogelstein, B. Genetic instability in colorectal cancers. Nature, 386:623-627, 1997.

53. Ohshima, K., Haraoka, S., Yoshioka, S., et al. Mutation analysis of mitotic checkpoint genes (hBUB1 and hBUBR1) and microsatellite instability in adult T-cell leukemia/lymphoma. Cancer Lett, 158:141-150, 2000.

54. Cahill, D. P., Lengauer, C., Yu, J., et al. Mutations of mitotic checkpoint genes in human cancers. Nature, 392:300-303, 1998.

55. Jin, D. Y., Spencer, F.Jeang, K. T. Human T cell leukemia virus type 1 oncoprotein Tax targets the human mitotic checkpoint protein MAD1. Cell, 93:81-91, 1998.

56. Michel, L. S., Liberal, V., Chatterjee, A., et al. MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells. Nature, 409:355-359, 2001.

57. Jallepalli, P. V. Lengauer, C. Chromosome segregation and cancer: cutting through the mystery. Nat Rev Cancer, 1:109-117, 2001.

58. Fodde, R., Kuipers, J., Rosenberg, C., et al. Mutations in the APC tumour suppressor gene cause chromosomal instability. Nat Cell Biol, 3:433-438, 2001.

59. Kaplan, K. B., Burds, A. A., Swedlow, J. R., et al. A role for the Adenomatous Polyposis Coli protein in chromosome segregation. Nat Cell Biol, 3:429-432, 2001.

60. Stoler, D. L., Chen, N., Basik, M., et al. The onset and extent of genomic instability in sporadic colorectal tumor progression. Proc Natl Acad Sci U S A, 96:15121-15126, 1999.

61. Ionov, Y., Peinado, M. A., Malkhosyan, S., Shibata, D.Perucho, M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature, 363:558-561, 1993.

62. Thibodeau, S. N., Bren, G.Schaid, D. Microsatellite instability in cancer of the proximal colon. Science, 260:816-819, 1993.

63. Hoang, J. M., Cottu, P. H., Thuille, B., et al. BAT-26, an indicator of the replication error phenotype in colorectal cancers and cell lines. Cancer Res, 57:300-303, 1997.

64. Jiricny, J. Marra, G. DNA repair defects in colon cancer. Curr Opin Genet Dev, 13:61-69, 2003.

65. Fishel, R., Ewel, A., Lee, S., Lescoe, M. K.Griffith, J. Binding of mismatched microsatellite DNA sequences by the human MSH2 protein. Science, 266:1403-1405, 1994.

66. Prolla, T. A., Pang, Q., Alani, E., Kolodner, R. D.Liskay, R. M. MLH1, PMS1, and MSH2 interactions during the initiation of DNA mismatch repair in yeast. Science, 265:1091-1093, 1994.

67. Boland, C. R., Thibodeau, S. N., Hamilton, S. R., et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res, 58:5248-5257, 1998.

68. Wu, Y., Berends, M. J., Mensink, R. G., et al. Association of hereditary nonpolyposis colorectal cancer-related tumors displaying low microsatellite instability with MSH6 germline mutations. Am J Hum Genet, 65:1291-1298, 1999.

69. Chung, D. C. Rustgi, A. K. The hereditary nonpolyposis colorectal cancer syndrome: genetics and clinical implications. Ann Intern Med, 138:560-570, 2003.

70. Fleisher, A. S., Esteller, M., Wang, S., et al. Hypermethylation of the hMLH1 gene promoter in human gastric cancers with microsatellite instability. Cancer Res, 59:1090-1095, 1999.

71. Leung, S. Y., Yuen, S. T., Chung, L. P., et al. hMLH1 promoter methylation and lack of hMLH1 expression in sporadic gastric carcinomas with high-frequency microsatellite instability. Cancer Res, 59:159-164, 1999.

72. Veigl, M. L., Kasturi, L., Olechnowicz, J., et al. Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. Proc Natl Acad Sci U S A, 95:8698-8702, 1998.

73. Aaltonen, L. A., Peltomaki, P., Leach, F. S., et al. Clues to the pathogenesis of familial colorectal cancer. Science, 260:812-816, 1993.

74. Toyota, M., Ahuja, N., Ohe-Toyota, M., et al. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A, 96:8681-8686, 1999.

75. Souza, R. F., Appel, R., Yin, J., et al. Microsatellite instability in the insulin-like growth factor II receptor gene in gastrointestinal tumours. Nat Genet, 14:255-257, 1996.

76. Rampino, N., Yamamoto, H., Ionov, Y., et al. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. Science, 275:967-969, 1997.

77. Schwartz, S., Jr., Yamamoto, H., Navarro, M., et al. Frameshift mutations at mononucleotide repeats in caspase-5 and other target genes in endometrial and gastrointestinal cancer of the microsatellite mutator phenotype. Cancer Res, 59:2995-3002, 1999.

78. Olschwang, S., Hamelin, R., Laurent-Puig, P., et al. Alternative genetic pathways in colorectal carcinogenesis. Proc Natl Acad Sci U S A, 94:12122-12127, 1997.

79. Duval, A. Hamelin, R. Genetic instability in human mismatch repair deficient cancers. Ann Genet, 45:71-75, 2002.

80. Papillon, J. Gerard, J. P. Role of radiotherapy in anal preservation for cancers of the lower third of the rectum. Int J Radiat Oncol Biol Phys, 19:1219-1220, 1990.

81. Minsky, B. D., Enker, W. E., Cohen, A. M.Lauwers, G. Local excision and postoperative radiation therapy for rectal cancer. Am J Clin Oncol, 17:411-416, 1994.

82. Hawkins, M. M., Wilson, L. M., Burton, H. S., et al. Radiotherapy, alkylating agents, and risk of bone cancer after childhood cancer. J Natl Cancer Inst, 88:270-278, 1996.

83. Pinedo, H. M. Peters, G. F. Fluorouracil: biochemistry and pharmacology. J Clin Oncol, 6:1653-1664, 1988.

84. Danenberg, P. V. Danenberg, K. D. Effect of 5, 10-methylenetetrahydrofolate on the dissociation of 5-fluoro-2'-deoxyuridylate from thymidylate synthetase: evidence for an ordered mechanism. Biochemistry, 17:4018-4024, 1978.

85. Heggie, G. D., Sommadossi, J. P., Cross, D. S., Huster, W. J.Diasio, R. B. Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine, and bile. Cancer Res, 47:2203-2206, 1987.

86. Diasio, R. B., Beavers, T. L.Carpenter, J. T. Familial deficiency of dihydropyrimidine dehydrogenase. Biochemical basis for familial pyrimidinemia and severe 5-fluorouracil-induced toxicity. J Clin Invest, 81:47-51, 1988.

87. Johnson, M. R., Hageboutros, A., Wang, K., et al. Life-threatening toxicity in a dihydropyrimidine dehydrogenase-deficient patient after treatment with topical 5-fluorouracil. Clin Cancer Res, 5:2006-2011, 1999.

88. van Kuilenburg, A. B., Muller, E. W., Haasjes, J., et al. Lethal outcome of a patient with a complete dihydropyrimidine dehydrogenase (DPD) deficiency after administration of 5-fluorouracil: frequency of the common IVS14+1G>A mutation causing DPD deficiency. Clin Cancer Res, 7:1149-1153, 2001.

89. Miwa, M., Ura, M., Nishida, M., et al. Design of a novel oral fluoropyrimidine carbamate, capecitabine, which generates 5-fluorouracil selectively in tumours by enzymes concentrated in human liver and cancer tissue. Eur J Cancer, 34:1274-1281, 1998.

90. Toffoli, G., Cecchin, E., Corona, G.Boiocchi, M. Pharmacogenetics of irinotecan. Curr Med Chem Anti-Canc Agents, 3:225-237, 2003.

91. Takasuna, K., Hagiwara, T., Hirohashi, M., et al. Involvement of beta-glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. Cancer Res, 56:3752-3757, 1996.

92. Tashiro, T., Kawada, Y., Sakurai, Y.Kidani, Y. Antitumor activity of a new platinum complex, oxalato (trans-l-1,2-diaminocyclohexane)platinum (II): new experimental data. Biomed Pharmacother, 43:251-260, 1989.

93. Mathe, G., Kidani, Y., Triana, K., et al. A phase I trial of trans-1-diaminocyclohexane oxalato-platinum (l-OHP). Biomed Pharmacother, 40:372-376, 1986.

94. Gamelin, E., Gamelin, L., Bossi, L.Quasthoff, S. Clinical aspects and molecular basis of oxaliplatin neurotoxicity: current management and development of preventive measures. Semin Oncol, 29:21-33, 2002.

95. Tucker, M. A., D'Angio, G. J., Boice, J. D., Jr., et al. Bone sarcomas linked to radiotherapy and chemotherapy in children. N Engl J Med, 317:588-593, 1987.

96. Pedersen-Bjergaard, J., Ersboll, J., Hansen, V. L., et al. Carcinoma of the urinary bladder after treatment with cyclophosphamide for non-Hodgkin's lymphoma. N Engl J Med, 318:1028-1032, 1988.

97. Felix, C. A. Secondary leukemias induced by topoisomerase-targeted drugs. Biochim Biophys Acta, 1400:233-255, 1998.

98. Gomez-Navarro, J., Curiel, D. T.Douglas, J. T. Gene therapy for cancer. Eur J Cancer, 35:2039-2057, 1999.

99. Midgley, R. Kerr, D. Colorectal cancer. Lancet, 353:391-399, 1999.

100. Thompson, J. A., Grunert, F.Zimmermann, W. Carcinoembryonic antigen gene family: molecular biology and clinical perspectives. J Clin Lab Anal, 5:344-366, 1991.

101. McCormick, F. Cancer gene therapy: fringe or cutting edge? Nat Rev Cancer, 1:130-141, 2001.

102. Harris, M. P., Sutjipto, S., Wills, K. N., et al. Adenovirus-mediated p53 gene transfer inhibits growth of human tumor cells expressing mutant p53 protein. Cancer Gene Ther, 3:121-130, 1996.

103. Opalka, B., Dickopp, A.Kirch, H. C. Apoptotic genes in cancer therapy. Cells Tissues Organs, 172:126-132, 2002.

104. van Etten, B., ten Hagen, T. L., de Vries, M. R., et al. Prerequisites for effective adenovirus mediated gene therapy of colorectal liver metastases in the rat using an intracellular neutralizing antibody fragment to p21-Ras. Br J Cancer, 86:436-442, 2002.

105. Moolten, F. L. Drug sensitivity ("suicide") genes for selective cancer chemotherapy. Cancer Gene Ther, 1:279-287, 1994.

106. Austin, E. A. Huber, B. E. A first step in the development of gene therapy for colorectal carcinoma: cloning, sequencing, and expression of Escherichia coli cytosine deaminase. Mol Pharmacol, 43:380-387, 1993.

107. Kilstrup, M., Meng, L. M., Neuhard, J.Nygaard, P. Genetic evidence for a repressor of synthesis of cytosine deaminase and purine biosynthesis enzymes in Escherichia coli. J Bacteriol, 171:2124-2127, 1989.

108. Mullen, C. A., Kilstrup, M.Blaese, R. M. Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. Proc Natl Acad Sci U S A, 89:33-37, 1992.

109. Huber, B. E., Austin, E. A., Good, S. S., et al. In vivo antitumor activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase. Cancer Res, 53:4619-4626, 1993.

110. Ohwada, A., Hirschowitz, E. A.Crystal, R. G. Regional delivery of an adenovirus vector containing the Escherichia coli cytosine deaminase gene to provide local activation of 5-fluorocytosine to suppress the growth of colon carcinoma metastatic to liver. Hum Gene Ther, 7:1567-1576, 1996.

111. Topf, N., Worgall, S., Hackett, N. R.Crystal, R. G. Regional 'pro-drug' gene therapy: intravenous administration of an adenoviral vector expressing the E. coli cytosine deaminase gene and systemic administration of 5-fluorocytosine suppresses growth of hepatic metastasis of colon carcinoma. Gene Ther, 5:507-513, 1998.

112. Block, A., Freund, C. T., Chen, S. H., et al. Gene therapy of metastatic colon carcinoma: regression of multiple hepatic metastases by adenoviral expression of bacterial cytosine deaminase. Cancer Gene Ther, 7:438-445, 2000.

113. Crystal, R. G., Hirschowitz, E., Lieberman, M., et al. Phase I study of direct administration of a replication deficient adenovirus vector containing the E. coli cytosine deaminase gene to metastatic colon carcinoma of the liver in association with the oral administration of the pro-drug 5-fluorocytosine. Hum Gene Ther, 8:985-1001, 1997.

114. Knox, R. J., Friedlos, F., Jarman, M.Roberts, J. J. A new cytotoxic, DNA interstrand crosslinking agent, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, is formed from 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) by a nitroreductase enzyme in Walker carcinoma cells. Biochem Pharmacol, 37:4661-4669, 1988.

115. Grove, J. I., Searle, P. F., Weedon, S. J., et al. Virus-directed enzyme prodrug therapy using CB1954. Anticancer Drug Des, 14:461-472, 1999.

116. Bridgewater, J. A., Springer, C. J., Knox, R. J., et al. Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrug CB1954. Eur J Cancer, 31A:2362-2370, 1995.

117. Drabek, D., Guy, J., Craig, R.Grosveld, F. The expression of bacterial nitroreductase in transgenic mice results in specific cell killing by the prodrug CB1954. Gene Ther, 4:93-100, 1997.

118. Chung-Faye, G., Palmer, D., Anderson, D., et al. Virus-directed, enzyme prodrug therapy with nitroimidazole reductase: a phase I and pharmacokinetic study of its prodrug, CB1954. Clin Cancer Res, 7:2662-2668, 2001.

119. Rubin, J., Galanis, E., Pitot, H. C., et al. Phase I study of immunotherapy of hepatic metastases of colorectal carcinoma by direct gene transfer of an allogeneic histocompatibility antigen, HLA-B7. Gene Ther, 4:419-425, 1997.

120. Fakhrai, H., Shawler, D. L., Gjerset, R., et al. Cytokine gene therapy with interleukin-2-transduced fibroblasts: effects of IL-2 dose on anti-tumor immunity. Hum Gene Ther, 6:591-601, 1995.

121. Galanis, E., Hersh, E. M., Stopeck, A. T., et al. Immunotherapy of advanced malignancy by direct gene transfer of an interleukin-2 DNA/DMRIE/DOPE lipid complex: phase I/II experience. J Clin Oncol, 17:3313-3323, 1999.

122. Sobol, R. E., Shawler, D. L., Carson, C., et al. Interleukin 2 gene therapy of colorectal carcinoma with autologous irradiated tumor cells and genetically engineered fibroblasts: a Phase I study. Clin Cancer Res, 5:2359-2365, 1999.

123. Schmidt-Wolf, I. G., Finke, S., Trojaneck, B., et al. Phase I clinical study applying autologous immunological effector cells transfected with the interleukin-2 gene in patients with metastatic renal cancer, colorectal cancer and lymphoma. Br J Cancer, 81:1009-1016, 1999.

124. Tsang, K. Y., Zaremba, S., Nieroda, C. A., et al. Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine. J Natl Cancer Inst, 87:982-990, 1995.

125. Conry, R. M., Khazaeli, M. B., Saleh, M. N., et al. Phase I trial of a recombinant vaccinia virus encoding carcinoembryonic antigen in metastatic adenocarcinoma: comparison of intradermal versus subcutaneous administration. Clin Cancer Res, 5:2330-2337, 1999.

126. Conry, R. M., Allen, K. O., Lee, S., et al. Human autoantibodies to carcinoembryonic antigen (CEA) induced by a vaccinia-CEA vaccine. Clin Cancer Res, 6:34-41, 2000.

127. Hodge, J. W., McLaughlin, J. P., Kantor, J. A.Schlom, J. Diversified prime and boost protocols using recombinant vaccinia virus and recombinant non-replicating avian pox virus to enhance T-cell immunity and antitumor responses. Vaccine, 15:759-768, 1997.

128. Marshall, J. L., Hawkins, M. J., Tsang, K. Y., et al. Phase I study in cancer patients of a replication-defective avipox recombinant vaccine that expresses human carcinoembryonic antigen. J Clin Oncol, 17:332-337, 1999.

129. Crystal, R. G. Transfer of genes to humans: early lessons and obstacles to success. Science, 270:404-410, 1995.

130. Ginsberg, H. S., Pereira, H. G., Valentine, R. C.Wilcox, W. C. A proposed terminology for the adenovirus antigens and virion morphological subunits. Virology, 28:782-783, 1966.

131. Hierholzer, J. C. Adenoviruses in the immunocompromised host. Clin Microbiol Rev, 5:262-274, 1992.

132. Green, M., Mackey, J. K., Wold, W. S.Rigden, P. Thirty-one human adenovirus serotypes (Ad1-Ad31) form five groups (A-E) based upon DNA genome homologies. Virology, 93:481-492, 1979.

133. Barnett, B. G., Crews, C. J.Douglas, J. T. Targeted adenoviral vectors. Biochim Biophys Acta, 1575:1-14, 2002.

134. Kovesdi, I., Brough, D. E., Bruder, J. T.Wickham, T. J. Adenoviral vectors for gene transfer. Curr Opin Biotechnol, 8:583-589, 1997.

135. Roy-Chowdhury, J. Horwitz, M. S. Evolution of adenoviruses as gene therapy vectors. Mol Ther, 5:340-344, 2002.

136. Graham, F. L. Prevec, L. Methods for construction of adenovirus vectors. Mol Biotechnol, 3:207-220, 1995.

137. Yang, Y., Su, Q.Wilson, J. M. Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. J Virol, 70:7209-7212, 1996.

138. Yang, Y. Wilson, J. M. Clearance of adenovirus-infected hepatocytes by MHC class I-restricted CD4+ CTLs in vivo. J Immunol, 155:2564-2570, 1995.

139. Gorziglia, M. I., Lapcevich, C., Roy, S., et al. Generation of an adenovirus vector lacking E1, e2a, E3, and all of E4 except open reading frame 3. J Virol, 73:6048-6055, 1999.

140. Griesenbach, U., Ferrari, S., Geddes, D. M.Alton, E. W. Gene therapy progress and prospects: cystic fibrosis. Gene Ther, 9:1344-1350, 2002.

141. Mack, C. A., Song, W. R., Carpenter, H., et al. Circumvention of anti-adenovirus neutralizing immunity by administration of an adenoviral vector of an alternate serotype. Hum Gene Ther, 8:99-109, 1997.

142. Moolten, F. L. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. Cancer Res, 46:5276-5281, 1986.

143. Moolten, F. L. Wells, J. M. Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors. J Natl Cancer Inst, 82:297-300, 1990.

144. Chiocca, E. A. Brain tumor gene therapy in mice with a novel "suicide" gene: the cyclophosphamide-activating CYP2B1 gene. Clin Neurosurg, 42:370-382, 1995.

145. Faulds, D. Heel, R. C. Ganciclovir. A review of its antiviral activity, pharmacokinetic properties and therapeutic efficacy in cytomegalovirus infections. Drugs, 39:597-638, 1990.

146. Cheng, Y. C., Grill, S. P., Dutschman, G. E., Nakayama, K.Bastow, K. F. Metabolism of 9-(1,3-dihydroxy-2-propoxymethyl)guanine, a new anti-herpes virus compound, in herpes simplex virus-infected cells. J Biol Chem, 258:12460-12464, 1983.

147. Field, A. K., Davies, M. E., DeWitt, C., et al. 9-([2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)guanine: a selective inhibitor of herpes group virus replication. Proc Natl Acad Sci U S A, 80:4139-4143, 1983.

148. Ashton, W. T., Karkas, J. D., Field, A. K.Tolman, R. L. Activation by thymidine kinase and potent antiherpetic activity of 2'-nor-2'-deoxyguanosine (2'NDG). Biochem Biophys Res Commun, 108:1716-1721, 1982.

149. Biron, K. K., Stanat, S. C., Sorrell, J. B., et al. Metabolic activation of the nucleoside analog 9-[(2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)guanine in human diploid fibroblasts infected with human cytomegalovirus. Proc Natl Acad Sci U S A, 82:2473-2477, 1985.

150. Smee, D. F. Interaction of 9-(1,3-dihydroxy-2-propoxymethyl)guanine with cytosol and mitochondrial deoxyguanosine kinases: possible role in anti-cytomegalovirus activity. Mol Cell Biochem, 69:75-81, 1985.

151. Ilsley, D. D., Lee, S. H., Miller, W. H.Kuchta, R. D. Acyclic guanosine analogs inhibit DNA polymerases alpha, delta, and epsilon with very different potencies and have unique mechanisms of action. Biochemistry, 34:2504-2510, 1995.

152. Wei, S. J., Chao, Y., Hung, Y. M., et al. S- and G2-phase cell cycle arrests and apoptosis induced by ganciclovir in murine melanoma cells transduced with herpes simplex virus thymidine kinase. Exp Cell Res, 241:66-75, 1998.

153. Halloran, P. J. Fenton, R. G. Irreversible G2-M arrest and cytoskeletal reorganization induced by cytotoxic nucleoside analogues. Cancer Res, 58:3855-3865, 1998.

154. Hamel, W., Magnelli, L., Chiarugi, V. P.Israel, M. A. Herpes simplex virus thymidine kinase/ganciclovir-mediated apoptotic death of bystander cells. Cancer Res, 56:2697-2702, 1996.

155. Krohne, T. U., Shankara, S., Geissler, M., et al. Mechanisms of cell death induced by suicide genes encoding purine nucleoside phosphorylase and thymidine kinase in human hepatocellular carcinoma cells in vitro. Hepatology, 34:511-518, 2001.

156. Tomicic, M. T., Thust, R.Kaina, B. Ganciclovir-induced apoptosis in HSV-1 thymidine kinase expressing cells: critical role of DNA breaks, Bcl-2 decline and caspase-9 activation. Oncogene, 21:2141-2153, 2002.

157. Wei, S. J., Chao, Y., Shih, Y. L., et al. Involvement of Fas (CD95/APO-1) and Fas ligand in apoptosis induced by ganciclovir treatment of tumor cells transduced with herpes simplex virus thymidine kinase. Gene Ther, 6:420-431, 1999.

158. Freeman, S. M., Abboud, C. N., Whartenby, K. A., et al. The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. Cancer Res, 53:5274-5283, 1993.

159. Takamiya, Y., Short, M. P., Moolten, F. L., et al. An experimental model of retrovirus gene therapy for malignant brain tumors. J Neurosurg, 79:104-110, 1993.

160. Culver, K. W., Ram, Z., Wallbridge, S., et al. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science, 256:1550-1552, 1992.

161. Bi, W. L., Parysek, L. M., Warnick, R.Stambrook, P. J. In vitro evidence that metabolic cooperation is responsible for the bystander effect observed with HSV tk retroviral gene therapy. Hum Gene Ther, 4:725-731, 1993.

162. Fick, J., Barker, F. G., 2nd, Dazin, P., et al. The extent of heterocellular communication mediated by gap junctions is predictive of bystander tumor cytotoxicity in vitro. Proc Natl Acad Sci U S A, 92:11071-11075, 1995.

163. Goodenough, D. A., Goliger, J. A.Paul, D. L. Connexins, connexons, and intercellular communication. Annu Rev Biochem, 65:475-502, 1996.

164. Vrionis, F. D., Wu, J. K., Qi, P., et al. The bystander effect exerted by tumor cells expressing the herpes simplex virus thymidine kinase (HSVtk) gene is dependent on connexin expression and cell communication via gap junctions. Gene Ther, 4:577-585, 1997.

165. Park, J. Y., Elshami, A. A., Amin, K., et al. Retinoids augment the bystander effect in vitro and in vivo in herpes simplex virus thymidine kinase/ganciclovir-mediated gene therapy. Gene Ther, 4:909-917, 1997.

166. Touraine, R. L., Ishii-Morita, H., Ramsey, W. J.Blaese, R. M. The bystander effect in the HSVtk/ganciclovir system and its relationship to gap junctional communication. Gene Ther, 5:1705-1711, 1998.

167. Mesnil, M., Piccoli, C.Yamasaki, H. A tumor suppressor gene, Cx26, also mediates the bystander effect in HeLa cells. Cancer Res, 57:2929-2932, 1997.

168. Dilber, M. S., Abedi, M. R., Christensson, B., et al. Gap junctions promote the bystander effect of herpes simplex virus thymidine kinase in vivo. Cancer Res, 57:1523-1528, 1997.

169. Imaizumi, K., Hasegawa, Y., Kawabe, T., et al. Bystander tumoricidal effect and gap junctional communication in lung cancer cell lines. Am J Respir Cell Mol Biol, 18:205-212, 1998.

170. Princen, F., Robe, P., Lechanteur, C., et al. A cell type-specific and gap junction-independent mechanism for the herpes simplex virus-1 thymidine kinase gene/ganciclovir-mediated bystander effect. Clin Cancer Res, 5:3639-3644, 1999.

171. Drake, R. R., Pitlyk, K., McMasters, R. A., et al. Connexin-independent ganciclovir-mediated killing conferred on bystander effect-resistant cell lines by a herpes simplex virus-thymidine kinase-expressing colon cell line. Mol Ther, 2:515-523, 2000.

172. Gagandeep, S., Brew, R., Green, B., et al. Prodrug-activated gene therapy: involvement of an immunological component in the "bystander effect". Cancer Gene Ther, 3:83-88, 1996.

173. Kianmanesh, A. R., Perrin, H., Panis, Y., et al. A "distant" bystander effect of suicide gene therapy: regression of nontransduced tumors together with a distant transduced tumor. Hum Gene Ther, 8:1807-1814, 1997.

174. Black, M. E., Newcomb, T. G., Wilson, H. M.Loeb, L. A. Creation of drug-specific herpes simplex virus type 1 thymidine kinase mutants for gene therapy. Proc Natl Acad Sci U S A, 93:3525-3529, 1996.

175. Black, M. E., Kokoris, M. S.Sabo, P. Herpes simplex virus-1 thymidine kinase mutants created by semi-random sequence mutagenesis improve prodrug-mediated tumor cell killing. Cancer Res, 61:3022-3026, 2001.

176. Qiao, J., Black, M. E.Caruso, M. Enhanced ganciclovir killing and bystander effect of human tumor cells transduced with a retroviral vector carrying a herpes simplex virus thymidine kinase gene mutant. Hum Gene Ther, 11:1569-1576, 2000.

177. Aghi, M., Kramm, C. M., Chou, T. C., Breakefield, X. O.Chiocca, E. A. Synergistic anticancer effects of ganciclovir/thymidine kinase and 5-fluorocytosine/cytosine deaminase gene therapies. J Natl Cancer Inst, 90:370-380, 1998.

178. Rogulski, K. R., Zhang, K., Kolozsvary, A., Kim, J. H.Freytag, S. O. Pronounced antitumor effects and tumor radiosensitization of double suicide gene therapy. Clin Cancer Res, 3:2081-2088, 1997.

179. Aghi, M., Chou, T. C., Suling, K., Breakefield, X. O.Chiocca, E. A. Multimodal cancer treatment mediated by a replicating oncolytic virus that delivers the oxazaphosphorine/rat cytochrome P450 2B1 and ganciclovir/herpes simplex virus thymidine kinase gene therapies. Cancer Res, 59:3861-3865, 1999.

180. Wildner, O., Blaese, R. M.Morris, J. C. Synergy between the herpes simplex virus tk/ganciclovir prodrug suicide system and the topoisomerase I inhibitor topotecan. Hum Gene Ther, 10:2679-2687, 1999.

181. Wildner, O., Blaese, R. M.Morris, J. C. Therapy of colon cancer with oncolytic adenovirus is enhanced by the addition of herpes simplex virus-thymidine kinase. Cancer Res, 59:410-413, 1999.

182. McMasters, R. A., Wilbert, T. N., Jones, K. E., et al. Two-drug combinations that increase apoptosis and modulate bak and bcl-X(L) expression in human colon tumor cell lines transduced with herpes simplex virus thymidine kinase. Cancer Gene Ther, 7:563-573, 2000.

183. Rainov, N. G., Fels, C., Droege, J. W., et al. Temozolomide enhances herpes simplex virus thymidine kinase/ganciclovir therapy of malignant glioma. Cancer Gene Ther, 8:662-668, 2001.

184. Kim, J. H., Kim, S. H., Brown, S. L.Freytag, S. O. Selective enhancement by an antiviral agent of the radiation-induced cell killing of human glioma cells transduced with HSV-tk gene. Cancer Res, 54:6053-6056, 1994.

185. Rogulski, K. R., Kim, J. H., Kim, S. H.Freytag, S. O. Glioma cells transduced with an Escherichia coli CD/HSV-1 TK fusion gene exhibit enhanced metabolic suicide and radiosensitivity. Hum Gene Ther, 8:73-85, 1997.

186. Kanazawa, T., Mizukami, H., Okada, T., et al. Suicide gene therapy using AAV-HSVtk/ganciclovir in combination with irradiation results in regression of human head and neck cancer xenografts in nude mice. Gene Ther, 10:51-58, 2003.

187. Atkinson, G. Hall, S. J. Prodrug activation gene therapy and external beam irradiation in the treatment of prostate cancer. Urology, 54:1098-1104, 1999.

188. Ramesh, R., Munshi, A., Marrogi, A. J.Freeman, S. M. Enhancement of tumor killing using a combination of tumor immunization and HSV-tk suicide gene therapy. Int J Cancer, 80:380-386, 1999.

189. Freeman, S. M., Ramesh, R., Shastri, M., et al. The role of cytokines in mediating the bystander effect using HSV-TK xenogeneic cells. Cancer Lett, 92:167-174, 1995.

190. Ramesh, R., Marrogi, A. J., Munshi, A., Abboud, C. N.Freeman, S. M. In vivo analysis of the 'bystander effect': a cytokine cascade. Exp Hematol, 24:829-838, 1996.

191. Caruso, M., Panis, Y., Gagandeep, S., et al. Regression of established macroscopic liver metastases after in situ transduction of a suicide gene. Proc Natl Acad Sci U S A, 90:7024-7028, 1993.

192. Ramesh, R., Munshi, A., Abboud, C. N., Marrogi, A. J.Freeman, S. M. Expression of costimulatory molecules: B7 and ICAM up-regulation after treatment with a suicide gene. Cancer Gene Ther, 3:373-384, 1996.

193. Majumdar, A. S., Zolotorev, A., Samuel, S., et al. Efficacy of herpes simplex virus thymidine kinase in combination with cytokine gene therapy in an experimental metastatic breast cancer model. Cancer Gene Ther, 7:1086-1099, 2000.

194. Caruso, M., Pham-Nguyen, K., Kwong, Y. L., et al. Adenovirus-mediated interleukin-12 gene therapy for metastatic colon carcinoma. Proc Natl Acad Sci U S A, 93:11302-11306, 1996.

195. Molnar-Kimber, K. L., Sterman, D. H., Chang, M., et al. Impact of preexisting and induced humoral and cellular immune responses in an adenovirus-based gene therapy phase I clinical trial for localized mesothelioma. Hum Gene Ther, 9:2121-2133, 1998.

196. Klatzmann, D., Cherin, P., Bensimon, G., et al. A phase I/II dose-escalation study of herpes simplex virus type 1 thymidine kinase "suicide" gene therapy for metastatic melanoma. Study Group on Gene Therapy of Metastatic Melanoma. Hum Gene Ther, 9:2585-2594, 1998.

197. Ram, Z., Culver, K. W., Oshiro, E. M., et al. Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. Nat Med, 3:1354-1361, 1997.

198. Sandmair, A. M., Loimas, S., Puranen, P., et al. Thymidine kinase gene therapy for human malignant glioma, using replication-deficient retroviruses or adenoviruses. Hum Gene Ther, 11:2197-2205, 2000.

199. Klatzmann, D., Valery, C. A., Bensimon, G., et al. A phase I/II study of herpes simplex virus type 1 thymidine kinase "suicide" gene therapy for recurrent glioblastoma. Study Group on Gene Therapy for Glioblastoma. Hum Gene Ther, 9:2595-2604, 1998.

200. Germano, I. M., Fable, J., Gultekin, S. H.Silvers, A. Adenovirus/herpes simplex-thymidine kinase/ganciclovir complex: preliminary results of a phase I trial in patients with recurrent malignant gliomas. J Neurooncol, 65:279-289, 2003.

201. Gambhir, S. S., Barrio, J. R., Phelps, M. E., et al. Imaging adenoviral-directed reporter gene expression in living animals with positron emission tomography. Proc Natl Acad Sci U S A, 96:2333-2338, 1999.

202. Min, J. J., Iyer, M.Gambhir, S. S. Comparison of [(18)F]FHBG and [(14)C]FIAU for imaging of HSV1-tk reporter gene expression: adenoviral infection vs stable transfection. Eur J Nucl Med Mol Imaging, 2003.

203. de Vries, E. F., van Dillen, I. J., van Waarde, A., et al. Evaluation of [18F]FHPG as PET tracer for HSVtk gene expression. Nucl Med Biol, 30:651-660, 2003.

204. Koehne, G., Doubrovin, M., Doubrovina, E., et al. Serial in vivo imaging of the targeted migration of human HSV-TK-transduced antigen-specific lymphocytes. Nat Biotechnol, 21:405-413, 2003.

205. Cheshire, D. R. Isaacs, W. B. Beta-catenin signaling in prostate cancer: an early perspective. Endocr Relat Cancer, 10:537-560, 2003.

206. Brown, A. M. Wnt signaling in breast cancer: have we come full circle? Breast Cancer Res, 3:351-355, 2001.

207. Bierie, B., Nozawa, M., Renou, J. P., et al. Activation of beta-catenin in prostate epithelium induces hyperplasias and squamous transdifferentiation. Oncogene, 22:3875-3887, 2003.

208. Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J.Skarnes, W. C. An LDL-receptor-related protein mediates Wnt signalling in mice. Nature, 407:535-538, 2000.

209. Oyama, T., Kanai, Y., Ochiai, A., et al. A truncated beta-catenin disrupts the interaction between E-cadherin and alpha-catenin: a cause of loss of intercellular adhesiveness in human cancer cell lines. Cancer Res, 54:6282-6287, 1994.

210. Klingensmith, J. Nusse, R. Signaling by wingless in Drosophila. Dev Biol, 166:396-414, 1994.

211. Miller, J. R. Moon, R. T. Signal transduction through beta-catenin and specification of cell fate during embryogenesis. Genes Dev, 10:2527-2539, 1996.

212. Cadigan, K. M. Nusse, R. Wnt signaling: a common theme in animal development. Genes Dev, 11:3286-3305, 1997.

213. Korinek, V., Barker, N., Morin, P. J., et al. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science, 275:1784-1787, 1997.

214. Behrens, J., Jerchow, B. A., Wurtele, M., et al. Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science, 280:596-599, 1998.

215. Ikeda, S., Kishida, S., Yamamoto, H., et al. Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. Embo J, 17:1371-1384, 1998.

216. Orford, K., Crockett, C., Jensen, J. P., Weissman, A. M.Byers, S. W. Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. J Biol Chem, 272:24735-24738, 1997.

217. Aberle, H., Bauer, A., Stappert, J., Kispert, A.Kemler, R. beta-catenin is a target for the ubiquitin-proteasome pathway. Embo J, 16:3797-3804, 1997.

218. Easwaran, V., Song, V., Polakis, P.Byers, S. The ubiquitin-proteasome pathway and serine kinase activity modulate adenomatous polyposis coli protein-mediated regulation of beta-catenin-lymphocyte enhancer-binding factor signaling. J Biol Chem, 274:16641-16645, 1999.

219. Li, L., Yuan, H., Weaver, C. D., et al. Axin and Frat1 interact with dvl and GSK, bridging Dvl to GSK in Wnt-mediated regulation of LEF-1. Embo J, 18:4233-4240, 1999.

220. Roose, J. Clevers, H. TCF transcription factors: molecular switches in carcinogenesis. Biochim Biophys Acta, 1424:M23-37, 1999.

221. Hsu, S. C., Galceran, J.Grosschedl, R. Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with beta-catenin. Mol Cell Biol, 18:4807-4818, 1998.

222. Prieve, M. G. Waterman, M. L. Nuclear localization and formation of beta-catenin-lymphoid enhancer factor 1 complexes are not sufficient for activation of gene expression. Mol Cell Biol, 19:4503-4515, 1999.

223. Bordonaro, M., Mariadason, J. M., Aslam, F., Heerdt, B. G.Augenlicht, L. H. Butyrate-induced apoptotic cascade in colonic carcinoma cells: modulation of the beta-catenin-Tcf pathway and concordance with effects of sulindac and trichostatin A but not curcumin. Cell Growth Differ, 10:713-720, 1999.

224. Mariadason, J. M., Corner, G. A.Augenlicht, L. H. Genetic reprogramming in pathways of colonic cell maturation induced by short chain fatty acids: comparison with trichostatin A, sulindac, and curcumin and implications for chemoprevention of colon cancer. Cancer Res, 60:4561-4572, 2000.

225. Baulida, J., Batlle, E.Garcia De Herreros, A. Adenomatous polyposis coli protein (APC)-independent regulation of beta-catenin/Tcf-4 mediated transcription in intestinal cells. Biochem J, 344 Pt 2:565-570, 1999.

226. Yang, S. Z., Kohno, N., Kondo, K., et al. Adriamycin activates E-cadherin-mediated cell-cell adhesion in human breast cancer cells. Int J Oncol, 15:1109-1115, 1999.

227. Easwaran, V., Pishvaian, M., SalimuddinByers, S. Cross-regulation of beta-catenin-LEF/TCF and retinoid signaling pathways. Curr Biol, 9:1415-1418, 1999.

228. Karin, M., Liu, Z.Zandi, E. AP-1 function and regulation. Curr Opin Cell Biol, 9:240-246, 1997.

229. Piechaczyk, M. Blanchard, J. M. c-fos proto-oncogene regulation and function. Crit Rev Oncol Hematol, 17:93-131, 1994.

230. Ziff, E. B. Transcription factors: a new family gathers at the cAMP response site. Trends Genet, 6:69-72, 1990.

231. Treier, M., Staszewski, L. M.Bohmann, D. Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain. Cell, 78:787-798, 1994.

232. Tsurumi, C., Ishida, N., Tamura, T., et al. Degradation of c-Fos by the 26S proteasome is accelerated by c-Jun and multiple protein kinases. Mol Cell Biol, 15:5682-5687, 1995.

233. Treisman, R. The serum response element. Trends Biochem Sci, 17:423-426, 1992.

234. Sheng, M., Thompson, M. A.Greenberg, M. E. CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science, 252:1427-1430, 1991.

235. Hill, C. S., Wynne, J.Treisman, R. Serum-regulated transcription by serum response factor (SRF): a novel role for the DNA binding domain. Embo J, 13:5421-5432, 1994.

236. Marnett, L. J., Rowlinson, S. W., Goodwin, D. C., Kalgutkar, A. S.Lanzo, C. A. Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. J Biol Chem, 274:22903-22906, 1999.

237. Smith, W. L., Garavito, R. M.DeWitt, D. L. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem, 271:33157-33160, 1996.

238. Tanabe, T. Tohnai, N. Cyclooxygenase isozymes and their gene structures and expression. Prostaglandins Other Lipid Mediat, 68-69:95-114, 2002.

239. Jaffe, B. M. Prostaglandins and cancer: an update. Prostaglandins, 6:453-461, 1974.

240. Eberhart, C. E., Coffey, R. J., Radhika, A., et al. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology, 107:1183-1188, 1994.

241. Kargman, S. L., O'Neill, G. P., Vickers, P. J., et al. Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. Cancer Res, 55:2556-2559, 1995.

242. DuBois, R. N., Radhika, A., Reddy, B. S.Entingh, A. J. Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. Gastroenterology, 110:1259-1262, 1996.

243. Williams, C. S., Luongo, C., Radhika, A., et al. Elevated cyclooxygenase-2 levels in Min mouse adenomas. Gastroenterology, 111:1134-1140, 1996.

244. Dubois, R. N., Abramson, S. B., Crofford, L., et al. Cyclooxygenase in biology and disease. Faseb J, 12:1063-1073, 1998.

245. Tippetts, M. T., Varnum, B. C., Lim, R. W.Herschman, H. R. Tumor promoter-inducible genes are differentially expressed in the developing mouse. Mol Cell Biol, 8:4570-4572, 1988.

246. Vane, J. R., Bakhle, Y. S.Botting, R. M. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol, 38:97-120, 1998.

247. Hsi, L. C., Angerman-Stewart, J.Eling, T. E. Introduction of full-length APC modulates cyclooxygenase-2 expression in HT-29 human colorectal carcinoma cells at the translational level. Carcinogenesis, 20:2045-2049, 1999.

248. Nettelbeck, D. M., Rivera, A. A., Davydova, J., et al. Cyclooxygenase-2 promoter for tumour-specific targeting of adenoviral vectors to melanoma. Melanoma Res, 13:287-292, 2003.

249. Li, Q., Guo, Y., Xuan, Y. T., et al. Gene therapy with inducible nitric oxide synthase protects against myocardial infarction via a cyclooxygenase-2-dependent mechanism. Circ Res, 92:741-748, 2003.

250. Nagi, P., Vickers, S. M., Davydova, J., et al. Development of a therapeutic adenoviral vector for cholangiocarcinoma combining tumor-restricted gene expression and infectivity enhancement. J Gastrointest Surg, 7:364-371, 2003.

251. Casado, E., Gomez-Navarro, J., Yamamoto, M., et al. Strategies to accomplish targeted expression of transgenes in ovarian cancer for molecular therapeutic applications. Clin Cancer Res, 7:2496-2504, 2001.

252. Yamamoto, M., Alemany, R., Adachi, Y., Grizzle, W. E.Curiel, D. T. Characterization of the cyclooxygenase-2 promoter in an adenoviral vector and its application for the mitigation of toxicity in suicide gene therapy of gastrointestinal cancers. Mol Ther, 3:385-394, 2001.

253. Richards, C. A., Austin, E. A.Huber, B. E. Transcriptional regulatory sequences of carcinoembryonic antigen: identification and use with cytosine deaminase for tumor-specific gene therapy. Hum Gene Ther, 6:881-893, 1995.

254. Boucher, D., Cournoyer, D., Stanners, C. P.Fuks, A. Studies on the control of gene expression of the carcinoembryonic antigen family in human tissue. Cancer Res, 49:847-852, 1989.

255. Lan, K. H., Kanai, F., Shiratori, Y., et al. Tumor-specific gene expression in carcinoembryonic antigen--producing gastric cancer cells using adenovirus vectors. Gastroenterology, 111:1241-1251, 1996.

256. Kijima, T., Osaki, T., Nishino, K., et al. Application of the Cre recombinase/loxP system further enhances antitumor effects in cell type-specific gene therapy against carcinoembryonic antigen-producing cancer. Cancer Res, 59:4906-4911, 1999.

257. DiMaio, J. M., Clary, B. M., Via, D. F., et al. Directed enzyme pro-drug gene therapy for pancreatic cancer in vivo. Surgery, 116:205-213, 1994.

258. Kurane, S., Krauss, J. C., Watari, E., et al. Targeted gene transfer for adenocarcinoma using a combination of tumor-specific antibody and tissue-specific promoter. Jpn J Cancer Res, 89:1212-1219, 1998.

259. Israeli, R. S., Powell, C. T., Corr, J. G., Fair, W. R.Heston, W. D. Expression of the prostate-specific membrane antigen. Cancer Res, 54:1807-1811, 1994.

260. Troyer, J. K., Beckett, M. L.Wright, G. L., Jr. Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. Int J Cancer, 62:552-558, 1995.

261. Lopes, A. D., Davis, W. L., Rosenstraus, M. J., Uveges, A. J.Gilman, S. C. Immunohistochemical and pharmacokinetic characterization of the site-specific immunoconjugate CYT-356 derived from antiprostate monoclonal antibody 7E11-C5. Cancer Res, 50:6423-6429, 1990.

262. Liu, H., Moy, P., Kim, S., et al. Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. Cancer Res, 57:3629-3634, 1997.

263. Chang, S. S., Reuter, V. E., Heston, W. D., et al. Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. Cancer Res, 59:3192-3198, 1999.

264. Silver, D. A., Pellicer, I., Fair, W. R., Heston, W. D.Cordon-Cardo, C. Prostate-specific membrane antigen expression in normal and malignant human tissues. Clin Cancer Res, 3:81-85, 1997.

265. Dumas, F., Gala, J. L., Berteau, P., et al. Molecular expression of PSMA mRNA and protein in primary renal tumors. Int J Cancer, 80:799-803, 1999.

266. Hakalahti, L., Vihko, P., Henttu, P., et al. Evaluation of PAP and PSA gene expression in prostatic hyperplasia and prostatic carcinoma using northern-blot analyses, in situ hybridization and immunohistochemical stainings with monoclonal and bispecific antibodies. Int J Cancer, 55:590-597, 1993.

267. Stamey, T. A., Yang, N., Hay, A. R., et al. Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. N Engl J Med, 317:909-916, 1987.

268. Young, C. Y., Montgomery, B. T., Andrews, P. E., et al. Hormonal regulation of prostate-specific antigen messenger RNA in human prostatic adenocarcinoma cell line LNCaP. Cancer Res, 51:3748-3752, 1991.

269. Pang, S., Taneja, S., Dardashti, K., et al. Prostate tissue specificity of the prostate-specific antigen promoter isolated from a patient with prostate cancer. Hum Gene Ther, 6:1417-1426, 1995.

270. Schuur, E. R., Henderson, G. A., Kmetec, L. A., et al. Prostate-specific antigen expression is regulated by an upstream enhancer. J Biol Chem, 271:7043-7051, 1996.

271. Latham, J. P., Searle, P. F., Mautner, V.James, N. D. Prostate-specific antigen promoter/enhancer driven gene therapy for prostate cancer: construction and testing of a tissue-specific adenovirus vector. Cancer Res, 60:334-341, 2000.

272. Chen, R. H. McCormick, F. Selective targeting to the hyperactive beta-catenin/T-cell factor pathway in colon cancer cells. Cancer Res, 61:4445-4449, 2001.

273. Lipinski, K. S., Djeha, A. H., Ismail, T., et al. High-level, beta-catenin/TCF-dependent transgene expression in secondary colorectal cancer tissue. Mol Ther, 4:365-371, 2001.

274. Kwong, K. Y., Zou, Y., Day, C. P.Hung, M. C. The suppression of colon cancer cell growth in nude mice by targeting beta-catenin/TCF pathway. Oncogene, 21:8340-8346, 2002.

275. McMasters, R. A., Saylors, R. L., Jones, K. E., et al. Lack of bystander killing in herpes simplex virus thymidine kinase-transduced colon cell lines due to deficient connexin43 gap junction formation. Hum Gene Ther, 9:2253-2261, 1998.

276. Nettelbeck, D. M., Jerome, V.Muller, R. Gene therapy: designer promoters for tumour targeting. Trends Genet, 16:174-181, 2000.

277. Miller, N. Whelan, J. Progress in transcriptionally targeted and regulatable vectors for genetic therapy. Hum Gene Ther, 8:803-815, 1997.

278. Ring, C. J., Harris, J. D., Hurst, H. C.Lemoine, N. R. Suicide gene expression induced in tumour cells transduced with recombinant adenoviral, retroviral and plasmid vectors containing the ERBB2 promoter. Gene Ther, 3:1094-1103, 1996.

279. Block, A., Milasinovic, D., Mueller, J., et al. Amplified Muc1-specific gene expression in colon cancer cells utilizing a binary system in adenoviral vectors. Anticancer Res, 22:3285-3292, 2002.

280. Nyati, M. K., Sreekumar, A., Li, S., et al. High and selective expression of yeast cytosine deaminase under a carcinoembryonic antigen promoter-enhancer. Cancer Res, 62:2337-2342, 2002.

281. Gamallo, C., Palacios, J., Moreno, G., et al. beta-catenin expression pattern in stage I and II ovarian carcinomas: relationship with beta-catenin gene mutations, clinicopathological features, and clinical outcome. Am J Pathol, 155:527-536, 1999.

282. Inagawa, S., Itabashi, M., Adachi, S., et al. Expression and prognostic roles of beta-catenin in hepatocellular carcinoma: correlation with tumor progression and postoperative survival. Clin Cancer Res, 8:450-456, 2002.

283. Rubinfeld, B., Robbins, P., El-Gamil, M., et al. Stabilization of beta-catenin by genetic defects in melanoma cell lines. Science, 275:1790-1792, 1997.

284. Lin, S. Y., Xia, W., Wang, J. C., et al. Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. Proc Natl Acad Sci U S A, 97:4262-4266, 2000.

285. Yano, H., Hara, A., Takenaka, K., et al. Differential expression of beta-catenin in human glioblastoma multiforme and normal brain tissue. Neurol Res, 22:650-656, 2000.

286. Gayet, J., Zhou, X. P., Duval, A., et al. Extensive characterization of genetic alterations in a series of human colorectal cancer cell lines. Oncogene, 20:5025-5032, 2001.

287. Koehne, C. H. Dubois, R. N. COX-2 inhibition and colorectal cancer. Semin Oncol, 31:12-21, 2004.

288. Barker, N., Morin, P. J.Clevers, H. The Yin-Yang of TCF/beta-catenin signaling. Adv Cancer Res, 77:1-24, 2000.

289. Chang, S. S., O'Keefe, D. S., Bacich, D. J., et al. Prostate-specific membrane antigen is produced in tumor-associated neovasculature. Clin Cancer Res, 5:2674-2681, 1999.

290. Foecking, M. K. Hofstetter, H. Powerful and versatile enhancer-promoter unit for mammalian expression vectors. Gene, 45:101-105, 1986.

291. Kiela, P. R., Hines, E. R., Collins, J. F.Ghishan, F. K. Regulation of the rat NHE3 gene promoter by sodium butyrate. Am J Physiol Gastrointest Liver Physiol, 281:G947-956, 2001.

292. Chu, E. C., Chai, J.Tarnawski, A. S. NSAIDs activate PTEN and other phosphatases in human colon cancer cells: novel mechanism for chemopreventive action of NSAIDs. Biochem Biophys Res Commun, 320:875-879, 2004.

293. Chesire, D. R., Ewing, C. M., Sauvageot, J., Bova, G. S.Isaacs, W. B. Detection and analysis of beta-catenin mutations in prostate cancer. Prostate, 45:323-334, 2000.

294. Uchida, A., O'Keefe, D. S., Bacich, D. J., Molloy, P. L.Heston, W. D. In vivo suicide gene therapy model using a newly discovered prostate-specific membrane antigen promoter/enhancer: a potential alternative approach to androgen deprivation therapy. Urology, 58:132-139, 2001.

295. Lee, S. E., Jin, R. J., Lee, S. G., et al. Development of a new plasmid vector with PSA-promoter and enhancer expressing tissue-specificity in prostate carcinoma cell lines. Anticancer Res, 20:417-422, 2000.

296. Chastel, C., Jiricny, J.Jaussi, R. Activation of stress-responsive promoters by ionizing radiation for deployment in targeted gene therapy. DNA Repair (Amst), 3:201-215, 2004.

297. Wu, Q., Moyana, T.Xiang, J. Cancer gene therapy by adenovirus-mediated gene transfer. Curr Gene Ther, 1:101-122, 2001.

298. Chuah, M. K., Collen, D.VandenDriessche, T. Biosafety of adenoviral vectors. Curr Gene Ther, 3:527-543, 2003.

299. Dihlmann, S., Klein, S.Doeberitz Mv, M. K. Reduction of beta-catenin/T-cell transcription factor signaling by aspirin and indomethacin is caused by an increased stabilization of phosphorylated beta-catenin. Mol Cancer Ther, 2:509-516, 2003.

300. Boon, E. M., Keller, J. J., Wormhoudt, T. A., et al. Sulindac targets nuclear beta-catenin accumulation and Wnt signalling in adenomas of patients with familial adenomatous polyposis and in human colorectal cancer cell lines. Br J Cancer, 90:224-229, 2004.

301. Brunori, M., Malerba, M., Kashiwazaki, H.Iggo, R. Replicating adenoviruses that target tumors with constitutive activation of the wnt signaling pathway. J Virol, 75:2857-2865, 2001.

302. McLaughlin, P. M., Trzpis, M., Kroesen, B. J., et al. Use of the EGP-2/Ep-CAM promoter for targeted expression of heterologous genes in carcinoma derived cell lines. Cancer Gene Ther, 11:603-612, 2004.

303. Dong, D., Dubeau, L., Bading, J., et al. Spontaneous and controllable activation of suicide gene expression driven by the stress-inducible grp78 promoter resulting in eradication of sizable human tumors. Hum Gene Ther, 15:553-561, 2004.

304. Steffens, S., Sandquist, A., Frank, S., et al. A neuroblastoma-selective suicide gene therapy approach using the tyrosine hydroxylase promoter. Pediatr Res, 56:268-277, 2004.

305. Barshishat, M., Polak-Charcon, S.Schwartz, B. Butyrate regulates E-cadherin transcription, isoform expression and intracellular position in colon cancer cells. Br J Cancer, 82:195-203, 2000.

306. Kufe, D. Weichselbaum, R. Radiation therapy: activation for gene transcription and the development of genetic radiotherapy-therapeutic strategies in oncology. Cancer Biol Ther, 2:326-329, 2003.

307. Fodde, R. Smits, R. Disease model: familial adenomatous polyposis. Trends Mol Med, 7:369-373, 2001.

308. Hecht, A. Kemler, R. Curbing the nuclear activities of beta-catenin. Control over Wnt target gene expression. EMBO Rep, 1:24-28, 2000.

309. Dranoff, G. GM-CSF-secreting melanoma vaccines. Oncogene, 22:3188-3192, 2003.

310. Nakano, H., Kishida, T., Asada, H., et al. Interleukin-21 triggers both cellular and humoral immune responses leading to therapeutic antitumor effects against head and neck squamous cell carcinoma. J Gene Med, 2005.

311. Saika, T., Kusaka, N., Mouraviev, V., et al. Therapeutic effects of adoptive splenocyte transfer following in situ AdIL-12 gene therapy in a mouse prostate cancer model. Cancer Gene Ther, 2005.

312. Gregorieff, A. Clevers, H. Wnt signaling in the intestinal epithelium: from endoderm to cancer. Genes Dev, 19:877-890, 2005.

313. Liang, H. D. Blomley, M. J. The role of ultrasound in molecular imaging. Br J Radiol, 76 Spec No 2:S140-150, 2003.

314. Penuelas, I., Haberkorn, U., Yaghoubi, S.Gambhir, S. S. Gene therapy imaging in patients for oncological applications. Eur J Nucl Med Mol Imaging, 2005.

315. Nakagawa, S., Massie, B.Hawley, R. G. Tetracycline-regulatable adenovirus vectors: pharmacologic properties and clinical potential. Eur J Pharm Sci, 13:53-60, 2001.

VITA

SAURABH KUMAR GUPTA

Work address
700 West Olney Road
Eastern Virginia Medical School
Department of Microbiology and Molecular Cell Biology
Noroflk, Virginia 23507

Education
Old Dominion University and Eastern Virginia Medical School
Norfolk, Virginia
Doctor of Philosophy, Biomedical Sciences (August 2001 – December 2005)

Indian Institute of Technology (formerly, University of Roorkee)
Roorkee, India
Masters in Biosciences (July 1995 – July 1997)

University of Delhi
Delhi, India
Bachelor of Sceince, Biology (July 1992 – July 1995)

Professional Experience
Research Associate (June 2003 – October 2005)
Eastern Virginia Medical School

Graduate Resaech assistant (August 2001 – June 2003)
Eastern Virginia Medical School

Graduate research Assistant (August 1999 – July 2003)
University of Arkansas for Medical Science
Little Rock, Arkansas – 72205

Research Fellow (July 1998 – July 1999)
National Center for Cell Sciences
Pune, India

Research Fellow (September 1997 – June 1998)
National Institute of immunology
New Delhi, India