METHODS TO ENHANCE AND CONFINE EXPRESSION OF GENES

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the field of gene therapy for cancer. More specifically, the present invention presents a method of controlling the expression of therapeutically valuable gene products via inducible promoters. The present invention provides a method whereby induced gene expression in the intended cell targets is enhanced and prolonged in a spatially and temporally regulable manner by means of heat or light inducible promoters. Moreover, the present invention provides a method whereby the background gene expression in non-targeted cells is reduced or eliminated.

Description of the Related Art

One of the major obstacles to the success of chemotherapy and radiation therapy for cancer is the difficulty in achieving tumor-

specific cell killing. The inability of radiation or cytotoxic chemotherapeutic agents to distinguish between tumor cells and normal cells necessarily limits the dosage that can be applied. As a result, disease relapse due to residual surviving tumor cells is frequently observed.

The use of gene therapy in cancer treatment presents many of the same disadvantages as chemotherapy and radiation therapy. Problems with current state-of-the-art gene therapy strategies include the inability to deliver the therapeutic gene specifically to the target cells. This leads to toxicity in cells that are not the intended targets. For example, manipulation of the p53 gene suppresses the growth of both tumor cells and normal cells, and intravenous administration of tumor necrosis factor alpha (TNFα) induces systemic toxicity with such clinical manifestations as fever and hypertension.

Attempts have been made to overcome these problems. These include such strategies as: the use of tissue-specific receptors to direct the genes to the desired tissues (Kasahara, N., *et al., Science*, 266:1373-1376 (1994)), the use of tissue-specific promoters to limit gene expression to specific tissues (e.g. use of the prostate specific antigen promoter) and the use of heat (Voellmy R., *et al., Proc. Natl. Acad. Sci. USA*, 82:4949-4953 (1985)) or ionizing radiation inducible enhancers and promoters (Trainman, R.H., *et al., Cell* 46: 567-574 (1986); Prowess, R., *et al., Proc. Natl. Acad. Sci. USA* 85, 7206-7210 (1988)) to enhance expression of the therapeutic gene in a temporally and spatially controlled manner. The heat inducible heat shock protein (HSP) promoter has been used to direct the expression of genes such as the cytokine IL-2.

Weichselbaum and colleagues were the first to discover the radiation inducible response of the early growth response (Egr-1) gene promoter. Accordingly, they have attempted to direct expression of such cytotoxic genes as TNF-α to tumor cells to enhance radiation cell killing by means of this promoter. Previously, systemic administration of the cytokine TNF-α as an adjuvant to ionizing radiation was initially reported to result in enhanced killing in a mouse xenograft tumor system. It has since been shown partially effective in human tumors. The effect of TNFα appears to be dosage-dependent, as its tumor-killing effect correlates with its serum concentration. However, systemic toxicity of TNFα restricts the dosage that can be applied and thus limits the usefulness of the treatment regimen. Attempts have also been made to deliver the TNFα gene to tumor cells via adenoviral vector and/or liposomes. Unfortunately, expression of the TNFα gene is not restricted to the tumor sites due to the 'leakiness' of the promoter.

In an attempt to localize the level of TNFα to the general area of radiation exposure and thereby reduce systemic toxicity, Weichselbaum and colleagues employed the radiation inducible Egr-1 promoter to activate the TNFα gene *in situ*. Earlier studies showed that the expression of certain immediate-early genes such as jun/fos and Egr-1 are activated in cells exposed to ionizing radiation (Sherman, M.L., *et al., Proc. Natl. Acad. Sci. USA*, 87: 5663-5666 (1997); Hallahan, D.E., *et al., Proc. Natl. Acad. Sci. USA*, 88: 2156-2160 (1991)). By placing the TNFα gene under the control of the Egr1 promoter (EGRp), the expression of the TNFα is enhanced in those cells harboring an EGRp-TNFα plasmid when exposed to ionizing radiation. *In vivo*, the

serum level of TNFα is greatly enhanced (Weichselbaum R.R., *et al.*, *Cancer Res.* 54: 4266-4269 (1994)) within a few hours after irradiation. The combined treatment with this plasmid and radiation leads to a partial regression of a xenografted tumor during the course of the treatment. The level of TNFα dropped precipitously within 24 hours; further decreases in serum level of TNFα coincided with regrowth of the tumors.

There are several possible reasons for the recurrence of the tumor upon cessation of therapy. The most obvious reason is probably the same limitation seen with chemotherapy or radiation therapy in general, viz., insufficient dosage levels. A major problem, which limits the amount of TNFα produced, is the weak and transient nature of the Egr-1 promoter. This promoter is intrinsically weak, with a maximum of less than three-fold increase in expression upon induction. Moreover, the induced expression is of necessity transient. This, coupled with the weakness of the promoter, permits only a brief exposure of the tumor cells to the TNFα.

Another factor that limits the production of sufficient dosage of TNFα is that not every tumor cell will have taken up the TNFα plasmid. While it has been suggested that repeated administration may help to improve the treatment outcome, it is not clear if the repeated delivery of a suboptimal low dosage of TNFα will be useful, the problems posed by an immune response notwithstanding. Although it might be conceivable to deliver larger doses of plasmids, the problem of promoter leakiness has hindered such an approach. It is known that a substantial basal level of activity (20-30%) can be detected with the Egr-1 promoter even in the absence

of ionizing radiation (Weichselbaum, *et al., supra*). This is not surprising, as the radiation response element, a CArG box, is part of the serum response element.

The HSP promoter is also rather leaky. In the absence of heat, this promoter exhibits a 25-30% background level of expression, not suitable for most cytotoxic genes. As this level of expression will be harmful to unirradiated normal cells that take up the gene. Hence, administration of this plasmid has been restricted to small doses of intra-tumoral injections to minimize systemic toxicity.

Therefore, while it may be advantageous to employ a spatially and temporally regulated promoter such as the HSP and Egr-1 promoters to enhance specificity of gene expression at the site of heat or radiation treatment, current versions of those promoters have serious problems that restrict their applicability. In order to apply these promoters for use in cancer therapy, it is necessary to eliminate or greatly reduce background expression in unheated or unirradiated cells. Ideally, the expression of cytotoxic genes should be limited to the area of external stimuli (heat or radiation). Additionally, to ensure a sufficient level of expression of therapeutic genes, the weak and transient nature of gene expression driven by these promoters must be improved.

It is important to note that even when an improved inducible vector system which can restrict the expression of a therapeutic gene to the area of external stimuli is developed, there is still the problem of expression in normal heated or irradiated bystander cells. Thus, it is critical to be able to further restrict the expression of therapeutic genes only to the intended targets, e.g., tumor cells.

The prior art is deficient in the lack of effective means of inhibiting unwanted toxic side effects of gene therapy treatments for cancer, as well as providing a method for enhancing and sustaining gene expression in targeted tumor cells in a controllable manner. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

The current invention provides the composition and methods for the controlled activation of DNA molecules for gene therapy. Activation of these DNA molecules leads to the production of protein products which then may provide opportunities for therapeutic manipulation of cells containing said DNA molecules. This may be achieved via alterations in cell growth and metabolism of the targeted cells and may include effects on neighboring cells via secretion of therapeutic products. The invention offers the options of sustained activation or activation regulable by the application of antibiotics. The invention further provides novel expression vectors for use in gene therapy of local and metastatic breast, ovarian and prostate cancer.

An original strategy to confine and enhance therapeutic gene expression to tumors spatially and temporally is also presented, in the form of an expression vector designed for use in local and metastatic breast, ovarian and prostate cancer.

In one embodiment of the present invention, there is provided a method for sustained and enhanced expression of a gene via activation of a heat or light inducible promoter. In a modification of this method, heat or light is used to activate the promoter, but

continued levels of gene expression are modulated by concentrations of an antibiotic (tetracycline or its derivatives), acting on a fusion protein with a tetracycline-responsive element.

In yet another embodiment of the present invention, there is provided a method of constructing the vectors for gene therapy activation modalities.

In another embodiment of the present invention, there are provided improved vectors for reducing background expression in unheated and unirradiated cells.

In another embodiment of the present invention, there are provided improved vectors for reducing expression in heated and irradiated normal bystander cells.

In another embodiment of the present invention, there are provided expression vectors for use in gene therapy treatment of local and metastatic breast and ovarian cancer.

In another embodiment of the present invention, there are provided expression vectors for use in gene therapy treatment of local and metastatic prostate cancer.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may

be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows a schematic representation of the plasmid, pDATH-X (Dominant negative, Antisense, TET-ON controllable Heat shock promoter plasmid) -p53, which consists of four cassettes as follows. (1) TET-ON is a fusion of the coding sequences for amino acids 1-207 of the tetracycline (tet) repressor and the C-terminus last 130 amino acid transcription activation domain of the VP16 protein of the herpes simplex virus (Gossen M., *et al., Science*, 268:1766-1769 (1995)). In Cassette 1, the TET-ON sequence is placed under the control of the HSP and the tet operator binding site and pCMV. (2) HSP is the heat shock promoter consisting of the heat shock response element (-260 to 30) of the human heat shock 70 gene promoter (Voellmy R., *et al., Proc. Natl. Acad. Sci. USA* 82: 4949-4953 (1985)) linked to the minimal CMV promoter, pCMV (Gossen M., *et al., Science*, 268:1766-1769 (1995)). In cassette 2, the therapeutic gene, X, is placed under the control of the tetp-pCMV promoter. (3) ptet is the tet operator consisting of the 19 base pair (bp) inverted repeats of the operator O2 of TN10 (Gossen M, and Bujard H., *Proc. Natl. Acad. Sci. USA* 89:5547-5551 (1992)) to which the tet repressor and TET-ON bind. In cassette 3, antisense TET-ON is placed under the control of the pCMV promoter. (4) Antisense TET-ON is an antisense sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG. In cassette 4, dominant negative TET-ON is placed under the control of the pCMV promoter. The

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Dominant negative TET-ON consists of the tet-repressor but without the VP16 transactivation domain, and it is placed under the control of the pCMV promoter. In the absence of heat or light, a background level of expression of the TET-ON sequence will result due to the leakiness of the minimal promoter pCMV.

Figure 2 depicts the pDATE vector. The plasmid, pDATE-X (Dominant negative, Antisense, TET-ON controllable EGR promoter expression plasmid) consists of four cassettes as follows: 1) in cassette 1, the TET-ON sequence is placed under the control of the EGRp, the tetracycline operator binding site and pCMV; 2) in cassette 2, the therapeutic gene, X, is placed under the control of the tetp-pCMV promoter; 3) in cassette 3, antisense TET-ON is placed under the control of the pCMV promoter; and 4) in cassette 4, dominant negative TET-ON is placed under the control of the pCMV promoter. "TET-ON" is a fusion of the coding sequences for amino acids 1-207 of the tet repressor and the C-terminus 130 amino acid transcription activation domain of the VP16 protein of the herpes simplex virus. "EGRp" is the radiation inducible promoter consisting of fragment -425 to +65 of the EGR-1 promoter containing four copies of the CArG domain. "ptet" is the tet operator consisting of the 19 bp inverted repeats of the operator O2 of TN10 to which the tet repressor and TET-ON bind, linked to the minimal CMV promoter, pCMV. "Antisense Tet-On" is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG. "Dominant negative TET-ON" consists of the coding sequences for amino acids 1-207 of the tet repressor placed under the control of the pCMV promoter. "M" is the chicken lysosomal matrix attachment site to isolate the position effects of each of the cassettes.

Figure 3 depicts the structure of the pRIBs-X (Radiation-Inducible, Breast-specific Promoter) expression vector. The pRIBS vector is comprised of four cassettes. Gene cassette 1 differs from previously described vectors only in that it contains "Gal-DBD-mx" which is a fusion open reading frame (ORF) encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein (Gal-DBD) fused to the basis helix-loop-helix-leucine zipper (bHLHLZ) domain of Max (mx, amino acids 8-112) followed by SV40 poly A. Gene cassette 2 is comprised of the minimal CMV promoter (mCMVp), "antisense Gal-DBD-mx", which is an antisense construct complementary to the Gal-DBD-mx sequence, "IRES", which is an internal ribosomal entry site and "Gal-DBD" which competes with the Gal-DBD-mx for the pGAL binding site. Gene cassette 3 is comprised of "VP16-TA-mc" which is a fusion open reading frame encoding at the N-terminus the first 11 amino acids of Gal4 (amino acids 1-147), followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the bHLHLZ domain of c-Myc (amino acids 350-439), followed by SV40 polyA. The resulting fusion gene, VP-16TA-mc, is placed under the control of the c-erbB-2 promoter "perbB2" up to the first ATG. Gene cassette 4 contains "GALp", consisting of five copies of a 17-mer DNA-binding site for Gal4. The TET-ON sequence is placed under the control of the GALp-ptet promoter and the therapeutic gene, X, is linked to the TET-ON via an IRES; Gene cassette 5 contains an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG, placed under the control of the pCMV promoter. Gene cassette 6 contains a dominant negative TET-ON consisting of the

coding sequences for amino acids 1-207 of the tet repressor placed under the control of the pCMV promoter.

Figure 4 shows the structure of the pRIPS-GFP (Radiation-Inducible, Prostate-specific Promoter) expression vector. The pRIPS vector is comprised of six cassettes. Gene cassette 1 differs from previously described vectors only in that it contains "Gal-DBD-mx" which is a fusion open reading frame encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein (Gal-DBD) fused to the basis helix-loop-helix-leucine zipper (bHLHLZ) domain of Max (mx, amino acids 8-112) followed by SV40 poly A. Gene cassette 2 is comprised of the minimal CMV promoter (mCMVp), "antisense Gal-DBD-mx", which is an antisense construct complementary to the Gal-DBD-mx sequence, "IRES", which is an internal ribosomal entry site and "Gal-DBD" which competes with the Gal-DBD-mx for the pGAL binding site. Gene cassette 3 is comprised of "VP16-TA-mc" which is a fusion open reading frame encoding at the N-terminus the first 11 amino acids of Gal4 (amino acids 1-147), followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the bHLHLZ domain of c-Myc (amino acids 350-439), followed by SV40 polyA. The resulting fusion gene, VP16-TA-mc, is placed under the control of the probasin gene promoter "pProbasin" up to the first ATG. Gene cassette 4 contains "GALp", consisting of five copies of a 17-mer DNA-binding site for Gal4. The TET-ON sequence is placed under the control of the GALp-ptet promoter and the therapeutic gene, X, is linked to the TET-ON via an IRES; Gene cassette 5 contains an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG, placed

under the control of the pCMV promoter. Gene cassette 6 contains a dominant negative TET-ON consisting of the coding sequences for amino acids 1-207 of the tet repressor placed under the control of the pCMV promoter.

Figure 5 is a schematic representation of the mode of action of pRIBS-GFP.

Figure 6 illustrates the leakiness of the HSP promoter. It summarizes the results of testing the heat inducible system containing the hsp70 promoter in the expression of therapeutic genes, p53 and TNFα. **Figure 6A** shows the plasmid construct for the two genes, p53 and TNFα. **Figure 6B** depicts p53 transcriptional activity. To analyze the inducibility of the hsp promoter, the plasmid pHSP.3p53CD1 or the control pHSP.3 vector alone was cotransfected with Post-2-CAT (containing a CAT coding sequence linked to a consensus p53 binding site) into the human ovarian carcinoma cell line SKOV3 which has a homozygous deletion of p53. At 36 hours after transfection, cells were either heated or unheated. CAT activity was measured 24 hours later. Little or no activity is seen with the SKOV3 parental untransfected cells (lane 2, heated; lane 1, unheated). Similarly, with the pHSP.3 vector alone, there is no activity with or without heat (lanes 3 and 4). With the pHSP.3p53 plasmid, there is a high level of CAT activity seen at 24 hrs after heating (lane 6). However, even without heating (lane 5), there is a substantial level of p53 expression (about 25%).

Figure 7 depicts the induction of TNFα by heat or photodynamic therapy (PDT). The coding sequence of TNFα was subcloned into the plasmid pHSP.3 and transfected into SKOV3 cells. Stable colonies were isolated by selection in G418. Cells were either heated at 45° C or untreated. At 6 hours after treatment, the level of

TNFα in the medium was measured with a Genzyme TNFα ELISA kit. TNFα shown to be induced four-fold by heat and three-fold by PDT and secreted. However, background expression was substantial (27%).

Figure 8 shows the expression kinetics of p53 in the H358 lung carcinoma cell line by the feed-forward reaction, where a,b,c,d and e represent the levels of p53 reached at 10 hours after the feed-forward reaction. Six hours after heat shock, transfected cells were treated with different doses of doxycycline. At various time points after the addition of doxycycline, the cells were stained with a p53 antibody. For each point, the digital images of fifty immunostained cells were captured using a Nikon microscope. The amount of protein expressed in each cell is proportional to the intensity of staining, expressed as I= 1/T (where T is a measure of the transmitted light/unit area. This plot shows the results of one such experiment using 0.01-0.1 μg/ml doxycycline.

Figure 9 depicts the pHIBS-X (Heat-Inducible, Breast-specific Promoter) expression vector. The pHIBS vector is comprised of six cassettes. Gene cassette 1 differs from the vectors described above only in that it contains "Gal-DBD-mx" which is a fusion open reading frame encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein (Gal-DBD) fused to the basis helix-loop-helix-leucine zipper (bHLHLZ) domain of Max (mx, amino acids 8-112) followed by SV40 poly A. The resulting fusion gene GAL-DBD-mx is controlled by the heat inducible HSP promoter. Gene cassette 2 is comprised of the minimal CMV promoter (mCMVp), "antisense Gal-DBD-mx", which is an antisense construct complementary to the Gal-DBD-mx sequence, "IRES", which is an internal ribosomal entry site and "Gal-DBD" which competes with the Gal-DBD-mx for the pGAL binding

site. Gene cassette 3 is comprised of "VP16-TA-mc" which is a fusion open reading frame encoding at the N-terminus the first 11 amino acids of Gal4 (amino acids 1-147), followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the bHLHLZ domain of c-Myc (amino acids 350-439), followed by SV40 polyA. The resulting fusion gene, VP-16TA-mc, is placed under the control of the c-erbB-2 promoter "perbB2" up to the first ATG. Gene cassette 4 contains "GALp", consisting of five copies of a 17-mer DNA-binding site for Gal4. The TET-ON sequence is placed under the control of the GALp-ptet promoter and the therapeutic gene, X, is linked to the TET-ON via an IRES; Gene cassette 5 contains an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG, placed under the control of the pCMV promoter. Gene cassette 6 contains a dominant negative TET-ON consisting of the coding sequences for amino acids 1-207 of the tet repressor placed under the control of the pCMV promoter.

Figure 10 illustrates the structure of the pHIPs-GFP (Heat-Inducible, Prostate-specific Promoter) expression vector. The pHIPS vector is comprised of six cassettes. Gene cassette 1 differs from previously described vectors only in that it contains "Gal-DBD-mx" which is a fusion open reading frame encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein (Gal-DBD) fused to the basis helix-loop-helix-leucine zipper (bHLHLZ) domain of Max (mx, amino acids 8-112) followed by SV40 poly A. The resulting fusion gene GAL-DBD-mx is controlled by the heat inducible HSP promoter. Gene cassette 2 is comprised of the minimal CMV promoter

(mCMVp), "antisense Gal-DBD-mx", which is an antisense construct complementary to the Gal-DBD-mx sequence, "IRES", which is an internal ribosomal entry site and "Gal-DBD" which competes with the Gal-DBD-mx for the pGAL binding site. Gene cassette 3 is comprised of "VP16-TA-mc" which is a fusion open reading frame encoding at the N-terminus the first 11 amino acids of Gal4 (amino acids 1-147), followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the bHLHLZ domain of c-Myc (amino acids 350-439), followed by SV40 polyA. The resulting fusion gene, VP16-TA-mc, is placed under the control of the probasin gene promoter (pProbasin) up to the first ATG. Gene cassette 4 contains "GALp", consisting of five copies of a 17-mer DNA-binding site for Gal4. The TET-ON sequence is placed under the control of the GALp-ptet promoter and the therapeutic gene, X, is linked to the TET-ON via an IRES; Gene cassette 5 contains an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG, placed under the control of the pCMV promoter. Gene cassette 6 contains a dominant negative TET-ON consisting of the coding sequences for amino acids 1-207 of the tet repressor placed under the control of the pCMV promoter.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "heat" is to mean heat energy generated by any means, including microwaves.

As used herein, the term "light" is to mean light energy with frequencies in the visible as well as the invisible spectrum, including ionizing radiation generated by any means. This would include a radiation source such as radionuclides capable of emitting gamma and or beta particles, or by a linear accelerator.

In accordance with the present invention, there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription and Translation" [B.D. Hames & S.J. Higgins eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

The present invention is directed towards a new method of gene therapy for confined areas such as tumors. In accordance with the above-mentioned object there is provided a mechanism for both constitutively active and regulable gene expression via plasmids containing elements which are heat and or light activated and responsive to presence and concentration of antibiotic (tetracycline and its derivatives). In regulating gene expression, heat or light initiates the expression, but the gene is constitutively expressed only in the presence of the antibiotic (tetracycline and its derivatives). Concentration of the antibiotic controls the level and duration of the gene expression.

For the confinement of gene expression to tumor cells, there are provided two mechanisms for the suppression of gene expression in normal cells that are bystander targets of heat or radiation. In the instance of normal cells not exposed to heat or light, which inadvertently take up the plasmid, expression of the therapeutic gene due to background activity of the promoter is suppressed by the constitutive expression of antisense and dominant negative DNA sequences to the heat or light inducible, antibiotic dependent transcriptional activator built into the plasmid. In the instance whereby normal cells that take up the plasmid are then exposed to heat or light, there is an additional mechanism for preventing the expression of the therapeutic gene. This is achieved by the use of a modified 'two hybrid' system where the antibiotic dependent transcriptional activator is itself under the control of both the expression of tissue-specific transcriptional activators and the exposure to heat or light. Expression of the therapeutic gene is therefore found only in cells that have been both exposed to heat or light and that express tissue-specific transcription factors.

In one embodiment of the present invention, there is provided a recombinant vector, pDATH-X (Dominant negative, Antisense, TET-ON controllable Heat shock promoter plasmid), for the purpose of reducing background levels of expression. This vector is comprised of the cassettes: (a) a fusion of the coding sequences for amino acids 1-207 of the tetracycline repressor and the C-terminus last 130 amino acid transcription activation domain of the VP16 protein of the herpes simplex virus; (b) a heat shock promoter consisting of heat shock response elements (-260 to 30) of the human heat shock 70 gene promoter linked to the minimal cytomegalovirus

promoter, pCMV; (c) a tet operator consisting of the 19 bp inverted repeats of the operator O2 of TN10 to which the tet repressor and TAKON bind; and (d) an antisense sequence consisting of the complementary sequence to the first 80 bases of the TAKON sequence including the ATG.

In another embodiment of the present invention provides a method of achieving sustained expression of a gene under control of a heat or light inducible promoter, comprising the step of: introducing the vector containing said gene into the host organism; and applying heat or light energy. In another embodiment of the invention, said host organism is a human.

In yet another embodiment of the invention, there is provided a recombinant vector, pDATE-X (Dominant negative, Antisense, TET-ON controllable EGR promoter expression plasmid), said vector comprising the cassettes: (a) in cassette 1, the TET-ON sequence is placed under the control of the EGRp, the tetracycline operator binding site and pCMV; (b) in cassette 2, the therapeutic gene X, is placed under the control of the tetp-pCMV promoter; (c) in cassette 3, antisense TET-ON is placed under the control of the pCMV promoter; and (d) in cassette 4, dominant negative TET-ON is placed under the control of the pCMV promoter.

Another embodiment of the present invention provides a recombinant vector, pRIBs-X, (Radiation-Inducible, Breast-specific Promoter) expression vector, said vector comprising the cassettes: (a) cassette 1 contains "Gal-DBD-mx" which is a fusion open reading frame encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein (Gal-DBD) fused to the basic helix-loop-helix-leucine zipper domain of Max (amino acids 8-112) followed by SV40

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poly A- the resulting fusion gene GAL-DBD-mx is controlled by the radiation inducible Egr-1 promoter; (b) cassette 2 is comprised of the minimal CMV promoter, "antisense Gal-DBD-mx", which is an antisense construct complementary to the Gal-DBD-mx sequence, "IRES", which is an internal ribosomal entry site and "Gal-DBD" which competes with the Gal-DBD-mx for the pGAL binding site; (c) cassette 3 is comprised of "VP16-TA-mc" which is a fusion open reading frame encoding at the N-terminus the first 11 amino acids of Gal4 (amino acids 1-147), followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the basic helix-loop-helix-leucine zipper domain of c-Myc (amino acids 350-439), followed by SV40 polyA - the resulting fusion gene, VP16-TA-mc, placed under the control of the c-erbB2 promoter "perB2" up to the first ATG; (d) cassette 4 contains "Galp", five copies of a17-mer DNA-binding site for Gal4. The TET-ON sequence is placed under the control of the GAPp-ptet promoter and the therapeutic gene, X, is linked to the TET-IN via an IRES; (e) cassette contains an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG, placed under the control of the pCMV promoter; and (f) cassette 6 contains a dominant negative TET-ON consisting of the coding sequences for amino acids 1-207.

There are further provided variants of the preceding vectors, wherein the perbB2 promoter is replaced with the whey acidic protein promoter or the stromelysin 3 promoter.

Another embodiment of the invention provides a method for the treatment of local and metastatic breast and ovarian cancer comprising: administration to the patient a pRIBs-X expression vector

(or a variant thereof) containing a cytotoxic gene. A representative cytotoxic gene is tumor necrosis factor alpha.

The present invention is also directed to a recombinant pRIPs-X (Radiation-Inducible, Prostate-specific Promoter) expression vector, said vector comprising the cassettes: (a) cassette 1 contains "Gal-DBD-mx" which is a fusion open reading frame encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein fused to the basic helix-loop-helix leucine zipper domain of Max (amino acids 8-112) followed by SV40 polyA - the resulting fusion gene GAL-DBD-mx is controlled by the radiation inducible Egr-1 promoter; (b) cassette 2 is comprised of the minimal CMV promoter, antisense Gal-DBD-mx, which is an antisense construct complementary to the Gal-DBD-mx sequence, IRES, which is an internal ribosomal entry site and Gal-DBD which competes with the Gal-DBD-mx for the pGAL binding site; (c) cassette 3 is comprised of "VP16-TA-mc", a fusion open reading frame encoding at the N-terminus the first 11 amino acids of Gal4, followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the basic helix-loop-helix leucine zipper domain of c-Myc (amino acids 350-439), followed by SV40 polyA - the resulting fusion gene, VP16-TA-mc, is placed under the control of the probasin gene promoter "pProbasin" up to the first ATG; (d) cassette 4 contains GALp, five copies of the 17-mer DNA-binding site for Gal4. The TET-ON sequence is placed under the control of the GALp-ptet promoter and the therapeutic gene, X, is linked to the TET-ON via an internal ribosomal entry site; (e) cassette 5 contains an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the

ATG, placed under the control of the pCMV promoter; and (f) cassette 6 contains a dominant negative TET-ON consisting of the coding sequence for amino acids 1-207. A variant of the preceding vector is also contemplated, wherein the probasin promoter is replaced with the prostate specific antigen promoter.

Another embodiment of the invention provides a method for the treatment of local and metastatic prostate cancer comprising: administration to the patient a pRIPs-X expression vector (or a variant thereof) containing a cytotoxic gene. A representative cytotoxic gene is tumor necrosis factor alpha.

In yet another embodiment of the present invention, there is provided a recombinant expression vector, pHIBs-X (Heat Inducible, Breast-specific promoter), said vector comprising the cassettes: (a) cassette 1 contains Gal-DBD-mx which is a fusion open reading frame encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein fused to the basic helix-loop-helix leucine zipper domain of Max (amino acids 8-112) followed by SV40 polyA - the resulting fusion gene GAL-DBD-mx is controlled by the heat inducible heat shock protein promoter; (b) cassette 2 is comprised of the minimal CMV promoter, antisense Gal-DBD-mx, a construct complementary to the Gal-DBD-mx sequence, an internal ribosomal entry site and Gal-DBD, which competes with the Gal-DBD-mx for the pGAL binding site; (c) cassette 3 is comprised of "VP16-TA-mc" which is a fusion open reading frame encoding at the N-terminus the first 11 amino acids (amino acids 1-147), followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the basic helix-loop-helix leucine zipper domain of c-Myc (amino acids

350-439), followed by SV40 polyA - the resulting fusion gene VP16-TA-mc is placed under the control of the c-erbB2 gene promoter "perbB2" up to the first ATG; (d) cassette 4 contains GALp, five copies of a 17-mer DNA-binding site for Gal4. The TET-ON sequence is placed under the control of the GALp-ptet promoter and the therapeutic gene, X, is linked to the TET-ON via an internal ribosomal entry site; (e) cassette 5 contains an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG, placed under the control of the pCMV promoter; and (f) cassette 6 contains a dominant negative TET-ON consisting of the coding sequences for amino acids 1-207. Variants of the preceding vector are contemplated, wherein the perbB2 promoter is replaced with the whey acidic protein promoter or the stromelysin 3 promoter.

The present invention is further directed to a method for the treatment of local and metastatic breast and ovarian cancer comprising: administration to the patient a pHIBs-X expression vector (or a variant thereof) containing a therapeutic gene. A representative therapeutic gene is tumor necrosis factor alpha.

Another embodiment of the invention provides a recombinant vector, pHIPs-X (Heat-Inducible, Prostate-specific Promoter), said vector comprising the cassettes: (a) cassette 1 contains Gal-DBD-mx which is a fusion open reading frame encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein fused to the basic helix-loop-helix leucine zipper domain of Max (amino acids 8-112) followed by SV40 polyA - the resulting fusion gene GAL-DBD-mx is controlled by the heat inducible heat shock protein promoter; (b) cassette 2 is comprised of the minimal CMV

promoter (mCMVp), antisense Gal-DBD-mx, a construct complementary to the Gal-DBD-mx sequence, an internal ribosomal entry site and Gal-DBD, which competes with the Gal-DBD-mx for the pGAL binding site; (c) cassette 3 is comprised of "VP16-TA-mc", a fusion open reading frame encoding at the N-terminus the first 11 amino acids of Gal4, followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the basic helix-loop-helix leucine zipper domain of c-Myc (amino acids 350-439), followed by SV40 polyA - the resulting fusion gene, VP16-TA-mc, is placed under the control of the probasin gene promoter "pProbasin" up to the first ATG; (d) cassette 4 contains GALp, five copies of a 17-mer DNA-binding site for Gal4. The TET-ON sequence is placed under the control of the GALp-ptet promoter and the therapeutic gene, X, is linked to the TET-ON via an internal ribosomal entry site; (e) cassette 5 contains an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG, placed under the control of the pCMV promoter; and (f) cassette 6 contains a dominant negative TET-ON consisting of the coding sequences for amino acids 1-207. A variant of the preceding vector is contemplated, wherein the probasin promoter is replaced with the prostate-specific antigen promoter.

In another embodiment of the invention, there is provided a method for the treatment of local and metastatic prostate cancer comprising: administration to the patient a pHIPs-X vector (or a variant thereof) containing a therapeutic gene. representative therapeutic gene is tumor necrosis alpha.

It is specifically contemplated that pharmaceutical compositions of the present invention may be prepared for the purpose of gene therapy. In such a case, the composition comprises a vector of the present invention and a pharmaceutically acceptable carrier. A person having ordinary skill in the art of cancer chemotherapy would readily be able to determine, without undue experimentation, appropriate dosages and routes of administration. For gene therapy, the gene of interest contained in one of the plasmid vectors of the present invention, could be delivered to the target cell via a viral vector or liposome.

The level of ordinary skill of the average scientist in the area of molecular cancer biology has increased substantially in recent years. A person having ordinary skill in this art would readily be able to construct and utilize the plasmids for this novel approach to gene therapy given the teachings of the present specification.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

The pDATE vector: Structure and mode of action

Figure 2 is a schematic depiction of the pDATE vector. The pDATE-X plasmid functions via a feed-forward reaction to amplify the expression of TET-ON and X. In the absence of radiation, background expression due to leakiness of the EGRp will result in the synthesis of TET-ON mRNA. Translation of this mRNA is reduced by the concomitant expression of antisense TET-ON RNA. Moreover, leaked-

through translated TET-ON protein is inactive without tetracycline. In the presence of tetracycline, the leaked (translated) TET-ON protein becomes active, but the feed-forward reaction is prevented by the constitutively expressed dominant negative TET-ON protein which competes for the same DNA binding site of the ptet promoter.

Two chicken lysosomal matrix attachment sites (MAR) are inserted to isolate the position effects of the cassettes (McKnight, R.A., *et al., Mol. Reprod. & Dev.*, 44:179-184 (1996)). While they may be unnecessary when the antisense and dominant negative TET-ON expressions are driven by the minimal CMV promoter, MARs may be needed if stronger promoters like the human ß actin promoter are to drive their expression.

When cells harboring the pDATE-X are exposed to radiation, an initial burst of TET-ON transcription occurs, leading to the synthesis of 2-4-fold above background level of TET-ON in greater excess than the dominant negative TET-ON. This excess TET-ON protein, in the presence of tetracycline, then binds to the tetp promoters to which the coding sequence of both TET-ON and X are linked and engages in a feed-forward reaction. This reaction is controlled by the level of tetracycline. As such, X expression is elevated and the duration lengthened until tetracycline is removed, at which point the half-life of the TET-ON protein will determine how long the feed-forward reaction can be restarted using tetracycline without further radiation exposure.

This vector makes use of a feed-forward reaction to achieve and maintain a high level of inducible gene expression. This feed-forward feature overcomes the transient nature and weakness of the inducible promoter. When the feed-forward reaction is limited to a few hours, there is a large difference in the level of TET-ON achieved

in heated and unheated cells. It is thus possible to adjust the difference in the level of amplified TET-ON in irradiated and unirradiated cells by enhancing the former with the alternate addition and removal of tetracycline. However, while the addition and removal of tetracycline can be precisely controlled in cell culture, it is difficult to do so *in vivo* due to the heterogeneity of tetracycline level in tissues and the variation in the absorption and removal of tetracycline *in vivo* in different individuals. Thus, it is critical to minimize the leak through expression of TET-ON with antisense and dominant negative cassettes so that the feed-forward reaction does not significantly amplify its level in unirradiated cells.

If necessary, the action of this vector can be further fine-tuned by replacing the pCMV minimal promoter with a much stronger promoter such as the human ß actin promoter to drive the expression of the antisense and the dominant negative TET-ON. In addition, the copy numbers of the antisense and the dominant negative coding sequences can be increased.

For *in vivo* induction of TET-ON expression, oxytetracycline will be used because of its short *in vivo* half-life. In humans, after a single oral dose peak plasma concentration of oxytetracycline is reached at 2-4 hours (see, e.g., Goodman & Gilman's *The Pharmacological Basis of Therapeutics*). The level of TET-ON expression as a function of oxytetracycline concentration can thus be monitored. Oxytetracycline is short acting with an *in vivo* half-life of only 9 hours (versus doxycycline which has a half-life of 18 hours). At the end of 24 hours, the oxytetracycline level is reduced to <25% of input (about 10-30 % are never absorbed and are excreted in the active form).

EXAMPLE 2

The pDATH vector: structure and mode of action

Figure 1 is a schematic depiction of the pDATH-X vector. This vector operates in identical fashion to the pDATE-X vector, except that the Egr-1 promoter is replaced with the HSP promoter and that heat is used in place of light/ionizing radiation.

EXAMPLE 3

Verification of the concept of amplifiable and sustained expression of TET-On and p53 with the feed-forward inducible promoter

To further validate the concept of heat inducible, tetracycline feed-forward amplification of gene expression, two plasmids were constructed. The plasmid "ptet-splice p53wt" was constructed by subcloning a wild-type p53 cDNA into the ptet-splice vector (Gibco BRL) which places p53 under the control of the tetp promoter (consists of the regulatory sequences from the tetracycline-resistance operon upstream of a minimal hCMV promoter). The plasmid "HSP-tetp-TET-ON" was constructed by replacing the CMV promoter in ptet-on (Clontech) with 300bp of the human heat shock protein promoter and the tetp promoter.

H358, a non-small cell lung carcinoma cell line with a homozygous deletion of p53, was grown in RPMI + 10% fetal calf serum. 10^7 exponentially growing cells were cotransfected with 50 μg of "ptet-splice p53wt" and 10 μg of "HSP-tetp-TET-ON" by electroporation using a BRL cell-Porator at 1180 μF and 240 V in 0.8 ml RPMI + 6 mM glucose. Transfected cells were plated out at 25%

confluence for 36 hours and then half of them were heat-shocked at 45°C for twenty minutes. Six hours after heat shock, cells were treated with different doses of doxycycline. At various time points after the addition of doxycycline, cells were stained immunohistochemically with the monoclonal p53 antibody DO-1 (Santa Cruz Biologicals) using an immunoperoxidase cell staining kit (Vector) and diaminobenzidine (DAB). For each point, the digital images of fifty immunostained cells were captured using a Nikon microscope. The amount of protein expressed in each cell is proportional to the intensity of staining which was expressed as I=1/T, where T is a measure of the transmitted light/unit area. Results of one such experiment at 0.01-0.1 μg/ml of doxycycline are shown in Fig. 8.

When 0.1 μg/ml of doxycycline was added at 6 hours after heating (when the level of induced TET-ON should have been at its peak), more than 12 fold amplification of p53 was reached in 10 hours (curves a and b, Figure 8). During this time, doxycycline also started a feed-forward reaction in the unheated cells as indicated by the substantial level of TET-ON. However, since the amplification started off from a lower level, the amplified level of TET-ON at 10 hours reached only a low level (curves c and d, Fig.8).

It is possible to regulate the level of induced p53 in the feed-forward system with an alternate regimen of tetracycline addition and removal. In the time it takes for TET-ON (e.g. Fig.8 level [c]) in the unheated cells to decline back to background level [e] after removal, the level of TET-ON in the heated cells, [a], would have declined by a similar proportion (which is equal to [c]-[e]). However, since this level ([a]-[c]-[e]) is much higher than in the unheated cells [e], the addition of tetracycline will re-start the feed-forward reaction

for the heated cells from a much higher level ([a]-([c]-[e])). As such, the level of background p53 in unheated cells can be kept at or below the low level reached at 10 hours ([c]) whereas the p53 level in heated cells will continue to escalate. Thus, while the TNFα and p53 expression driven by the HSP directly is transient, the expression driven by the feed-forward system is on for as long as tetracycline is available. Since the regimen of tetracycline addition *in vivo* will be determined by the decay rate of tetracycline *in vivo*, it is important to know the half-life of the TET-ON in tumor cells.

In vivo, the pharmacokinetics of tetracycline is heterogeneous for different tissues. Preferential concentration of tetracycline in specific tissues will lead to higher background expression of TET-ON in some tissues. For example, in humans, 10-35% of oxytetracycline is removed via the kidney, a substantial amount of which is excreted in the active form. Therefore, it is desirable to minimize the background expression levels at the onset to prevent run away amplification in the unintended tissues. The pDATE and pDATH inducible systems use a constitutively expressed antisense TET-ON to suppress the background level of TET-ON translation and a dominant negative TET-ON to compete with leak-through expressed TET-ON to suppress the background expression. With the suppressed background, the timing of tetracycline addition is only affected by the desired level and duration of the expression of the therapeutic genes and not by the need to suppress the level of background expression in normal unirradiated cells.

EXAMPLE 4

Reduction in background levels of expression

Employing the 300 bp HSP promoter, the background level of expression without heat or light is about 25% of the level seen with heat or light. To reduce this, the HSP was linked from -80 to +30 to the minimal pCMV promoter. The pCMV promoter is preferred due to its lower background expression. Additionally, it permits greater amplification of the expression of the therapeutic gene, independent of the constraints of the weaker HSP promoter, which is used to initiate the reaction with a burst of heat or light.

To further overcome the problem of background expression, two cassettes in the plasmid pDATH are introduced. An antisense to TAKON is placed under the control of the pCMV promoter. The constitutively produced antisense binds to any TAKON sense mRNA from the background transcription and prevents its being translated. An additional block on background transcription is provided in cassette #4 in which a dominant negative TAKON with the DNA binding site, but not the transcription activation domain, is placed under the control of the pCMV. This results in background transcription driving the production of TAKON and dominant negative TAKON, which then compete for the ptet binding site.

EXAMPLE 5

Monitoring of p53 expression levels

To ensure that a suitable level of antisense TAKON RNA and dominant negative TAKON protein is produced, levels of p53

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expression are monitored to calibrate copy number and strength of the promoter needed in order to reduce background. First, cell lines harboring pDATH are isolated in the absence of tetracycline. The level of p53 or a cotransfected ptet-EGFP is then monitored to determine the copy number of antisense TAKON and dominant negative TAKON that needs to be incorporated into pDATH to reduce background expression.

EXAMPLE 6

The expression vector pRIBs for treatment of local and metastatic breast and ovarian cancer

As mentioned *supra*, genes placed under the control of such promoters as the radiation inducible promoter of the Egr-1 gene are often expressed only transiently and at low levels. This renders them unsuitable for use in cancer therapy. To overcome these problems, the expression vector pRIBs-X (Radiation-Inducible, Breast-specific Promoter) was designed.

Gene expression levels were optimized using a feed-forward reaction with the tetracycline-dependent transactivator, Tet-On, placed under the control of a tetracycline promoter (tetp), followed by the GAL-4 promoter (pGAL). Transient transcription initiated at pGAL leads to synthesis of a low level of Tet-On, which then binds to tetp in the presence of tetracycline. Tet-On then amplifies its own expression and that of the therapeutic gene linked to it via a feed-forward reaction. The expression of therapeutic genes is controlled by six gene cassettes in the pRIBs vector (Figure 3). In cassette 1, the fusion gene GAL-DBD-mx (HLH-LZ domain of max fused to the DNA-binding domain of GAL-4) is regulated by EGRp. Background expression of GAL-DBD-mx is

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suppressed by a constitutively expressed antisense GAL-DBD-mx and a dominant negative GAL-DBD in cassette 2. In cassette 3, the transcription activation domain of the herpes simplex viral protein VP16 is fused to the HLH-LZ domain of c-Myc. The resulting fusion gene, VP16-TA-mc, placed under the control of the c-erbB-2 promoter, is expressed in breast tumor cells overexpressing c-erbB-2. GAL-DBD-mx fusion protein binds to and activates transcription from the pGAL promoter (cassette 4) by recruiting the VP16-TA-mc proteins.

In unirradiated cells, the translation of the background GAL-DBD-mx mRNA is reduced and the dominant negative GAL-DBD (without mx) competitively occupies the GALp in cassette 4, blocking Tet-On expression. Upon irradiation, GAL-DBD-mx is transiently induced 3-4 fold and temporarily overcomes the suppression by cassette 2. The GAL-DBD-mx recruits the VP-16-TA-mc (a fusion gene of the VP16 transactivation domain and the leucine zipper of myc under the control of the c-erbB-2 promoter) to the GALp and activates a low level of Tet-On transcription starting the feed-forward reaction.

In a treatment scheme using pRIBs-TNFα, for example, can be delivered systemically in a liposome complex or as a recombinant virus to tumor and normal cells alike. Without radiation and tetracycline, TNFα is not expressed. Oxytetracycline is then administered systemically followed by X-ray irradiation of known metastatic tumor sites. As a result, TNFα expression is induced in the tumor sites by the X-ray and amplified and maintained by oxytetracycline. Even though not all tumor cells may take up pRIBs-TNFα, tumor cells in the vicinity of those that do are exposed to the very high local concentration of TNFα secreted. The design of pRIBs-TNFα confers TNFα expression in the breast tumor cells only and not in the

irradiated normal cells that were in the path of the X-ray. As such, systemic toxicity, if any, is limited to the low level of TNFα diffused from the tumor cells. In addition to, or instead of, TNF α, another therapeutic gene, designated X, can be used with the pRIBS vector.

The structure of pRIBs-GFP-1 is shown in Figure 3 and the mode of action summarized in Figure 5. In unirradiated cells, background GAL-DBD-mx expression and function are suppressed by cassette 2 in two ways. The antisense to GAL-DBD-mx suppresses the translation of background GAL-DBD-mx mRNA whereas the GAL-DBD protein acts as a dominant negative inhibitor by competing with GAL-DBD-mx for the pGAL promoter. In irradiated cells, GAL-DBD-mx expression is transiently induced three to 4 fold, overcoming the suppression by cassette 2. The GAL-DBD-mx recruits the VP-16-TA-mc (a fusion gene of the VP16 transactivation domain and the leucine zipper of Myc under the control of the c-erbB-2 promoter) to the GALp and activates the transient expression of the transactivator TET-ON. In the presence of tetracycline, Tet-ON is activated and it binds to and transactivates the tetp promoter (Gossen, M., *et al.*, *Science*, 268:1766-1769 (1995)), amplifying its own level and GFP in a feed-forward reaction. Background expression of TET-ON and GFP is null in the absence of radiation or tetracycline.

EXAMPLE 7

Generation of cell lines and xenografts stably expressing pRIBs-GFP

Two pRIBs-GFP plasmids, pRIBs-GFP-1 and pRIBs-GFP-4, with one and four copies of antisense and dominant negative gene cassettes, respectively, were constructed and stably transfected into the

fibrosarcoma cell line HTB152 and the breast tumor cell lines SK-BR-3 and MDAMB231 for *in vitro* analysis. $5x10^6$ cells are xenografted into SCID mice. While all three human cell lines form poorly differentiated tumors, only SK-BR-3 expresses a high level of c-erbB-2. Indeed, anti-erbB-2 intracellular single-chain antibody which down-regulates cell surface erbB-2, induces apoptosis only in SK-BR-3 but not MDA-MB-231 (Chumakov A.M., *et al., Oncogene* 8:3005-3011 (1993)).

The pRIBs-GFP-1 and -4 plasmids are thus used as models to optimize the conditions for testing treatment of metastatic breast tumor xenografts in nude mice with cytotoxic genes. As cytotoxic genes linked to EGRp are induced only in irradiated cells, toxicity to unirradiated cells is eliminated. However, it is important to prevent expression of cytotoxic genes in normal cells that are in the pathway of the X-ray. The three cell lines, which differ in c-erbB-2 expression, show that controlling VP16-TA-mc expression with a tissue- or tumor-specific promoter confines expression to irradiated breast tumor cells only and not the irradiated normal cells of the vital organs where the metastatic tumor cells reside.

The pRIBs-GFP plasmids are assembled as shown in Figure 3. The GAL-DBA-mx and the VP16-TA-mc are modified from the mammalian two hybrid system (Fearon, E. R., *et al., Proc. Natl. Acad. Sci. USA*, 89:7958-7962 (1992)). Two plasmids, pRIBs-GFP-1 and pRIBs-GFP-4, with 1 and 4 copies of antisense and dominant negative GAL-DBD driven by the minimal CMV promoter are tested.

All three cell lines are cotransfected with pRIBs-GFP and a SVneo plasmid. Cell lines stably expressing pRIBs-GFP-1 and pRIBs-GFP-4 are isolated by selection in G418. For *in vivo* analysis, $5x10^6$ cells of each of the cell lines stably expressing the pRIBs-GFP plasmids are implanted into the flank of SCID mice (four per group) and allowed

to grow to 0.5 cm in diameter. The expression of GFP *in vitro* and in the xenografts without radiation or oxytetracycline is analyzed by extracting the proteins into EBC buffer from the pulverized tumor and the amount of protein is quantitated by RIA.

The inducible level of GFP *in vitro* is measured by Western analysis and quantitated by RIA after irradiating the cells at 0-4 Gy with a Varian Clinac 2000 X-ray generator followed by administration of 0-2 μg/ml of oxytetracycline. Data using HSPp showed that the feed-forward reaction is very efficient and 0.01 μg/ml is sufficient to induce a nine-fold increase of p53 expression in 10 hours. For *in vivo* analysis, tumors are exposed to 0-4 Gy/X-ray. Six hours after radiation, 0-15 μg/g of oxytetracycline is injected intraperitoneally. At 3 hour intervals (for 24 hours) after an injection, tumor mass is removed and the amount of TET-ON and GFP measured relative to the total amount of actin proteins. To achieve a higher or lower level of GFP, the experiments are repeated with the level of TET-ON modified by adjusting the dose of oxytetracycline. The rate of oxytetracycline removal by excretion is monitored by analyzing plasma concentration at three hour intervals.

EXAMPLE 8

Targeting metastatic breast tumors with WAPp or ST3p

The c-erbB-2 promoter had been chosen to initially validate the pRIB-X concept because human cancers overexpressing c-erbB-2 are associated with poor prognosis. It is unlikely, however, that one particular promoter will address the problem of treating different breast tumors. Therefore it is also important to target GAL-DBD-mx expression to metastatic breast tumors with the whey acidic protein promoter, WAPp (McKnight, R.A., *et al., Mol. Reprod. & Dev.*, 44:179-184 (1996)) or

the stromelysin 3 promoter, ST3p (Ahmad, A., *et al., Int. J. of Cancer*, 73:290-296 (1997)). WAPp targets expression to breast epithelial cells while ST3p targets expression to matrix-metalloproteinase-secreting stromal cells adjacent to tumors.

pRIBs is reconstructed by replacing the c-erbB-2 promoter with either WAPp or ST3p. Breast and other tumor cell lines are screened for high and low expression of WAP and ST3. Cell lines differing in their expression of WAP and/or ST3 are used to test the expression of GFP.

The WAP promoter has been shown to be very specific for lactating mammary epithelial cells in transgenic animals (Tzeng YJ., *et al., Oncogene* 16(16):2103-2114 (1998)) and the stromelysin 3 promoter, ST3p, has been shown to be expressed only in stromal fibroblasts adjacent to cancer cells. Evidence suggests that production in stromal cell of matrix-metalloproteinases (including ST3), implicated in the process of tumor metastasis, is stimulated by the cancer cells. Thus, the targeting of VP16-TA-mc to the stromal cells will lead to the expression and release of therapeutic gene products in the vicinity of the metastatic tumor cells. It must be noted that additional treatment specificity is attained by delivering pRIBs-X with liposomes coated with antibodies to c-erbB2.

EXAMPLE 9

The expression vector pRIPs for treatment of local and metastatic prostate cancer

As mentioned *supra*, genes placed under the control of such promoters as the radiation inducible promoter of the Egr-1 gene are

often expressed only transiently and at low levels. This renders them unsuitable for use in cancer therapy. To overcome these problems, the expression vector pRIPs-X (Radiation-Inducible, Prostate-specific Promoter) was designed.

The pRIPS vector is comprised of six cassettes. Gene cassette 1 differs from previously described vectors only in that it contains "Gal-DBD-mx" which is a fusion ORF encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein (Gal-DBD) fused to the basis helix-loop-helix-leucine zipper (bHLHLZ) domain of Max (mx, amino acids 8-112) followed by SV40 poly A. Gene cassette 2 is comprised of the minimal CMV promoter (mCMVp), "antisense Gal-DBD-mx", which is an antisense construct complementary to the Gal-DBD-mx sequence, "IRES", which is an internal ribosomal entry site and "Gal-DBD" which competes with the Gal-DBD-mx for the pGAL binding site. Gene cassette 3 is comprised of "VP16-TA-mc" which is a fusion ORF encoding at the N-terminus the first 11 amino acids of Gal4 (amino acids 1-147), followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the bHLHLZ domain of c-Myc (amino acids 350-439), followed by SV40 polyA. The resulting fusion gene, VP16-TA-mc, is placed under the control of the probasin gene promoter "pProbasin" up to the first ATG. Gene cassette 4 contains "GALp", consisting of five copies of a 17-mer DNA-binding site for Gal4. The TET-ON sequence is placed under the control of the GALp-ptet promoter and the therapeutic gene, X, is linked to the TET-ON via an IRES; Gene cassette 5 contains an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence

including the ATG, placed under the control of the pCMV promoter. Gene cassette 6 contains a dominant negative TET-ON consisting of the coding sequences for amino acids 1-207 of the tet repressor placed under the control of the pCMV promoter. In other variants of pRIPs-X, pProbasin is replaced by PSA, the promoter region of the prostate specific antigen, or other prostate-specific genes.

EXAMPLE 10

The expression vector pHIBs-X for treatment of local and metastatic breast and ovarian cancer

The expression vector pHIBs-X was designed and is comprised of six cassettes. Gene cassette 1 differs from previously described vectors only in that it contains "Gal-DBD-mx" which is a fusion ORF encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein (Gal-DBD) fused to the basis helix-loop-helix-leucine zipper (bHLHLZ) domain of Max (mx, amino acids 8-112) followed by SV40 poly A. The resulting fusion gene GAL-DBD-mx is controlled by the heat inducible HSP promoter. Gene cassette 2 is comprised of the minimal CMV promoter (mCMVp), "antisense Gal-DBD-mx", which is an antisense construct complementary to the Gal-DBD-mx sequence, "IRES", which is an internal ribosomal entry site and "Gal-DBD" which competes with the Gal-DBD-mx for the pGAL binding site. Gene cassette 3 is comprised of "VP16-TA-mc" which is a fusion ORF encoding at the N-terminus the first 11 amino acids of Gal4 (amino acids 1-147), followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the bHLHLZ domain of c-Myc (amino acids 350-439), followed by SV40 polyA. The resulting fusion

gene, VP-16TA-mc, is placed under the control of the c-erbB-2 promoter "perbB2" up to the first ATG. Gene cassette 4 contains "GALp", consisting of five copies of a 17-mer DNA-binding site for Gal4. The TET-ON sequence is placed under the control of the GALp-ptet promoter and the therapeutic gene, X, is linked to the TET-ON via an IRES; Gene cassette 5 contains an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG, placed under the control of the pCMV promoter. Gene cassette 6 contains a dominant negative TET-ON consisting of the coding sequences for amino acids 1-207 of the tet repressor placed under the control of the pCMV promoter.

The pHIBs-X expression vector is identical to the pRIBs-X plasmid except for gene cassette 1 where the Egr-1 promoter in pRIBs-X is replaced by the HSP 70 promoter. pHIBs-X specifically targets local and metastatic breast and ovarian tumors when the tumors are exposed to heat.

EXAMPLE 11

The expression vector pHIPs-X for treatment of local and metastatic prostate cancer

Figure 10 illustrates the structure of the pHIPs-GFP (Heat-Inducible, Prostate-specific Promoter) expression vector. This vector is comprised of six cassettes. Gene cassette 1 differs from previously described vectors only in that it contains "Gal-DBD-mx" which is a fusion ORF encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein (Gal-DBD) fused to the basis helix-loop-helix-leucine zipper (bHLHLZ) domain of Max (mx, amino acids 8-112) followed by SV40 poly A. The resulting fusion gene GAL-DBD-mx

is controlled by the heat inducible HSP promoter. Gene cassette 2 is comprised of the minimal CMV promoter (mCMVp), "antisense Gal-DBD-mx", which is an antisense construct complementary to the Gal-DBD-mx sequence, "IRES", which is an internal ribosomal entry site and "Gal-DBD" which competes with the Gal-DBD-mx for the pGAL binding site. Gene cassette 3 is comprised of "VP16-TA-mc" which is a fusion ORF encoding at the N-terminus the first 11 amino acids of Gal4 (amino acids 1-147), followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the bHLHLZ domain of c-Myc (amino acids 350-439), followed by SV40 polyA. The resulting fusion gene, VP16-TA-mc, is placed under the control of the probasin gene promoter (pProbasin) up to the first ATG. Gene cassette 4 contains "GALp", consisting of five copies of a 17-mer DNA-binding site for Gal4. The TET-ON sequence is placed under the control of the GALp-ptet promoter and the therapeutic gene, X, is linked to the TET-ON via an IRES; Gene cassette 5 contains an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG, placed under the control of the pCMV promoter. Gene cassette 6 contains a dominant negative TET-ON consisting of the coding sequences for amino acids 1-207 of the tet repressor placed under the control of the pCMV promoter.

The pHIPs-X expression vector is identical to the pRIPs-X plasmid except for gene cassette 1 where the Egr-1 promoter in pRIBs-X and pRIPs-X is replaced by the HSP 70 promoter. pHIPs-X specifically targets local and metastatic prostate tumors when the tumors are exposed to heat.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

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WHAT IS CLAIMED IS:

1. A recombinant vector, pDATH-X (Dominant negative, Antisense, TET-ON controllable Heat shock promoter plasmid), said vector comprising the cassettes:

(a) a fusion of the coding sequences for amino acids 1-207 of the tetracycline repressor and the C-terminus last 130 amino acid transcription activation domain of the VP16 protein of the herpes simplex virus;

(b) a heat shock promoter consisting of heat shock response elements (-260 to 30) of the human heat shock 70 gene promoter linked to the minimal cytomegalovirus promoter, pCMV;

(c) a tet operator consisting of the 19 bp inverted repeats of the operator O2 of TN10 to which the tet repressor and TAKON bind; and

(d) an antisense sequence consisting of the complementary sequence to the first 80 bases of the TAKON sequence including the ATG.

2. A method of achieving sustained expression of a gene under control of a heat or light inducible promoter, comprising the step of:

introducing the vector containing said gene into the host organism; and

applying heat or light energy.

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3. The method of claim 2, where said host organism is a human.

4. A recombinant vector, pDATE-X (Dominant negative, Antisense, TET-ON controllable EGR promoter expression plasmid), said vector comprising the cassettes:

(a) cassette 1 comprising the TET-ON sequence under the control of the EGRp, the tetracycline operator binding site and pCMV;

(b) cassette 2 comprising a therapeutic gene X under the control of the tetp-pCMV promoter;

(c) cassette 3 comprising antisense TET-ON under the control of the pCMV promoter; and

(d) cassette 4 comprising dominant negative TET-ON under the control of the pCMV promoter.

5. A recombinant vector, pRIBs-X, (Radiation-Inducible, Breast-specific Promoter) expression vector, said vector comprising the cassettes:

(a) cassette 1 comprising "Gal-DBD-mx" which is a fusion open reading frame encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein (Gal-DBD) fused to the basic helix-loop-helix-leucine zipper domain of Max (amino acids 8-112) followed by SV40 poly A, wherein the resulting fusion gene GAL-DBD-mx is controlled by the radiation inducible Egr-1 promoter;

(b) cassette 2 comprising the minimal CMV promoter, "antisense Gal-DBD-mx", which is an antisense construct

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complementary to the Gal-DBD-mx sequence, an internal ribosomal entry site (IRES) and "Gal-DBD" which competes with the Gal-DBD-mx for the pGAL binding site;

(c) cassette 3 comprising "VP16-TA-mc" which is a fusion ORF encoding at the N-terminus the first 11 amino acids of Gal4 (amino acids 1-147), followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the basic helix-loop-helix-leucine zipper domain of c-Myc (amino acids 350-439), followed by SV40 polyA, wherein the resulting fusion gene, VP16-TA-mc, is under the control of the c-erbB2 promoter "perB2" up to the first ATG;

(d) cassette 4 comprising "Galp", five copies of a17-mer DNA-binding site for Gal4, wherein a TET-ON sequence is placed under the control of the GAPp-ptet promoter and a therapeutic gene X is linked to the TET-IN via an IRES;

(e) cassette comprising an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG under the control of the pCMV promoter; and

(f) cassette 6 comprising a dominant negative TET-ON consisting of the coding sequences for amino acids 1-207.

6. The recombinant vector of claim 5, wherein the perbB2 promoter of cassette 3 is replaced with the whey acidic protein promoter.

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7. The recombinant vector of claim 5, wherein the perbB2 promoter of cassette 3 is replaced with the stromelysin 3 promoter.

8. The recombinant vector of claim 5, wherein said gene X is a gene encoding tumor necrosis factor alpha.

9. A method of treating local and metastatic breast and ovarian cancer comprising the step of:

administering the expression vector of claim 5 to an individual in need of such treatment.

10. A method of treating local and metastatic breast and ovarian cancer comprising the step of:

administering the expression vector of claim 6 to an individual in need of such treatment.

11. A method of treating local and metastatic breast and ovarian cancer comprising the step of:

administering the expression vector of claim 7 to an individual in need of such treatment.

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12. A recombinant pRIPs-X (Radiation-Inducible, Prostate-specific Promoter) expression vector, said vector comprising the cassettes:

(a) cassette 1 comprising "Gal-DBD-mx" which is a fusion open reading frame encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein fused to the basic helix-loop-helix leucine zipper domain of Max (amino acids 8-112) followed by SV40 polyA, wherein the resulting fusion gene GAL-DBD-mx is controlled by the radiation inducible Egr-1 promoter;

(b) cassette 2 comprising the minimal CMV promoter, antisense Gal-DBD-mx, which is an antisense construct complementary to the Gal-DBD-mx sequence, IRES, which is an internal ribosomal entry site and Gal-DBD which competes with the Gal-DBD-mx for the pGAL binding site;

(c) cassette 3 comprising "VP16-TA-mc", a fusion open reading frame encoding at the N-terminus the first 11 amino acids of Gal4, followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the basic helix-loop-helix leucine zipper domain of c-Myc (amino acids 350-439), followed by SV40 polyA, wherein the resulting fusion gene, VP16-TA-mc, is under the control of the probasin gene promoter "pProbasin" up to the first ATG;

(d) cassette 4 comprising GALp, five copies of the 17-mer DNA-binding site for Gal4, wherein the TET-ON sequence is under the control of the GALp-ptet promoter and a therapeutic gene X is linked to the TET-ON via an internal ribosomal entry site;

(e) cassette 5 comprising an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80

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bases of the TET-ON sequence including the ATG, under the control of the pCMV promoter; and

(f) cassette 6 comprising a dominant negative TET-ON consisting of the coding sequence for amino acids 1-207.

13. The recombinant vector of claim 12, wherein said probasin promoter of cassette 3 is replaced with the prostate specific antigen promoter.

14. The recombinant vector of claim 12, wherein said gene X is tumor necrosis factor alpha.

15. A method of treating local and metastatic prostate cancer comprising the step of:

administering the expression vector of claim 12 to an individual in need of such treatment.

16. A method of treating local and metastatic prostate cancer comprising the step of:

administering the expression vector of claim 13 to an individual in need of such treatment.

17. A recombinant expression vector, pHIBs-X (Heat Inducible, Breast-specific promoter), said vector comprising the cassettes:

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(a) cassette 1 comprising Gal-DBD-mx which is a fusion open reading frame encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein fused to the basic helix-loop-helix leucine zipper domain of Max (amino acids 8-112) followed by SV40 polyA, wherein the resulting fusion gene GAL-DBD-mx is controlled by the heat inducible heat shock protein promoter;

(b) cassette 2 comprising the minimal CMV promoter, antisense Gal-DBD-mx, a construct complementary to the Gal-DBD-mx sequence, an internal ribosomal entry site and Gal-DBD, which competes with the Gal-DBD-mx for the pGAL binding site;

(c) cassette 3 comprising "VP16-TA-mc" which is a fusion open reading frame encoding at the N-terminus the first 11 amino acids (amino acids 1-147), followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the basic helix-loop-helix leucine zipper domain of c-Myc (amino acids 350-439), followed by SV40 polyA, wherein the resulting fusion gene VP16-TA-mc is under the control of the c-erbB2 gene promoter "perbB2" up to the first ATG;

(d) cassette 4 contains GALp, five copies of a 17-mer DNA-binding site for Gal4, wherein the TET-ON sequence is under the control of the GALp-ptet promoter and a therapeutic gene, X, is linked to the TET-ON via an internal ribosomal entry site;

(e) cassette 5 comprising an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG, under the control of the pCMV promoter; and

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(f) cassette 6 comprising a dominant negative TET-ON consisting of the coding sequences for amino acids 1-207.

18. The recombinant vector of claim 17, wherein the perbB2 promoter of cassette 3 is replaced with the whey acidic protein promoter.

19. The recombinant vector of claim 17, wherein the perbB2 promoter of cassette 3 is replaced with the stromelysin 3 promoter.

20. The method of claim 17, wherein said therapeutic gene is tumor necrosis factor alpha.

21. A method of treating local and metastatic breast and ovarian cancer comprising the step of:

administering the expression vector of claim 17 to an individual in need of such treatment.

22. A method of treating local and metastatic breast and ovarian cancer comprising the step of:

administering the expression vector of claim 18 to an individual in need of such treatment.

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23. A method of treating local and metastatic breast and ovarian cancer comprising the step of:

administering the expression vector of claim 19 to an individual in need of such treatment.

24. A recombinant vector, pHIPs-X (Heat-Inducible, Prostate-specific Promoter), said vector comprising the cassettes:

(a) cassette 1 comprising Gal-DBD-mx which is a fusion open reading frame encoding the N-terminus (amino acids 1-147) DNA-binding domain of the yeast GAL4 protein fused to the basic helix-loop-helix leucine zipper domain of Max (amino acids 8-112) followed by SV40 polyA, wherein the resulting fusion gene GAL-DBD-mx is controlled by the heat inducible heat shock protein promoter;

(b) cassette 2 comprising the minimal CMV promoter (mCMVp), antisense Gal-DBD-mx, a construct complementary to the Gal-DBD-mx sequence, an internal ribosomal entry site and Gal-DBD, which competes with the Gal-DBD-mx for the pGAL binding site;

(c) cassette 3 comprising "VP16-TA-mc", a fusion open reading frame encoding at the N-terminus the first 11 amino acids of Gal4, followed by the nuclear localization signal of the SV40 large T antigen, the 130 amino acid C-terminus transactivation domain of the herpes simplex viral protein VP16, the basic helix-loop-helix leucine zipper domain of c-Myc (amino acids 350-439), followed by SV40 polyA, wherein the resulting fusion gene, VP16-TA-mc, is under the control of the probasin gene promoter "pProbasin" up to the first ATG;

(d) cassette 4 comprising GALp, five copies of a 17-mer DNA-binding site for Gal4, wherein the TET-ON sequence is under the

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control of the GALp-ptet promoter and a therapeutic gene, X, is linked to the TET-ON via an internal ribosomal entry site;

(e) cassette 5 comprising an antisense TET-ON which is a sequence consisting of the complementary sequence to the first 80 bases of the TET-ON sequence including the ATG, under the control of the pCMV promoter; and

(f) cassette 6 comprising a dominant negative TET-ON consisting of the coding sequences for amino acids 1-207.

25. The recombinant vector in claim 24, wherein the probasin promoter is replaced with the prostate-specific antigen promoter.

26. The recombinant vector of claim 24, wherein said therapeutic gene is tumor necrosis alpha.

27. A method of treating local and metastatic prostate cancer comprising the step of:

administering the expression vector of claim 24 to an individual in need of such treatment.

28. A method of treating local and metastatic prostate cancer comprising the step of:

administering the expression vector of claim 25 to an individual in need of such treatment.

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