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Review Article

Modular Cre/lox System and Genetic Therapeutics for Colorectal Cancer

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The Cre/lox system is a powerful tool for targeting therapeutic effectors in a wide variety of human disorders. I review a Cre/lox Wnt-targeted system that has shown promise against Wnt-positive colorectal cancer cell lines. In addition to Wnt-specific targeting of cell death inducers, the modular nature of this gene therapy model system can be exploited by designing positive and negative feedback loops to either amplify or inhibit Wnt activity for experimental or therapeutic benefit. I discuss the structural components and performance parameters of the system, the implication of these findings with respect to cancer stem cells, as well as the general applicability of this system to any disorder characterized by differential gene expression. I also consider the issue of gene delivery as well as in vivo testing requirements necessary for the further characterization and development of this system.

1. Introduction

Wnt signaling is characterized by the stabilization of active, dephosphorylated β-catenin, which, when complexed with the T-cell factor/lymphocyte enhancer factor (Tcf-Lef) family of transcription factors, upregulates the expression of genes whose promoters contain Tcf/Lef-binding sites [1–6]. As a result of mutations in the *adenomatous polyposis coli* (*APC*) or *β-catenin* genes, the majority of human colorectal cancers (CRCs) exhibit constitutive Wnt pathway activity, which has been implicated as a crucial step in the initiation and development of colonic tumorigenesis [7–9]. Hyperactivation of Wnt activity, which can be achieved by exposure of CRC cells to inhibitors of histone deaceylases (HDACis) such as butyrate, induces apoptosis of these cells and thus may constitute an important preventative and therapeutic strategy against CRC [10]. However, in some cases, hyperactivation of Wnt activity may not be the most efficient treatment of choice. CRC cells can develop resistance to HDACis; thus, in some patients, early stage CRCs may be resistant to the effects of butyrate derived from dietary fiber [11]. In addition, novel therapeutic strategies are essential for advanced/metastatic CRC. In general, the life expectancy for individuals with advanced CRC is only ∼2 years [12]. Furthermore, in many advanced cases, surgical intervention is no longer possible, and even where surgery is performed, methodologies to ensure full eradication of neoplastic cells and to suppress recurrence would be desirable. An alternative strategy against CRC is the use of CRC-targeted gene therapy methodologies, particularly those targeting aberrant Wnt signaling activation.

Gene therapy approaches for CRC [13] have utilized genes (a) inducing cell death [14–22], (b) encoding for enzymes converting prodrugs to their active forms [23–26], and (c) necessary for viral replication, which kills neoplastic cells [27, 28]. Cancer-specific gene therapy depends on differences in gene expression between neoplastic and normal cells; therefore, expression of a gene must be both specific to abnormal cells and strong enough to efficiently inhibit cell proliferation or induce cell death. Wnt-targeted therapeutic expression, in which expression and activity of the relevant gene product occurs specifically in Wnt-positive cells, has been successfully demonstrated [13, 21, 25, 27, 28]. This approach is particularly useful in CRC, since the majority of CRCs exhibit deregulated Wnt activity and the vast majority of normal colonic cells are Wnt negative. Therefore, Wnt-specific expression of therapeutic genes would direct therapeutic activity to the Wnt-positive cancer cells while sparing the bulk of the normal cells.

However, studies on utilizing Wnt-targeted therapeutic expression have been limited in that they did not undertake a systematic analysis of the factors influencing the effectiveness and specificity of Wnt-mediated gene therapy. For example, CRC cells intrinsically vary in their levels of Wnt activity [29–35]. Variability in Wnt activity is likely an in vivo property of CRCs which may complicate Wnt-directed gene therapy. Despite this, Wnt-targeted therapeutic gene expression holds great promise for anti-CRC gene therapy approaches [21], by specifically inducing cell death in Wnt-expressing malignant colonic cells, while sparing normal cells. Further, given that Wnt deregulation is one of the earliest events in colonic tumorigenesis [1–6], Wnt-targeted gene therapy can also be adapted as a preventive strategy to eliminate early neoplasms (adenomas) that exhibit deregulated Wnt activity. However, given the inherent variability of Wnt activity in colonic neoplasms, this targeting strategy requires optimization through the use of a system that enhances both the efficiency and the specificity of effector gene expression. The Cre/lox system [36] represents such an approach to enhance expression from tissue- or disease-specific promoter activity.

2. Structural Components of System

The ability of the Cre/lox system to enhance expression from specific promoters [31, 32, 37–39] has been exploited to construct a modular Wnt-targeted therapeutic system [33]. The rationale behind the Cre/lox methodology is as follows. The enzyme Cre recombinase excises gene sequences placed in between tandem consensus sequences called loxP sites. A stop cassette, flanked by tandem loxP sites ("floxed"), is placed between a strong promoter and the gene of interest (i.e., the effector). The stop cassette contains a SV40 polyadenylation site that serves to end the mRNA at that point and thus prevents inclusion of the downstream effector sequences in the mature transcript [[31, 36], and references therein]. In addition, an unpaired splice donor site and an extra translation start site is downstream of the polyadenylation site in order to abrogate normal RNA processing and translation in the event that inefficient use of the SV40 polyadenylation site extends the transcript past that site [[31], and references therein]. In the absence of Cre recombinase, the effector is not expressed due to the presence of the retained stop cassette. However, in the presence of Cre recombinase, the stop cassette is excised, allowing for efficient effector expression from the strong constitutive promoter. Specificity is generated through the use of a tissue/disease-specific and/or inducible promoter to drive Cre expression. Thus, the Cre/lox approach is a "two-step" expression system, in which specificity of expression at the initial Cre expression step allows for high-level, efficient expression at the secondary effector expression step (Figure 1). Because of the advantages of the Cre/lox system in maximizing both efficient and specific gene expression, this methodology is generally superior to alternatives in which effectors are directly expressed from Wnt-sensitive promoters [21, 34, 39]. Low levels of Cre recombinase are sufficient to catalyze the excision of high concentrations of floxed DNA sequences; therefore, expression from highly specific promoters driving Cre recombinase expression is amplified by the enzymatic activity of the Cre recombinase. In most cases, the Cre and loxP/stop cassette components of the system are in different vectors, which are introduced together into the cells (i.e., cotransfected). However, it is possible to combine the two components into a single vector as long as both transcription units are kept separate [31].

Wnt-specific expression of Cre recombinase will therefore result in Wnt-specific expression of the floxed gene of interest. The floxed gene could be, for example, an inducer of cell death. Thus, if Wnt activity is primarily characteristic of neoplastic cells, then Wnt-specific activation of the Cre/lox system will drive highly specific expression of the cell death inducer, killing cancer cells while sparing their normal counterparts. The modular model system under consideration here (Figure 1) utilized a variety of Wnt-specific promoters to drive Cre expression. Cre recombinase activity, in turn, promoted the expression of the floxed effectors, which were a luciferase reporter, FADD or diphtheria toxin A-chain (DT-A) cell death genes, or effectors capable of up- or downregulating Wnt activity. FADD, which induces cell death through activation of caspase 8 as part of the Fas-mediated apoptotic pathway [35], had been previously used as an effector in a Wnt-targeted system [21]. The DT-A, which induces cell death by ADP ribosylation of elongation factor 2 and irreversible inhibition of protein synthesis, had also been established as a potent effector gene in several gene therapy systems [14–18]. Other effectors that were utilized in this system included Lef-VP16, which upregulates Wnt signaling [40], or dominant negative Tcf4 (DN-Tcf4), which downregulates Wnt activity [5]. The Wnt-specific promoters consisted of minimal TK, Fos, CMV, or TATA-box promoters coupled to either modular Cre/lox Wnt-mediated multimerized wild-type ("TOP") or mutant, inactive ("FOP") Tcf-Lef binding sites. The TOP promoters therefore were Wnt-sensitive and directed Wnt-specific Cre expression; the FOP promoters were insensitive to Wnt activity and exhibited only background expression from the minimal promoter.

A promoterless Cre construct served as a negative control (Figure 1).

The following example illustrates how this system would work in principle. Assume that the effector vector contains floxed luciferase reporter. The Cre expression vectors would be in other separate plasmid vectors. For the purposes of illustration, we will assume that these Cre vectors utilize the CMV minimal promoter (TOPCMV-CRE, FOPCMV-CRE, and the promoterless 0-CRE). The floxed luciferase vector would be cotransfected into Wnt-positive CRC cells along with one of the three Cre expression constructs. TOPCMV-CRE contains the Wnt-sensitive TOP promoter containing Tcf-binding sites. Therefore, in Wnt-positive CRC cells, TOPCMV-CRE would express levels of Cre recombinase sufficient to remove the stop cassettes from most of the cotransfected floxed luciferase plasmids, resulting in high levels of luciferase expression and activity. However, in cells cotransfected with FOPCMV-CRE and the floxed luciferase vector, very little luciferase activity is expected;

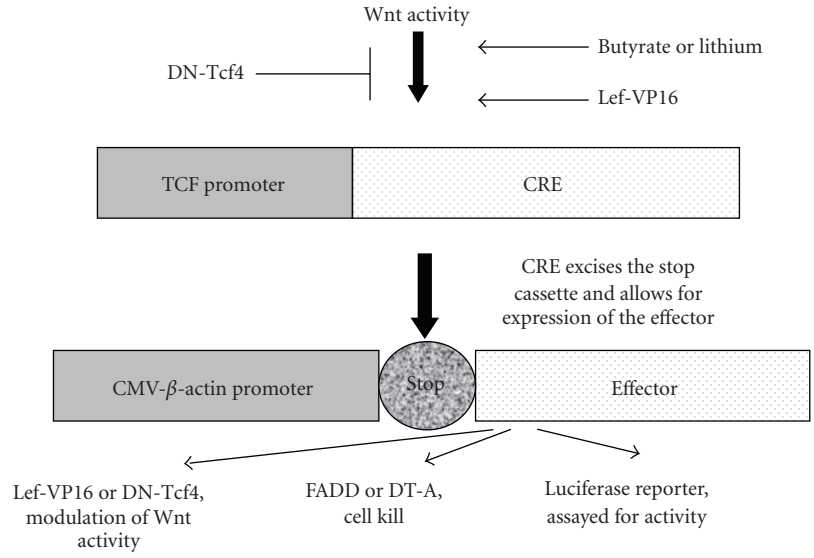

Figure 1: Schematic of the model system. Wnt signaling, which can be up- or downregulated by pharmacological or genetic modulators, promotes expression from Wnt-sensitive promoters, driving production of Cre recombinase. Cre excises stop cassettes from floxed expression vectors, allowing for production of effectors. Included in the effectors are genes that can positively or negatively feed back to influence Wnt activity and, hence, Cre expression. This model system can be generally applicable to any human disorder characterized by aberrant gene expression involving disorder-specific transcription factors, reproduced as per copyright agreement from [33].

the FOP promoter, containing mutant binding sites, is not activated by Wnt signaling. Therefore, very low levels of Cre recombinase are produced by the weak background activity of the minimal CMV promoter fragment of FOPCMV-CRE. 0-CRE, lacking a promoter, should not express any Cre at all.

There are several issues of importance with respect to the structural design and performance parameters of this modular system. In human patients, the environment in which this therapy system would operate would consist of both aberrant Wnt-positive cells and normal Wnt-negative cells (effects of this system on normal Wnt-positive cells are discussed below). However, CRC cell lines used to test the system in vitro are, typically, Wnt-positive only. To test the specificity of the system in vitro, it is necessary to mimic the lack of Wnt activity of normal cells in neoplastic CRC cells that possess constitutively activated Wnt signaling. This was addressed by utilizing the TOP/FOP promoter system (Figure 1). Cre expression driven by TOP promoters will be Wnt-specific; hence, effector expression resulting from Cre excision of the stop cassette will also be Wnt-specific. Cre expression from the mutant FOP promoters will be insensitive to Wnt activity; therefore, in cells transfected with FOP-CRE and floxed effector constructs, the expression of the effector will also be insensitive to Wnt activity. Note the similarity between (a) introduction of TOP-CRE and floxed effector into Wnt-negative normal cells in vivo and (b) introduction of FOP-CRE and floxed effector into Wnt-positive CRC cell lines in vitro; in both cases, Cre expression and consequent effector activity will be low or nonexistent. In the first case (i.e., example a), this is because there is no Wnt activity in the normal cells to stimulate Cre expression. In the second case (i.e., example b), Wnt activity is present, but the mutant FOP promoter is insensitive to this Wnt activity and very little CRE is produced. Therefore, the lack of effector expression from the FOP-CRE constructs in Wnt-positive cells in vitro models the lack of effector activity which would be exhibited by the wild-type TOP promoters in Wnt-negative cells in vivo.

Second, the terms "efficiency" and "specificity" must be defined for this system. Efficiency refers to the maximization of effector expression; on the other hand, specificity refers to targeting effector expression in a Wnt-specific manner. In vivo, optimizing efficiency and specificity would mean the highest possible effector expression in targeted Wnt-positive cells coupled with the least possible effector expression in Wnt-negative cells. In vitro, maximal efficiency of effector expression will be observed with the wild-type TOP promoters driving Cre expression; specificity will be observed in the difference between effector expression resulting from use of the TOP versus FOP promoters.

Third, it is crucial to understand the fundamental differences in the dynamics of a luciferase reporter system compared with a cell kill model. In the specific case of luciferase reporter assays, I have observed that the activity of the reporter generally correlates with the level of expression of the reporter gene in a linear fashion (Figure 2(a)); thus, the more luciferase produced, the higher the luciferase activity [33]. In contrast, once a level of FADD or DT-A sufficient to kill cells with ~100% certainty is reached, further expression of that gene is superfluous (Figure 2(b)). A given cell can only be killed once; expression of FADD or DT-A beyond the level required to kill the cell cannot further increase the efficiency of cell death. Therefore, for cell kill, a roughly linear correlation between expression and activity exits only within a relatively narrow range of effector expression. At the lower end of the range of effector expression, effector levels are

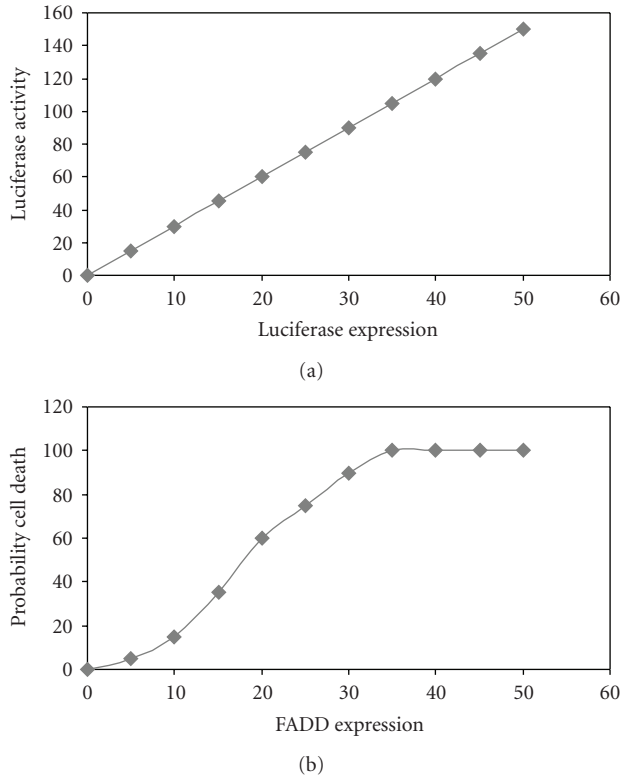

Figure 2: Reporter activity differs from cell death induction. (a) Graph modeling the relationship between luciferase expression and luciferase reporter activity, within the linear range of detection of a luminometer; an essentially linear relationship is depicted. (b) Graph modeling the relationship between concentration of the cell death inducer FADD and probability of cell death for cells in the transfected cell population. An essentially sigmoidal curve is depicted, as the probability of cell death cannot exceed 100% regardless of increasing levels of FADD. For both (a) and (b) arbitrary units of measurement are shown for the purposes of illustration.

too low to result in any detectable cell kill. At higher levels of effector expression, the probability of cell kill reaches ~ 100%; beyond that level of expression, saturation of effector activity occurs.

On the other hand, cell death, while also quantitative, is a "yes or no" binary measurement, and the probability of death of any given cell is intrinsically saturable; increasing effector expression beyond what is required for ~100% cell kill will have no further effects. Therefore, a graph of luciferase effector expression versus luciferase activity is expected to result in an upwardly sloping straight line (Figure 2(a)), while a graph of FADD effector concentration versus probability of cell kill is expected to level off at higher effector concentrations, producing an essentially sigmoidal curve (Figure 2(b)). This would suggest that using reporter systems to model cell death may yield misleading findings, since the dynamics of these effectors are different.

Gene therapy systems for CRC have been developed in which effectors are directly linked to Wnt-specific promoters, without the additional step of Cre-mediated amplification of expression [21, 34]. Although the efficacy of these systems has been demonstrated, they lack the fine control of effector expression exhibited by the more complex Cre/lox system [33, 34]. This fine control is made possible by the amplification of effector expression that results from the catalytic activity of Cre recombinase. Theoretically, four molecules of Cre recombinase can repeatedly catalyze the excision of stop cassettes from many molecules of floxed effector; thus, relatively small amounts of Cre produced from low concentrations of Cre-expressing vectors can result in high levels of therapeutic effector gene expression. It is therefore possible to titrate the Cre vector to low concentrations that minimize nonspecific background expression, while maintaining high levels of specific, disease-targeted effector expression. Separating Wnt-specificity (Cre vector) from strong effector expression (floxed vector) allows the Cre/lox system to combine high specificity with high efficiency. On the other hand, *direct* expression of the therapeutic gene bypasses the Cre-mediated amplification step. Here, high levels of effector expression will require higher concentrations of the gene therapy vector, resulting in greater background expression of the effector and a consequent loss of specificity.

3. Activity of the Cre/Lox Wnt Model System

The Cre/lox system described (Figure 1) was shown to induce efficient and specific Wnt-targeted effector activity when the CMV minimal promoter was coupled to TOP sequences and used to drive Cre expression, which in turn activates FADD expression to kill the targeted cells (Figure 3). In these experiments, a vector expressing luciferase from a constitutive promoter was cotransfected with the Cre and floxed effector gene therapy vectors. Luciferase activity was used as a marker of cell transfection to determine the relative number of remaining cells.

In these experiments, cell type differences were observed; efficient and specific cell kill in the HCT-116 CRC cell line required cotreatment with both butyrate and lithium chloride, while SW620 CRC cells exhibited efficient cell kill in both the presence and absence of butyrate [33]. These differences between HCT-116 and SW620 CRC cells in response to Wnt-specific Cre/lox-mediated effector activity suggest that a given genetic therapeutic strategy may not be optimal for all CRCs. HCT-116 and SW620 CRC cells may therefore represent subtypes of CRCs in vivo which would exhibit differential responses to Wnt-targeted gene therapy.

This modular Cre/lox system was able to rescue suboptimal concentrations of transfected Cre expression vectors, and induce efficient cell death, by utilizing floxed Lef-VP16 alongside floxed FADD (Figure 4) [33]. In the absence of floxed Lef-VP16, very low levels of Cre expression could not sufficiently activate FADD expression to induce cell death. In the presence of floxed Lef-VP16, the low levels of Cre expression activated Lef-VP16 expression. Lef-VP16 bound to the TOP promoter and, in turn, induced higher levels of Cre expression. These greater levels of Cre expression resulted in more efficient expression of FADD and increased cell death.

Thus, given that the floxed Lef-VP16 was shown to act in positive feedback fashion to enhance Wnt activity in a Wnt-specific manner, these data suggest that weak Wnt signals can be amplified by an Lef-VP16 effector, resulting in efficient and Wnt-specific effector expression over a wide range of endogenous Wnt activity levels. Alternatively, utilizing floxed DN-Tcf as the effector, Wnt-specific down regulation of Wnt activity, constituting a negative feedback system, was demonstrated (Figure 5) [33]. Downregulation of Wnt activity may useful for CRC resistant to the ability of butyrate to enhance Wnt activity and apoptosis [10, 11, 30].

4. Modular Nature of System

A novel feature of the Wnt-targeted Cre/lox system is its modular nature. This is best represented by Lef-VP16 and DN-Tcf4 as effectors to up- or downregulate Wnt signaling in a Wnt-dependent manner (Figures 4 and 5). Positive or negative feedback loops can be applied to any signaling pathway which generates aberrant gene expression, by creating a floxed dominant negative form of a key transcription factor of that pathway (negative feedback regulation) or by fusing the DNA binding domain of a transcription factor to VP16 (positive feedback regulation). One objective of negative feedback regulation could include directly inhibiting an aberrantly activated signaling pathway for therapeutic effect; this self-downregulation method can be applied to any situation in which a dominant-negative protein inhibits a signaling pathway essential for cell growth and/or survival. Alternatively, positive feedback regulation could entail stimulating the relevant signaling pathway in order to enhance the efficiency of the Cre/lox system, resulting in increased effector expression. Another aspect of this modular system is the ability to use aberrant activation of one signaling pathway to induce targeted activation of a more general, second signaling pathway. Thus, for example, a cancer specific signaling pathway can be used to drive Cre recombinase expression, which would then activate a transcription factor (the floxed effector) involved in a second signaling pathway, for example, inducing differentiation or some other desired effect. In summary, virtually any physiological process characterized by specific gene expression, and by defined transcription factors, can be (therapeutically) modulated by the Cre/lox system. In particular, positive and negative feedback loops, made possible with this modular system, are well suited for "fine tuning" transcriptional activity, leading to more precise and tightly controlled amplification or repression of relevant signaling pathways.

5. Safer Gene Delivery Options

The design of an effective gene therapy vector system is only part of the requirement for effective therapy; an efficient and safe delivery system must also be designed and evaluated. Viral vectors have been the primary method for delivering genes into host cells and achieving gene expression; however, viral vectors suffer from several drawbacks, including toxicity, immunogenicity, and the potential replication of competent viruses. Furthermore, several deaths have been likely associated with the use of viral vectors in gene therapy trials [41–43]. Thus, the development of nonviral gene therapy delivery systems is necessary to make gene therapy approaches safer and more practical for the human patient. An effective nonviral vector must efficiently enter and transfect a cell without stimulating an immune response, and must also be nontoxic. Biodegradable nanoparticles are generally superior to other nonviral vectors, since they are comparable in size to viruses and are therefore internalized in a similar manner, while being large enough to carry genetic material such as plasmid DNA [44]. L-tyrosine polyphosphate (LTP) nanoparticles have been produced and have shown considerable promise in initial studies [44]. Plasmid DNA is released from these nanoparticles over a period of 14 days, optimal for intracellular delivery of therapeutic gene sequences, and these nanoparticles exhibit a lack of toxicity during this time period [44]. Further, using known coupling techniques [45–47], nanoparticles can be complexed with antibodies, thus targeting gene delivery to cells expressing specific cell surface markers, enhancing both the efficacy and safety of the system. Nanoparticle systems are well suited for use with the Cre/lox system; LTP nanoparticles have been tested with plasmids [44] and will be able to carry both the Cre and lox components of a modular gene therapy system. Given the nontoxic nature of the this gene delivery system, repeated and overlapping applications of Cre/lox system-containing nanoparticles are likely to result in significant levels of targeted cell death with relatively low levels of side effects due to immunogenicity or toxicity.

6. Wnt-Targeted Therapy and CRC Stem Cells

Cancer stem cells are a subpopulation of neoplastic cells that exhibit the defining characteristics of stem cells: the ability to self-proliferate and the ability to differentiate into varied cell types. The leading theory of cancer stem cells [48] suggests that these cells give rise to tumors and are responsible for metastases and relapse, the latter occurring when treatment eliminates the bulk of relatively differentiated tumor cells, while leaving the cancer stem cell population unaffected. The "migrating cancer stem cell" (MCS) concept of Brabletz et al. [49] may be of relevance to Wnt-directed therapy. These authors make a distinction between stationary cancer stem (SCS) cells, which are anchored in the epithelial tissue and do not migrate, and MCS cells, which possess the same proliferative and differentiating potential as the SCS cells, but exhibit a migratory phenotype. MCS cells are derived from their stationary counterparts by acquiring a transient epithelial to mesenchymal transition (EMT), which allows them to disseminate. This dissemination can either be short-range, resulting in expansion of the primary tumor, or long-range through blood and/or lymphatic vessels, resulting in metastases. Once established in the new location, these cells may undergo a reversal of EMT, or a mesenchymal to epithelial transition (MET), which reverses the growth arrest (and resistance to apoptosis) that characterizes the EMT phenotype. This MET ultimately results in enhanced

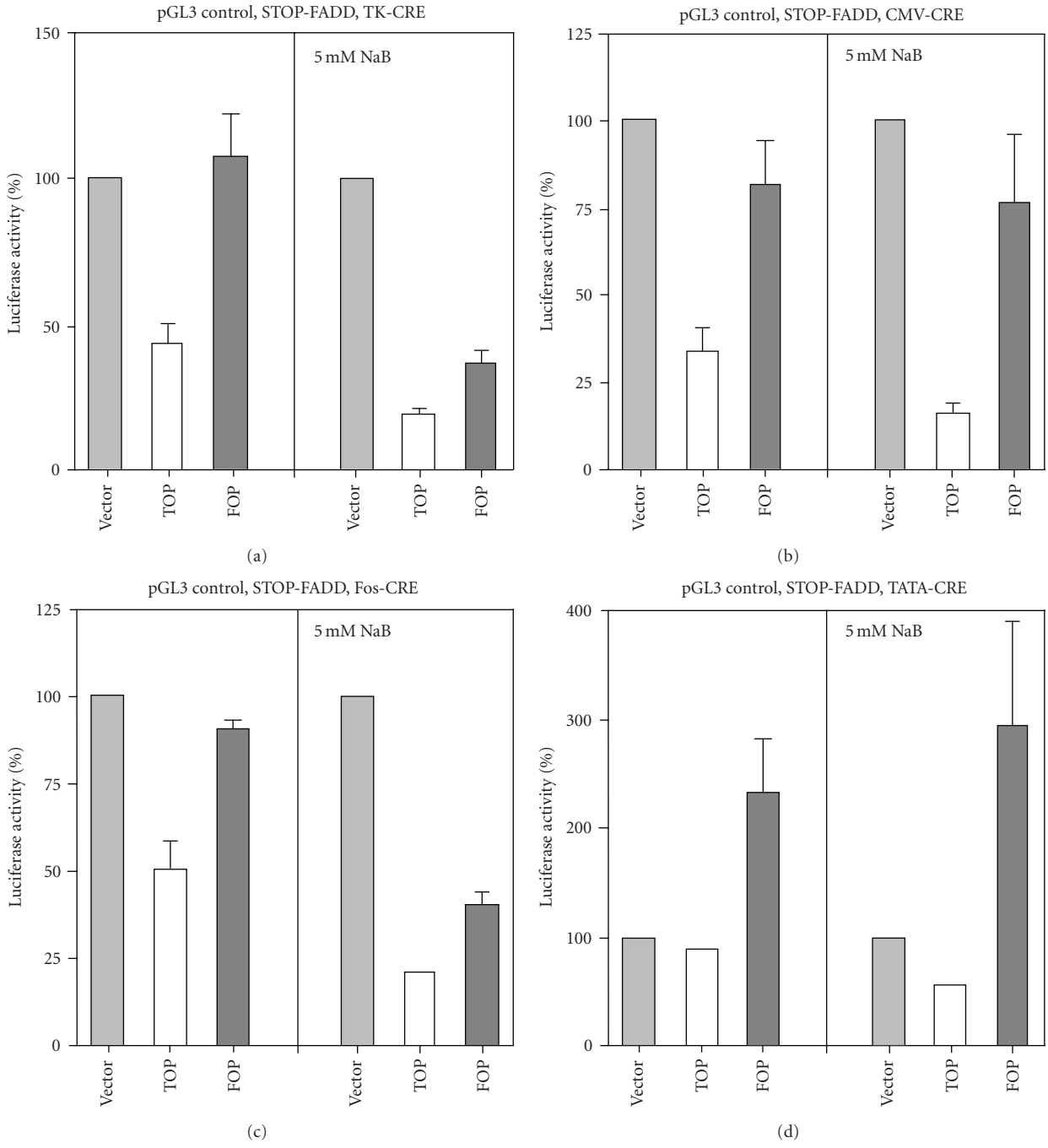

Figure 3: Wnt-specific SW620 cell kill. SW620 cells in a 24-well plate were transfected with 0.05 μg of pGL3-Ctl, 0.5 μg of STOP-FADD, and (a) 0.05 μg of TOP/FOPTK-CRE, (b) 0.0067 μg of TOP/FOPCMV-CRE, (c) 0.1 μg of TOP/FOPFos-CRE, or (d) 0.15 μg of TOP/FOPTATA-CRE, with equivalent amounts of the 0-CRE control expression vector per well, and untreated or treated with 5 mM butyrate, and assayed for luciferase expression 48 hours later. Luciferase readings were normalized to protein concentration determined for each sample. The input plasmids for each experiment are listed at the top of each graph. Results are from three independent experiments. Bars indicate standard deviations, reproduced as per copyright agreement from [33].

proliferation, differentiation, and growth of the metastasis [49]. Wnt activity is intimately involved in these processes, as this signaling pathway has been linked to stem cell generation and maintenance [50, 51] as well as the establishment of EMT [49]. Several Wnt target genes promote the stem cell phenotype, among them survivin, which induces proliferation while inhibiting apoptosis; another set of Wnt target genes, such as SLUG, L1CAM, and LAMC2, are associated with EMT [49]. The first set of "stemness" genes are expressed early in colonic tumorigenesis and likely require

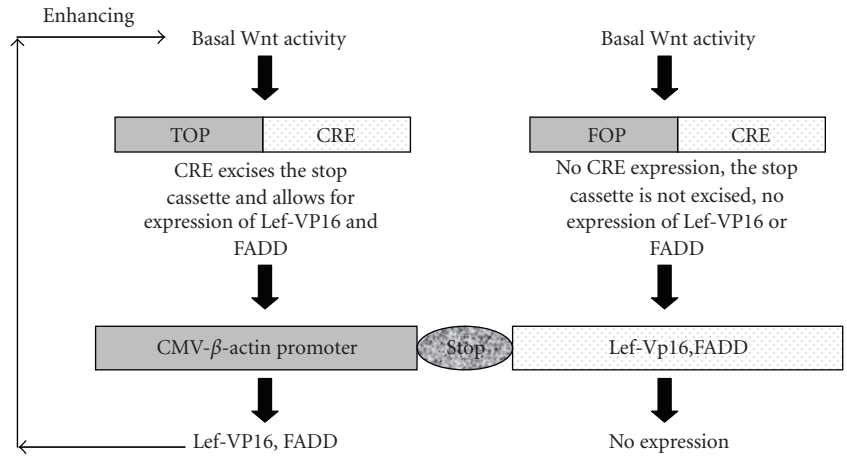

Figure 4: Positive upregulation using the modular Cre/lox system. Floxed effectors can include positive upregulators of the transcriptional activity driving Cre expression. For example, in this model system, Lef-VP16, which upregulates Wnt activity, is expressed from a floxed vector in a Wnt activity-specific manner. The Lef-VP16 feeds back to stimulate Wnt activity, resulting in higher levels of Cre expression and, hence, more production of the floxed effectors, which includes not only the Lef-VP16 itself but also gene products resulting in, for example, cell death. Positive feedback increases the overall efficiency of the system, amplifying the original transcriptional signal, and can be used to enhance the effectiveness of the system under suboptimal conditions, adapted from [33].

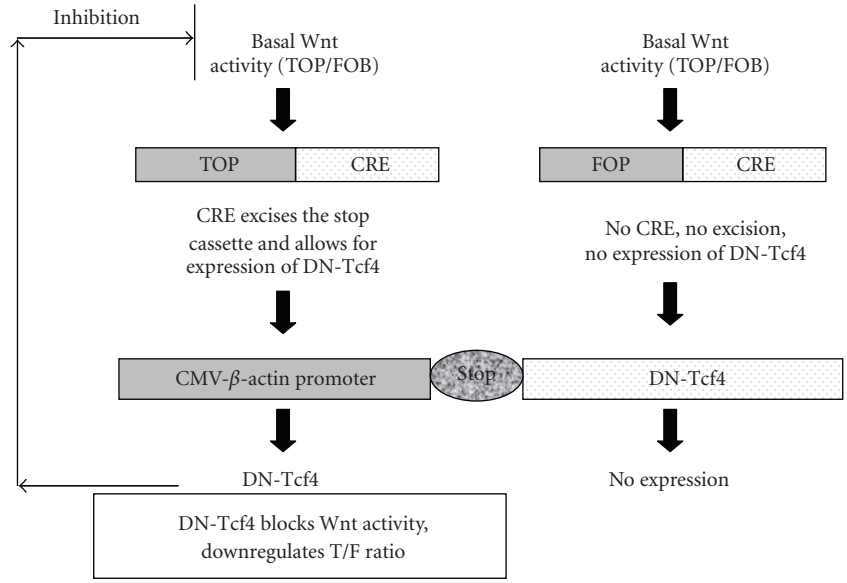

Figure 5: Negative downregulation using the modular Cre/lox system. Floxed effectors can include negative repressors of the transcriptional activity driving Cre expression. For example, in this model system, DN-Tcf4, which inhibits Wnt activity, is expressed from a floxed vector in a Wnt activity-specific manner. The DN-Tcf4 feeds back to repress Wnt activity in a Wnt activity-specific manner. Generalized systems of this nature can be applied to other disorders in which constitutive high activation of cell signaling causes disease, adapted from [33].

low levels of constitutive Wnt activity; the expression of these genes is maintained throughout the process of tumorigenesis [49]. However, EMT-related genes are transiently upregulated in the invasive-front cells; these are likely MCS cells that express high levels of nuclear beta-catenin and Wnt activity. EMT-related gene expression is subsequently downregulated during MET, leading to the differentiation of cells which exhibit lower levels of nuclear beta-catenin [49]. Wnt-targeted gene therapy should therefore represent a reasonable approach for targeting CRC stem cells, since these cells both (a) exhibit Wnt signaling and (b) are apparently more dependent on Wnt activity for maintenance and progression compared to the more differentiated cells within the tumor. The fact that MCS cells exhibit high levels of nuclear beta-catenin suggests greater levels of Wnt activity in these cells. Therefore, it may be possible to design Wnt-targeted "calibrated" so that they produce effector output in the presence of high, aberrant Wnt activity in MCS cells but not in the presence of lower levels of Wnt activity that characterize normal stem cells in the colonic crypt. This

approach would target the dangerous cancer stem cells, while leaving normal stem cells relatively unaffected. Further, Wnt target genes associated with EMT can be utilized to target therapeutic expression to CRC stem cells.

7. In Vivo Cre/Lox Function, Differences with In Vitro Evaluation

In general, successful gene therapy for the elimination of abnormality (e.g., neoplastic cells, abnormal gene expression, and expression of mutant proteins) is more difficult to achieve than is the restoration of diminished normal function. In the former case, in vivo therapeutic success often requires ~100% elimination of the abnormality (e.g., ~100% elimination of neoplastic cells, to prevent recurrence), while, in the latter case, even partial restoration of normal gene expression and normal function may be sufficient to alleviate or eliminate disease.

There are significant differences between gene therapy (including Cre/lox) approaches for the in vitro (cell culture) and in vivo (animal models, humans) situations. Any given in vitro experiment usually utilizes a single cell type growing as a monolayer, while in vivo work deals with whole animals with integrated organ systems and a wide variety of tissue types arranged in a complex three dimensional architecture. Further, a given cell type in culture may be either normal or abnormal (e.g., cancer cell line), but an in vivo disease model, or human patient, will most often consist of a small fraction of abnormal cells within a great mass of normal tissues. Thus, issues of targeting and side effects are more important in vivo than in vitro. CRC, a disease for which Wnt-targeted therapeutic expression would be particularly effective, illustrates these points.

For example, in contrast to a CRC cell line in culture, an in vivo tumor consists of a mass of neoplastic cells within an organism that most likely otherwise consists of healthy tissues and organ systems. Not all CRCs are Wnt positive, although the great majority is; those that are not will require genetic targeting other than that of Wnt activity. Of those CRCs that are Wnt positive, the levels of Wnt signaling may vary, requiring different approaches for Wnt-targeted gene therapy. As an in vitro analogy, SW620 cells exhibited efficient and specific Wnt-targeted cell kill by Cre/lox-mediated FADD expression in the presence or absence of butyrate, while HCT-116 cells required cotreatment with butyrate and lithium chloride to allow for efficient and specific Cre/lox-mediated cell death [33]. Even more fundamentally, normal cells, including intestinal stem cells, exhibit low basal Wnt activity levels. Therefore, given the efficiency of the Cre/lox system to induce gene expression, the possibility exists that a Wnt-targeted system may result in off-target normal cell death, inducing unwanted side effects. To some extent, it may be possible to design relatively insensitive Wnt responsive promoters, so that Cre expression will only occur at a relatively high threshold level of Wnt activity exhibited by CRC cells, but not exhibited by the weakly Wnt-positive stem cells of intestinal crypts.

However, it is possible that some degree of off-target cell death of normal Wnt positive cells is inevitable with a Wnt-targeted gene therapy system. Side effects from such treatment may be analogous to that observed with chemotherapy or radiation and can be acceptable if significant therapeutic benefit occurs. As part of in vivo testing of the Wnt-targeted Cre/lox system in mouse models of CRC, it will be important to monitor side effects of treatment, particularly focused on cells/tissues known to require basal Wnt signaling (e.g., intestinal stem cells, hair follicles). Wild-type mice should be evaluated alongside their cancer model counterparts, to determine if Wnt-targeted effector expression results in unwanted morbidity and/or mortality, and to what extent this occurs. The specific sites of these off target effects must be identified; determination of the mechanism(s) by which these side effects occur will assist in developing approaches to minimize off targeting and its associated morbidity/mortality. Wnt-targeted therapy must be shown to exhibit a sound safety profile in mouse models before clinical trials can begin in human subjects.

It also remains to be determined whether the more complex positive and negative feedback loops described in Figures 4 and 5 of the current review would be as effective in vivo as they are in vitro. To effectively create such feedback systems in vivo will require delivery of all components of the Cre/lox system into the same cells at the same time, which is in large part a function of the delivery methods utilized. Cotransfection in vitro is not difficult, but the same approach in vivo may require that all components of the system should be incorporated into the same delivery vehicle.

Delivery of the gene therapy vectors (see also Section 5, above) is another area in which in vitro and in vivo experiments are expected to significantly differ. Cells in culture can be transfected by means not feasible or safe for whole organisms. Viral delivery is effective both in vitro and in vivo; however, effective viral gene delivery into a cell culture monolayer will be significantly easier to achieve than into most, or all, of the cells of a solid tumor in vivo. Methods for introducing viral vectors more directly into the targeted tissue of mouse models, such as injection at the tumor site, may not be feasible or desirable in human patients. Systemic application (e.g., i.v.) of the viral vectors is the most likely clinical approach, but viral vectors also present safety problems for human subjects [41–43]. Nonviral delivery systems may eventually represent an effective solution to many of these problems. For example, nanoparticles are not only non-immunogenic and nontoxic [44] but can be complexed with antibodies to cell surface antigens that are relatively specific to cancer cells, improving targeting efficiency and specificity. Nonviral gene delivery vehicles can be designed to encapsulate multiple vectors at the same time, allowing for more efficient introduction of all system components concomitantly into target cells. For gastrointestinal disease, oral formulations of these nonviral delivery vehicles can be designed to target release at the appropriate point in the gastrointestinal tract. In summary, much work is required for designing delivery systems for in vivo gene therapy, particularly delivery approaches appropriate for human subjects.

In vivo testing of the modular, Wnt-targeted Cre/lox system is expected to be feasible given that the Cre/lox system has a long history of in vivo use, particularly in mice. The role of the Cre/lox system in creating conditional gene knockouts in transgenic mice is well known. For example, and relevant to this review's emphasis on CRC, the Fearon group has created a mouse model in which Cre/lox-mediated knockout of the *Apc* allele in an intestinal-targeted manner resulted in intestinal neoplasms, including adenoma-carcinoma progression in the large bowel [52]. In general, the Cre/lox system has shown the ability to direct highly specific and targeted gene expression in mouse models.

Of greater relevance to the current review, the Cre/lox system has shown in vivo therapeutic efficacy in various mouse models of human disease. Dopamine deficient mice have been created by the functional knockout of the Tyrosine hydroxylase (Th) gene through insertion of floxed stuffer sequences. These mice exhibit fatal neurological disorders that can be rescued by Cre-mediated targeted expression of Th in the mouse brain [53]. Activated hepatic stellae cells are involved in extracellular matrix deposition subsequent to liver injury and thus represent a target for antifibrotic therapy [54]. The collagen 1A2 gene promoter driving Cre expression was shown to target effector gene expression and activity in activated hepatic stellae cells, with limited non-specific expression in hepatocytes [54]. Targeted clearance of activated hepatic stellae cells was achieved in vivo, in mouse models of liver injury, utilizing collagen 1A2 promoter-Cre coupled to a floxed HSV-1 thymidine kinase effector gene, in combination with ganciclovir treatment [54]. Thymidine kinase activates ganciclovir via phosphorylation, allowing for targeted cell death; the combination of Cre-mediated thymidine kinase expression and ganciclovir treatment is a typical model of Cre/lox-mediated prodrug activation [39].

Carcinoembryonic antigen (CEA) is overexpressed in gastrointestinal cancer; Ueda et al., utilizing a floxed lacZ effector under the control of Cre expressed from a CEA promoter, demonstrated in vivo tissue specific lacZ expression [38]. In this and subsequent studies from this research group, adenovirus was used as the delivery vehicle to introduce the Cre/lox vectors into mouse models of gastrointestinal cancer. Thus, Ueda et al. created a gene therapy model system in which CEA promoter-Cre activity controlled expression of floxed cytosine deaminase, targeting conversion of the nontoxic prodrug 5-fluorocytosine to 5-fluorouracil in CEA expressing cells. This system was initially demonstrated to be efficacious against an orthotopic gastric carcinoma mouse model [32] and was later applied to CRC [55]. Here, an orthotopic mouse model of CRC was developed which progresses to liver metastases, similar to what is observed in advanced human CRC. Adenovirus vectors containing CEA promoter-Cre and floxed cytosine deaminase were injected into the abdominal cavity of the mice, followed by treatment with 5-fluorocytosine [55]. This treatment regimen completely repressed the development of liver metastases, reduced primary tumor burden, and extended mouse survival [55], clearly demonstrating in vivo efficacy of Cre/lox gene therapy in this mouse model. These findings were similar to that of an earlier study that demonstrated effective Cre/lox-mediated therapeutic activity against peritonitis carcinomatosa caused by implantation of CEA expressing LoVo CRC cells in mice [39]. Adenovirus vectors containing CEA promoter-Cre and floxed HSV-1 thymidine kinase were coinjected into the mice via the intraperitoneal route, followed by treatment with ganciclovir. This treatment regimen markedly reduced tumor burden in the mice, without significant weight loss [39]. The in vivo advantage of the Cre/lox amplification step, as compared to weak direct expression of thymidine kinase, was also clearly demonstrated in this study; Cre/lox-mediated thymidine kinase expression reduced tumor weight to 22% of that observed with direct thymidine kinase expression from the CEA promoter [39]. Furthermore, the Cre/lox system enhanced specificity of expression as well as strength of expression. Thus, an adenoviral vector nonspecifically and directly expressing thymidine kinase from a strong constitutive promoter resulted in death of the mice from side effects such as diarrhea, weight loss, and liver malfunction [39]. These findings underscore the ability of the Cre/lox system to leverage Cre mediated amplification of highly specific gene expression, resulting in robust and specifically targeted in vivo therapeutic effects that avoids excessive off-targeting and associated morbidity/mortality.

An adenoviral Cre/lox system was also successfully used to direct therapeutic gene expression of HSV-1 thymidine kinase in astrocytomas, which, when combined with ganciclovir, significantly reduced tumor growth [56], extending the practical utility of viral-delivered Cre/lox therapeutics to another cancer type. Therefore, Cre/lox-mediated gene therapy has clearly shown efficacy in mouse models, albeit with adenoviral delivery systems not well suited for human use [41–43]. The challenge is to combine various gene therapy approaches (e.g., modular Wnt-targeted Cre/lox systems) with nonviral delivery vehicles for evaluation in mouse models (e.g., models of CRC) in advance of human clinical trials.

8. Summary of Practical Issues, Utility, and Future In Vivo Testing

Wnt-targeted gene therapy systems can be of particular practical utility in the treatment of advanced/metastatic CRC, and/or, as an adjunct or an alternative to surgery; they may also be particularly attractive as an alternative to total colonectomy. In addition, a gene therapy approach could be useful in the treatment of patients with *familial adenomatous polyposis coli* (FAP), as an alternative to prophylactic surgery. The modular Wnt-targeted Cre/lox system described in this review must be tested in an animal model, focusing on efficacy and specificity, general applicability, and of course, determination of the most effective delivery system. The APC^{min} mouse is an effective model of CRC initiation and early progression; this mouse strain represents a useful experimental tool to examine whether Wnt-targeted therapeutic expression can suppress the initiation and growth of Wnt-mediated intestinal neoplasms. Orthotopic mouse models of

CRC are particularly useful analogues of advanced CRC and metastasis in humans, as they often result in lymph node and liver metastases, and thus mimic advanced stage human disease [32, 55]. Thus, these mice can be utilized to test the efficacy of the Wnt-targeted Cre/lox therapy system against advanced/metastatic CRC, the most likely clinical target of this system. In addition, the efficacy of this gene therapy system against the viability of primary CRC cells in culture, obtained from human patients after resection, should be determined along with the necessary rodent studies. It will be crucial to determine whether Wnt-targeted therapeutics have negative side effects on normal cells that exhibit Wnt activity, such as the stem cell compartment of intestinal crypts. Viral and nonviral (e.g., nanoparticle) delivery systems for these gene therapy vectors should be directly compared in the same in vivo studies, to evaluate both efficiency of gene delivery as well as safety/toxicity.

With respect to projected human use, biopsies of CRC tissue or of colonic tissue of patients with FAP can be evaluated for the presence of Wnt-activating mutations, as well as for levels of nuclear, active β-catenin via immunocytochemistry. Such measurements will ascertain the presence of, and the potential level of, Wnt activity in the neoplastic cells; determination of the presence of Wnt-activation in the neoplastic tissues will allow for the use of Wnt-targeted genetic therapeutics. Estimation of the level of the Wnt activity in the cells, by analysis of active β-catenin in the tissues, can assist in the choice of the optimal gene therapeutic approach (e.g., cell death and/or inhibition of Wnt activity). Optimally, a nonviral system can be utilized to introduce the genetic material into the cells; further, more specific targeting of the nonviral delivery system to the neoplastic tissues can enhance efficacy and specificity. The presence of particular cell surface markers overexpressed in the neoplastic cells compared to normal tissues can be determined from the biopsied tissues. Antibodies to these cell surface markers can be complexed to nanoparticles, targeting gene delivery to aberrant cells that are characterized by the relevant cell surface marker profile. Thus, careful examination of tissue obtained from the patient can provide information that will allow for the Wnt-targeted strategy best suited for treating that individual's specific neoplasm.

9. Conclusion

A Cre/lox Wnt-targeted gene therapy model system has been developed and tested in vitro. Levels of effector expression in this system can be finely regulated by the choice of promoter and by the use of both pharmacological and genetic factors. Importantly, the modular nature of the system allows for a variety of gene expression circuits to be established, including positive and negative feedback loops. Further, it may be possible to utilize a promoter that has a specific threshold of activation, so that the effector gene will only be expressed in the presence of the higher levels of Wnt activity found in MSC cells, leaving normal colonic stem cells unaffected. The modular nature of this system suggests that it may be generally applicable to a wide variety of disease states that exhibit differential gene expression through aberrant signaling pathways. To complement this system, it will be important to develop nonviral methods that combine efficiency of gene delivery with a superior safety profile. In vivo testing, including determination of effective and safe delivery systems, is the logical next step in furthering the development of this system. The findings of these projected in vivo studies will advance our understanding of the theoretical and practical issues related to Wnt-targeted anti-CRC genetic therapeutics and provide the foundation for further development leading to clinical trials in individuals with late stage/metastatic CRC. Modular gene therapy systems may eventually represent a major and novel advance in the treatment of advanced cancer.

Abbreviations

HDAC: Histone deacetylase inhibitors
Tcf/Lef: T-cell factor/lymphocyte enhancer factor
APC: Adenomatous polyposis coli
CRC: Colorectal cancer
DN-Tcf4: Dominant negative Tcf4
LiCl: Lithium chloride
DT-A: Diphtheria toxin A-chain
CSCs: Cancer stem cells
MCS: Migratory cancer stem cell
SCS: Stationary cancer stem cell
EMT: Epithelial to mesenchymal transition
MET: Mesenchymal to epithelial transition
UTR: Untranslated region.

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