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# Molecular approaches to chemo-radiotherapy

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**Abstract**

Although radiotherapy is used to treat many solid tumours, normal tissue tolerance and inherent tumour radioresistance can hinder successful outcome. Cancer gene therapy is one approach being developed to address this problem. However, the potential of many strategies are not realised owing to poor gene delivery and a lack of tumour specificity. The use of treatment-, condition- or tumour-specific promoters to control gene-directed enzyme prodrug therapy (GDEPT) is one such method for targeting gene expression to the tumour. Here, we describe two systems that make use of GDEPT, regulated by radiation or hypoxic-responsive promoters. To ensure that the radiation-responsive promoter is be activated by clinically relevant doses of radiation, we have designed synthetic promoters based on radiation responsive CArG elements derived from the Early Growth Response 1 (*Egr1*) gene. Use of these promoters in several tumour cell lines resulted in a 2–3-fold activation after a single dose of 3 Gy. Furthermore, use of these CArG promoters to control the expression of the herpes simplex virus (HSV) thymidine kinase (*tk*) gene in combination with the prodrug ganciclovir (GCV) resulted in substantially more cytotoxicity than seen with radiation or GCV treatment alone. Effectiveness was further improved by incorporating the GDEPT strategy into a novel molecular switch system using the Cre/loxP recombinase system of bacteriophage P1. The level of GDEPT bystander cell killing was notably increased by the use of a fusion protein of the HSVtk enzyme and the HSV intercellular transport protein vp22. Since hypoxia is also a common feature of many tumours, promoters containing hypoxic-responsive elements (HREs) for use with GDEPT are described. The development of such strategies that achieve tumour targeted expression of genes via selective promoters will enable improved specificity and targeting thereby addressing one of the major limitations of cancer gene therapy. © 2002 Published by Elsevier Science Ltd.

*Keywords:* Radiation; Gene therapy; Hypoxia; Promoters; CArG elements; GDEPT

## 1. Combination gene therapy and radiotherapy

Radiotherapy is the treatment modality of choice for most solid malignancies. However, the tolerance of surrounding normal tissues to treatment-induced injury often restricts the dose that can be delivered to the tumour to achieve cure. This problem can be partly overcome using physical techniques such as conformal or intensity modulated radiotherapy that deliver the dose to a more precisely defined tumour volume. An alternative approach is to combine radiotherapy with a pharmacological- or gene-based component, which improves the effectiveness of the radiation dose delivered to the tumour by increasing the therapeutic ratio between normal and malignant cells. Pharmacological approaches to modify tumour radiosensitivity are well established, and include for example the use of chemical radiosensitisers and inhibitors of cellular repair processes and tumour cell proliferation [1]. In contrast, the combination of radiotherapy and gene therapy technologies is an evolving discipline. Gene delivery, insufficient specificity and tumour targeting often hamper the efficacy of gene therapy approaches, but new concepts are evolving to overcome these [2,3]. This review will focus on one of these problems, describing two different strategies to selectively target the tumour, one mediated by radiation and the second by hypoxia. Both exploit selective gene promoters to activate gene-directed enzyme prodrug therapy (GDEPT) systems for use as adjuvant or concomitant with radiotherapy. GDEPT involves the delivery to the target cells of a foreign gene encoding a non-toxic enzyme which activates specific prodrugs to toxic agents at the site of conversion [4].

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## 2. Radiation-mediated gene therapy (RMGT)

Radiation-mediated gene therapy exploits the fact that in the majority of patients receiving radiotherapy the radiation is directed to the tumour volume, providing some degree of tumour localisation for controlling the expression of therapeutic genes. Temporal and spatial control of gene expression can therefore be achieved for any genes delivered to the tumour and for any tumour types treated with radiotherapy [5–7].

### 2.1. Radiation-induced gene expression

A number of cell cycle control and DNA repair genes (e.g. $p21^{WAF1/CIP1}$ and Early Growth Response 1 (*Egr1*) [8–10]) are upregulated following exposure to ionising radiation [11–15]. Some of these genes, such as *WAF1* [10] and *GADD45* [16] operate within p53-dependent pathways, and thus may not function efficiently in the many tumours exhibiting mutant p53 phenotypes. However, since activation of *Egr1* is p53-independent, its promoter has been adopted for the regulation of therapeutic genes in the majority of the experimental models of RMGT. A 425 nucleotide region (denoted E425 here) [17] of the murine *Egr1* gene was inserted upstream of the cDNA of the tumoricidal cytokine tumour necrosis factor-α (TNF-α) and delivered to human epidermoid carcinoma xenografts (SQ-20B) using a cell carrier [8], liposomes [5] or an adenoviral vector (Ad5 [18,19]). The combination of a fractionated radiation dose schedule of 40 Gy (5 Gy fractions twice a day for 4 days) and intratumorally delivered Ad5.Egr.TNF resulted in increased tumour regression by up to 90% of control values compared with maxima of 50% for vector or radiation alone [7,19]. Similarly, Joki and colleagues [20] and Takahashi and colleagues [21] used a plasmid construct containing the E425 promoter to regulate the expression of the herpes simplex virus thymidine kinase (HSV*tk*) suicide gene. Glioma cells containing this vector were sensitised to the anti-herpes viral agent ganciclovir (GCV), a substrate for HSVtk, after exposure to a single radiation dose of 20 Gy.

The radiation-responsive regions of the *Egr1* promoter have been identified as 10 nucleotide motifs of the consensus sequence $CC(A/T)_6GG$, also known as CArG elements [22]. However, only the CArG elements grouped in the 5′ distal 'enhancer' region, appear to contribute to the radiation responsiveness of *Egr1*. Radiation-produced reactive oxygen intermediates (ROIs) are believed to be critical in activating the *Egr1* promoter via these CArG motifs [23]. In addition, CArG elements are also known to be involved in the regulation of a number of immediate-early genes (e.g. *Egr1*, *c-fos*, *β-actin*) following mitogenic stimulation, and are thus often referred to as serum-response elements. This growth factor-induced response is governed by the binding of serum-response factor. Whether this transcription factor is also involved in the activation of the *Egr1* gene by ionising radiation has not as yet been determined. The human and murine *Egr1* promoters also contain putative binding sites for the Sp1 transcription factor, the Fos-Jun heterodimer AP1, as well as for cyclic adenosine monophosphate (AMP) and Egr1 itself [24–27], all of which may influence radiation-mediated promoter induction.

### 2.2. Synthetic radiation-responsive gene promoters

In order to produce a radiation-responsive promoter without such potentially antagonistic binding sites, we have developed synthetic alternatives based on isolated CArG elements [28]. These promoters consist of an enhancer region, containing the CArG elements themselves, adjacent to a basal promoter (i.e. from the cytomegalovirus (CMV) immediate early gene) containing the essential transcriptional initiation apparatus, such as the CCAAT/TATA boxes (see Fig. 1 [28]). The enhancer is produced by cloning complementary, single-stranded, oligo-deoxyribonucleotides (ODNs) containing the CArG sequences. The synthetic promoter is positioned directly upstream of the coding sequence of the enhanced green fluorescent protein (EGFP) reporter gene [29,30] in a plasmid vector. Target cells are transfected with the plasmid construct, irradiated and subsequent EGFP production measured by flow cytometry. A synthetic promoter containing four CArG elements was shown to be responsive to radiation doses as low as 1 Gy and was more radiation-responsive (3.1-fold ± 0.2) than the wild-type *Egr1* counterpart (1.86-fold ± 0.2) at an optimal 3 Gy dose (Fig. 2 [28,31]). Multiple doses of 3 Gy resulted in greater levels of induced expression than seen after a single dose. Promoters containing different numbers, sequences and arrangements of CArG elements were also evaluated. Increasing the number of CArG elements in the promoter improved the specific radio-responsiveness and reduced basal expression (data not shown). We have also demonstrated that alteration of CArG element core sequences can substantially affect the response, either positively or negatively. Although the transcription factors involved in the induction have yet to be identified, the binding of such proteins or protein complexes to the CArG elements are likely be influenced by their spatial arrangement. This is currently being investigated in our laboratory.

A GDEPT approach was then used to demonstrate the potential of CArG-activated promoters. This consisted of the HSV*tk*/GCV suicide gene system and a tumour cell growth inhibition assay. The HSV*tk*/GCV system, currently adopted in clinical trials [32], is the most well known example of enzyme/prodrug combination in cancer GDEPT. The killing effect of the HSV*tk*/

GCV is only seen in proliferating cells in which HSVtk can monophosphorylate GCV, which is then further phosphorylated by cellular kinases, to cause termination of DNA synthesis and cell death [33]. The HSV*tk*/GCV system is therefore particularly suitable for the eradication of rapidly dividing tumour cells invading non-proliferating tissue. However, HSV*tk*/GCV would not be the combination of choice to target the slowly dividing hypoxic population in solid tumours, which have been shown to contribute to the resistance of human tumours to chemo- and radiotherapy. Alternative enzyme/prodrug combinations able to function in non-proliferating cells would be for example carboxypeptidase 2 (CPG2) and CMDA [34,35] or horseradish peroxidase (HRP) and indole-3-acetic acid (IAA) [4,36]. The selective enhancement of radiation-mediated toxicity using GDEPT has been demonstrated both *in vitro* and *in vivo*, with some degree of synergy between radiation and gene therapy noted [37–42]. Non-transduced cells adjacent to HSV*tk*-containing cells can also be killed via a

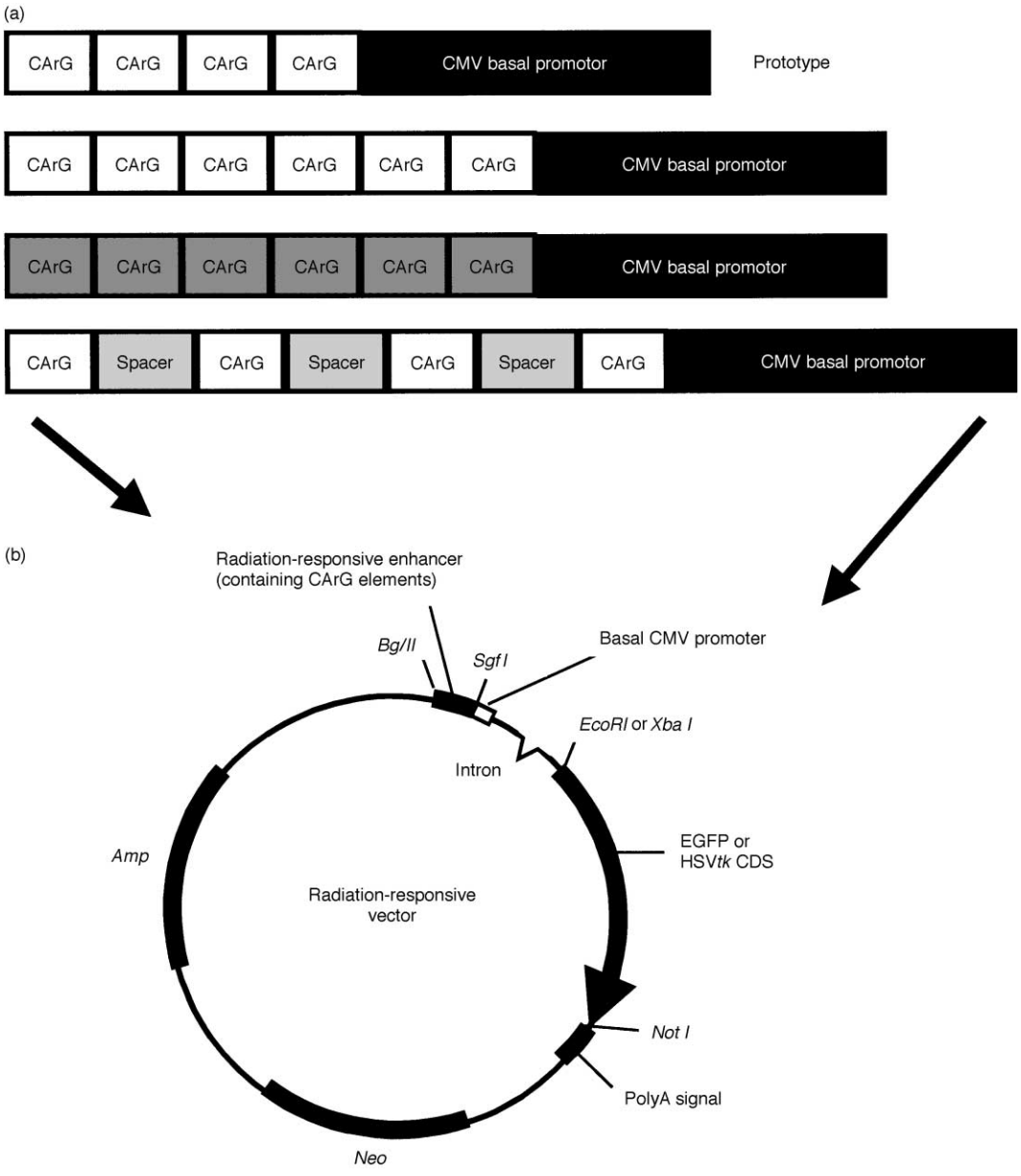


Fig. 1. (a) Strategies adopted to improve the radiation responsiveness of the synthetic CArG enhancer; increasing the number of CArG elements from the four used in the prototype (28); altering the CArG element 'core' sequences; introducing spacers between CArG elements to aid binding of any transcription factors involved in the radiation response. (b) Schematic representation of the radiation-responsive plasmid vector (based on PCI-*neo*; Promega) showing chimeric enhancer/promoter and reporter (EGFP) or suicide (HSV*tk*) coding sequences (CDS). Restriction sites used for cloning components are italicised. The intron is present to increase expression of the CDS immediately downstream. The SV40 polyadenylation signal allows efficient processing of RNA transcripts. *Amp* and *Neo* genes are used as selectable markers for establishing clones in bacterial and mammalian cell cultures respectively. CMV, cytomegalovirus; EGFP, enhanced green fluorescent protein.

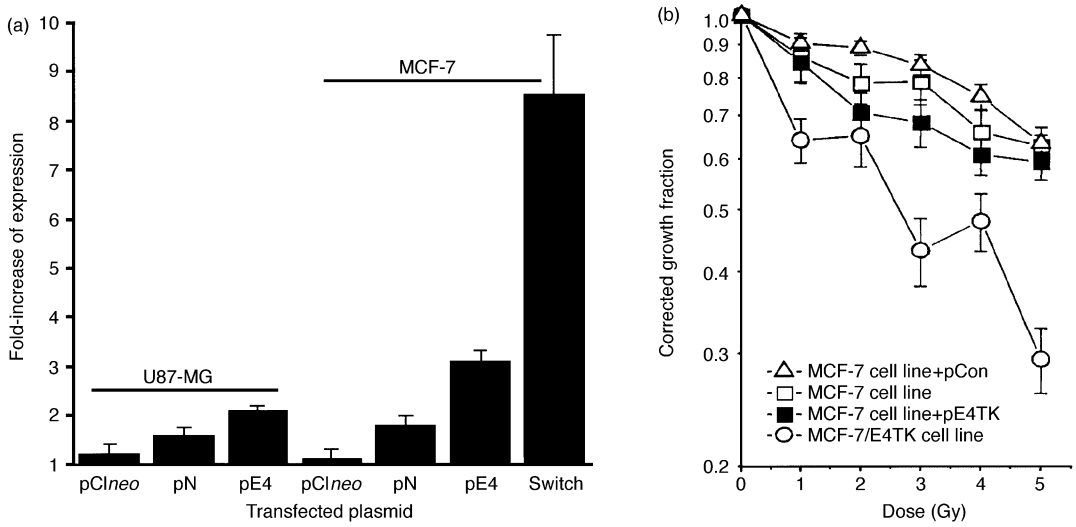


Fig. 2. (a) Fold- increase of enhanced green fluorescent protein (EGFP) expression in U87-MG glioma and MCF-7 breast adenocarcinoma cells 40 h after irradiation with a single dose of 3 Gy. Before irradiation, cells were transfected with plasmids containing the *EGFP* gene controlled by either the synthetic enhancer containing four CArG elements (pE4); the wild-type human Egr1 enhancer (labelled 'switch') (pN); or a control plasmid (pCI*neo*) [28]. MCF-7 cells were also co-transfected with two plasmids that allowed Cre/loxP-mediated recombination. Radiation activates the production of Cre via the 4 CArG promoter (pE4Cre; see Figure 3, Ref. [31]). Cre then causes recombination in the second plasmid (pSGFP) resulting in constitutive expression of EGFP from the CMV promoter. The synthetic enhancer was significantly better at inducing EGFP expression than the wild-type enhancer. Furthermore, when E4 was incorporated into the 'promoter switch' system, a substantially higher level of expression was achieved. (b) The effect of radiation on cell growth following transfection. Open squares, MCF-7 cells; open triangles, MCF-7 cells transiently transfected with the pCI*neo* control plasmid (pCon); solid squares, MCF-7 cells transiently transfected with pE4TK plasmid; open circles, MCF-7/E4TK established cell line (see also Ref. [28]).

bystander mechanism [43]. This 'bystander effect' is thought to be partly attributable to the diffusion of toxin molecules through gap junctions [44–46]. *In vivo*, the host immune system is believed to enhance this effect [47,48]. Bystander killing will be vital for prodrug-mediated gene therapy since the most efficient delivery systems will be unable to target all the cells in a tumour. Wu and colleagues [49] noted that complete tumour cell killing was achieved when only 10% of cultured melanoma cells were expressing HSV*tk* in the presence of GCV. Furthermore, it has been shown that significant tumour regression could be achieved in animal models when only 10% of tumour cells were producing HSV*tk* [50].

In our own studies, when tumour cell cultures were transfected with CArG-based vectors (~20% of cells) and grown in the presence of GCV after a single 2 Gy trigger dose, cell survival was reduced to ~70%, compared with 90% for mock-transfected cultures treated similarly [28]. If this finding were to be translated into a therapeutic dose schedule in a typical 70 Gy treatment (35 fractions of 2 Gy), the resultant equivalent dose directed to the tumour would be almost 100 Gy.

The potential of using the synthetic radiation-responsive promoters was also evaluated in a preliminary study *in vivo* in a MCF-7 breast adenocarcinoma xenograft model. Modified cell lines were established from plasmid-transfected MCF-7 cells by G418 selection and cloning. One of these stable cell lines transfected with a synthetic promoter containing four CArG elements regulating the expression of HSV*tk* was used to establish xenografts in nude mice. 50 μM GCV was administered interperitoneally (i.p.) 2 days prior, during and for three days after three daily 3-Gy fractions of X-rays. The effect of each treatment was compared by assessing the time required for tumours to reach to 500 $mm^3$ (Table 1). An increase in tumour growth delay was evident for those tumours treated with GCV combined with irradiation, compared with GCV or radiation treatment alone. Despite the small size of this study, it clearly demonstrated that the synthetic radiation-responsive promoters are functional *in vivo*, an observation that supported our earlier studies in whole tumours using EGFP as a reporter system.

We have also investigated the use of an intercellular transport protein (the HSV protein vp22) to enhance the bystander effect. As reported by Elliot and O'Hare [51], vp22 is an efficient intercellular transporter, able to transfer into neighbouring cells and translocate to the nucleus of the target cell. The fusion protein product of the chimeric *vp22/EGFP* gene was efficiently exported into as many as 200 nuclei of cells surrounding the original transfectant. Chimeric vp22 proteins are known to function in a wide range of cell lines [52] and deliver proteins that modify the cell response to radiation damage [53]. This active transport has been achieved using vp22/HSVtk fusion proteins that retain enzyme

Table 1
Measurement of growth delay (days to reach 500 mm$^3$ after treatment) of MCF-7 and MCF-7/pE4TK tumour xenografts in nude mice[a]

| Xenograft | Radiation treatment | GCV dosing (IP) | Days to 500 mm$^3$ |
|---|---|---|---|
| MCF-7 | Sham | None | 8.9±2 |
| MCF-7 | Sham | 8×50 μM | 11.2±5 |
| MCF-7 | 3×3 Gy | None | 21.4±4 |
| MCF-7 | 3×3 Gy | 8×50 μM | 23.1±4 |
| MCF-7/pE4TK | Sham | None | 11.3±6 |
| MCF-7/pE4TK | Sham | 8×50 μM | 14.7±5 |
| MCF-7/pE4TK | 3×3 Gy | None | 24.9±5 |
| MCF-7/pE4TK | 3×3 Gy | 8×50 μM | 33.1±3 |

[a] Treatment commenced when tumours were of the same size. MCF-7/pE4TK cells were established by transfecting MCF-7 cells with plasmids containing HSV*tk* under the control of the radiation-responsive four CArG promoter, and then enriched by neomycin selection so that all cells contained the HSV*tk* gene. Tumours were divided into groups and either sham-irradiated, irradiated with three doses of 3 Gy (24 h interval), given intraperitoneal (i.p.) injections of ganciclovir (GCV, 8 daily doses of 50 μM) or vehicle controls, or combinations of the treatments. The largest tumour growth delay, indicating the highest toxicity, was seen in irradiated tumours that were combined with GCV treatment. These data indicate that the synthetic promoter is functional *in vivo*.

activity to transduce surrounding cells making them targets for GCV-mediated cell killing [54]. Moreover, vp22 transport is independent of intercellular connections [51]. This is particularly attractive for its use in tumour cells where cell–cell communication is often considerably impaired. We have shown that a vp22/EGFP protein can be exported from the plasmid-transfected cell to its neighbours, leading to their fluorescence as evidenced by microscopy. When the *EGFP* gene was replaced with HSV*tk*, tumour cell killing in the vp22/HSV*tk*-transfected populations was increased >5-times compared with HSV*tk* alone. Thus, the use of the vp22 protein may have the potential to address the important limitation of gene delivery [55].

In order to ensure long-term, high-level expression of therapeutic genes following the modest radiation-induction of the CArG promoters, we have devised a novel 'molecular switch' system based on the Cre/LoxP recombinase of bacteriophage P1 (Fig. 3 [31,56]). Using this strategy, the radiation-responsive promoter drives the expression of the *Cre* recombinase gene, which in turn activates a transcriptionally-silenced tumour sensitising gene by loxP site-mediated recombination. Therefore, a single activating dose of radiation could lead to HSV*tk* gene expression via the strong constitutive CMV promoter, thereby producing a substantial amplification of the activation signal. Indeed, in dual-plasmid transfection experiments using the EGFP reporter system, we demonstrated an 8.2-fold (±1.2) increase in numbers of brightly fluorescent cells after a radiation trigger, compared with unirradiated control cultures (Fig. 2 and [31]). In addition, these cells were on average nearly 15 times brighter than those produced when the CArG promoter directly (without the Cre/

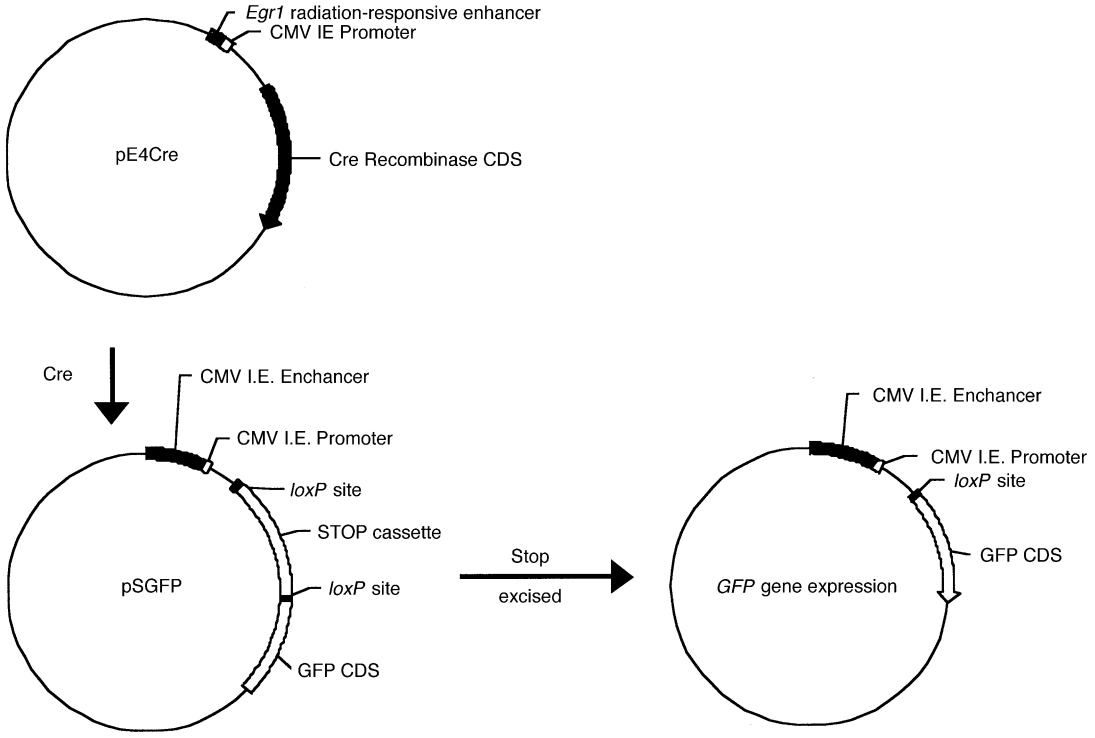


Fig. 3. Schematic representation of the 'molecular switch'. Irradiation induces Cre recombinase expression via the E4 promoter. Cre excises the transcriptional stop cassette from pSGFP via the loxP sites. The resultant recombinant plasmid now expresses green fluorescent protein (GFP) from the strong constitutive CMV IE promoter [31]. CDS, coding sequences.

loxP switch) regulated EGFP expression. This translates to an approximately 40-fold induction of EGFP production via the switch, compared with 3.1 (±0.25)-fold when the synthetic promoter regulated EGFP expression directly. This system has now been engineered to function in a single vector, thus avoiding the necessity of having to target two vectors to each tumour cell. The use of a constitutive secondary promoter (e.g. CMV) should also ensure sustained expression of the therapeutic gene within the tumour volume after the radiation stimulus has been withdrawn. However, since this system incorporates two controlling gene promoters, the CMV promoter could be replaced by tumour-, tissue-, condition or micro-environment specific promoters [3,57,58] providing the potential for even greater specificity, selectivity and indeed safety.

## 3. Hypoxia-mediated gene therapy

The presence of hypoxia is a negative prognostic indicator for outcome following radiotherapy in a range of human tumour sites [59–63]. The absence of a cell killing component that can be attributed to radiation-mediated oxygen radicals is unlikely to be the only reason for the resistance of hypoxic tissue to the lethal effects of radiotherapy [64]. Resistance may also arise from modifications to gene expression induced as a direct consequence of the hypoxic environment, such as a more aggressive locoregional disease and enhanced invasive capacity [65–67]. Rather than devise therapeutic strategies of overcoming hypoxic resistance, a number of groups have chosen to exploit the hypoxic nature of tumours to gain a therapeutic advantage [4,57,68–72]. The adaptive response to cellular hypoxia involves the modulation of the synthesis of multiple proteins controlling processes such as glucose homeostasis, angiogenesis, vascular permeability and inflammation. These include, for example, phosphoglycerate kinase 1 (PGK1), erythropoietin (EPO), lactate dehydrogenase A (LDHA), vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) [73]. The DNA regulatory elements controlling the expression of oxygen-responsive genes have been defined in many cases, and involve the specific binding and trans-activation by various inducible, phosphorylation-dependent and/or redox sensitive transcription factors, including Hypoxia Inducible Factor 1 (HIF1), Activator Protein 1 (AP1), Nuclear Factor κB (NF κB), p53 and the Heat Shock Transcription Factor. Published evidence indicates that only HIF1 is specifically oxygen-responsive [73], while the other transcriptional systems appear to contribute to the response to hypoxia *via* related redox and metabolic changes.

Affinity purification and molecular cloning of HIF1 showed it to function as a heterodimer consisting of two basic-helix-loop-helix proteins, HIF1α and HIF1β (previously identified as ARNT, aryl receptor nuclear translocator, which is part of the xenobiotic response) [74]. Although both subunits are constitutively expressed, HIF1α is the hypoxia-regulated component *via* post-translational stabilisation and transactivation by several additional factors [75,76]. To modulate gene expression, HIF1 specifically binds to hypoxia-responsive elements (HREs), enhancers containing the core sequence 5′-(A/G)CGT(G/C)(G/C)-3′, localised at varying distances and orientations of the coding region of several hypoxia-regulated genes. The HRE/HIF1 regulation system was shown to be common to all mammalian cells and human tissues tested to date [76] and the HIF1α subunit was found to be overexpressed in 68% of the tumour types analysed [77]. The high frequency of HIF1 expression across many human tumours of diverse tissue origin represents a possible therapeutic target for HRE-directed gene therapy of the hypoxic environment. It has been shown that marker gene expression regulated by the murine PGK1 HRE could be induced in hypoxic tumour cells [68]. Following the demonstration of the successful use of radiation [28] or hypoxic-responsive gene promoters [68,78] alone, we have assessed the function of chimeric promoters containing radiation-responsive CArG elements in combination with HREs (data not shown). These dual enhancer constructs functioned in response to radiation or hypoxia alone and also in the presence of both stimuli.

## 4. Other gene therapy approaches

A number of radiotherapy and gene therapy strategies have been developed. High therapeutic potential was recently demonstrated for radiation therapy in combination with a trimodal approach consisting of a replication-competent oncolytic adenovirus containing a *cytosine deaminase*/HSV*tk* fusion gene [42]. This combination of radiation therapy, lytic viral therapy and double suicide gene therapy produced significant tumour regression in an experimental C33A tumour xenograft model.

Manipulating the expression of the sodium and iodide symporter (*NIS*) gene increased the uptake of radiolabelled iodide in an *in vitro* spheroid model [79–81]. The overexpression of such membrane transporter proteins increases the selectivity of [$^{131}$I]-metaiodobenzylguanidine therapy for neuroblastoma and ionic [$^{131}$I]-administration for differentiated thyroid tumours. In contrast, gene therapy has also been exploited to decrease the side-effects of radiation-induced damage in normal tissue. For example, intratracheal injection of a vector carrying the human superoxide dismutase (*SOD2*) transgene prior to irradiation prevented radiation-

induced damage of transduced tissues, resulting in decreased radiation-induced oesophageal stricture [82] and decreased late pulmonary fibrosis [83]. These examples of combining radiotherapy and gene therapy illustrate the breadth of ideas being investigated. Moreover, they highlight the benefit of adopting a combinational approach in that treatment can be tailored to address individual situations.

Ultimately, the success of gene therapy will dependent on the efficient delivery of transgenes to the tumour site. However, the development of strategies or vectors that can offer, precise tumour targeting will undoubtedly play a significant role in the outcome of future gene therapies for cancer.

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