Radiol Oncol 2008; 42(3): 115-35. doi:10.2478/v10019-008-0009-1

review

Targeted gene therapy in radiotherapy

Urska Kamensek and Gregor Sersa

Department of Experimental Oncology, Institute of Oncology Ljubljana, Slovenia

The dramatic pace in development of gene therapy over the past decades has made it a realistic alternative for the treatment of cancer. Radiotherapy, on the other hand, is one of the most commonly used and well established cancer treatment modalities. The latest improvements in the physical targeting ability of radiotherapy and understanding of the molecular mechanism involved in the cellular response to ionizing radiation have presented an opportunity to combine radiotherapy with gene therapy. This review article will focus on gene therapy strategies that can be used to enhance the effectiveness of radiotherapy, with an emphasis on transcriptional targeting approaches.

Key words: gene therapy; radiotherapy; transcriptional targeting

Developments in radiotherapy

Radiotherapy is the use of ionizing radiation in the treatment of malignant tumors. It is one of the main treatment modalities for many forms of cancer, with more than half of all cancer patients receiving radiation therapy at some point in their treatment.[1]

Biological and technological advances have brought notable improvements in radiotherapy over the years. Biologically based advances include improvements in fractionation schedules, treatment planning and combining radiotherapy with other treatment modalities such as surgical tumor debulking, chemotherapy and, most recently, gene therapy.[1]

Received 21 May 2008
Accepted 18 June 2008

Correspondence to: Prof. Gregor Sersa, Department of Experimental Oncology, Institute of Oncology Ljubljana, SI-1000 Ljubljana, Slovenia. E-mail: gsersa@onko-i.si

Recent progress in diagnostic imaging, such as computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and molecular imaging techniques, have led to the development of conformal (CRT) and intensity modulated radiotherapy (IMRT), which is the most advanced form of conformal radiotherapy. CRT and IMRT have enabled more precise dose delivery, conforming closely to the shape of the tumor and thus improving the therapeutic index;[2] namely better local tumor control without compromising normal tissue. Although higher doses of radiation can produce better tumor control, the dose is limited by the possibility of normal tissue damage surrounding the tumor *i.e.* in the irradiation field.

Despite a marked progression in the efficacy of radiotherapy, there is still a need for improvement of this treatment modality. Because radiotherapy is a local treatment, tumor cells outside the immediate field of radiation and those that have metastasized

Table 1. Strategies of gene therapy

Gene therapy strategy		Genes
Genetic replacement or correction therapy		*p53, CTS1, MDA7, Bcl-2-, BclXL-, survivin-* antisense...
Suicide gene therapy (gene chemotherapy)	Endogenous precursors	*HSV-tk, CD, CD/HSV-tk fusion, HRP, IAA*
	Exogenous precursors	*iNOS*
Gene based immunotherapy		*TNF-α, IFN-γ, IL-12..., tumor associated antigens (PSA)*
Vascular-targeted gene therapy		*VEGF*-antisense, *soluble Flt-1, endostatin, angiostatin, vazostatin, TNF-α, IL-12*

CTS1, Chimeric Tumor Suppressor 1 (synthetic variant of wild-type *p53*); *HSV-tk, Herpes Simplex Virus thymidine kinase*; *CD, Cytosine Deaminase*; *HRP, Horseradish Peroxidase*; *IAA, Indol-3-Acetic Acid*; *iNOS, inducible Nitric Oxide Synthase*; *TNF- α, Tumor Necrosis Factor-α; IL-12, Interleukin-12*; *PSA, Prostate Specific Antigen*; *VEGF, Vascular Endothelial Growth Factor*.

out of the primary tumor are not destroyed. In addition, there are some radio-resistant cells within a single tumor mass that may survive despite a relatively high radiation dose. The efficacy of radiotherapy is also limited by chronic and intermittent hypoxia in the tumors.

To increase the efficacy of radiotherapy, while minimizing its side effects, developments have been made in combining radiotherapy with chemotherapy and, lately, gene therapy.

Gene therapy and its combination with radiotherapy

Gene therapy consists of the transfer of exogenous genes, called transgenes, into human somatic cells and the expression of these genes in transfected cells for a therapeutic purpose. In cancer treatment, this means either correction of genetic defects, characteristic of cancer cells, or induction of targeted tumor cell death.[3] In gene correction or replacement approach, a defective or inactivated tumor suppressor gene is replaced, for example to increase radiation-induced apoptosis (wild-type *p53* replacement therapy) or high oncogene expression levels are repressed with the use of antisense, ribozymes or siRNA technology. However, because cancer is a consequence of countless genetic mutations, most anti-tumor therapies aim to destroy cancer cells, rather than correct these complex defects. Strategies to destroy cancer cells can be exerted in different ways; by gene directed chemotherapy, potentiation of immune response and targeting of the tumor vasculature (Table 1).

Recent advances in gene therapy approaches have allowed researchers to successfully combine gene therapy with radiotherapy.[4,5] There are many potential benefits of combining radiotherapy with gene therapy:

- Gene therapy and radiotherapy techniques have different mechanisms of action and they target best at different parts of the cell cycle, which may result in an additive effect (Figure 1).
- Gene therapy can cause radiosensitization, which means that a synergistic (supra-additive) anti-tumor effect is possible (Figure 1).
- Radiation can enhance the "bystander effect" of gene therapy, meaning that more

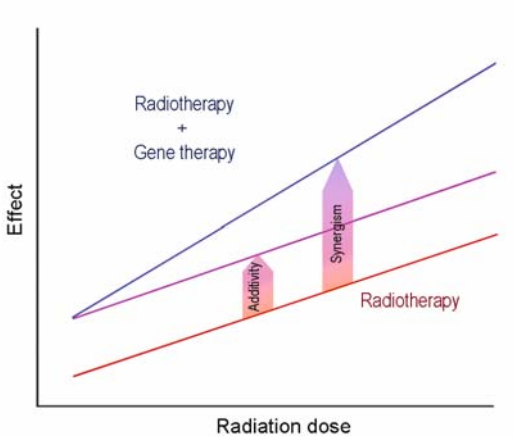

Figure 1. The effect of combined radio- and gene therapy can be either the same as the sum of individual monotherapies (additive) or greater (synergistic).

cells are affected by gene therapy than are transfected initially. This is probably because of the release of products of therapeutic genes from radiation damaged cells and the activation of an anti-tumor immune response.[6]

- Radiation can increase the efficacy of gene delivery and expression.[7-9]
- The targeting ability of radiotherapy is exploited in an approach that uses radiation-inducible promoters to control the timing and location of gene expression within the irradiated tumor volume.[10]

This last approach, called transcriptional targeting, is especially promising since any therapeutic gene with radio-sensitizing properties can be chosen, and will be the main focus of this review. Therapeutic genes used in combination with radiation-inducible promoters can be alienated in the last three categories of gene therapy shown in Table 1: suicide gene therapy, gene based immunotherapy and vascular-targeted therapy.

Suicide gene therapy

Radiosensitizers are chemical or biological agents that increase the sensitivity of tumor cells to radiotherapy. In the past, attempts have been made with radiosensitizers mimicking oxygen effect, with hyperbaric oxygen breathing, with the administration of carbogene and nicotinamid and, most successfully, with chemotherapeutic agents.[11,12] However, the dose of chemotherapy agents required to give a sufficient anti-tumor effect often results in severe systemic toxicity.[13]

The quest to find non-toxic agents that selectively sensitize tumors to radiotherapy has led to innovations in so called suicide gene therapy or gene chemotherapy. The basic principle in suicide gene therapy involves the transfer of a gene encoding an enzyme that converts an otherwise none or mildly toxic substance of either exogenous (pro-drugs) or endogenous origin into a cytotoxic agent that kills cancer cells.[14]

Exogenous precursors. In the first suicide gene therapy strategy, called gene directed enzyme pro-drug therapy (GDEPT), a gene encoding a drug metabolizing enzyme is delivered to a tumor, followed by systemic administration of the pro-drug, which is then metabolized and converted by the expressed enzyme into a cytotoxic substance specifically within the site of transfection.[14,15] Genes used in this strategy originate from viruses, bacteria, or fungi and are foreign to the transfected mammalian cells.

There is a range of enzyme pro-drug combinations available for effective GDEPT, many have also been combined with radiotherapy.[16] The most widely investigated radio-GDEPT combination, involves *herpes simplex virus thymidine kinase* (*HSV-tk*) and the pro-drug gancyclovir (GCV). The viral thymidin kinase enzyme phosphorylates gancyclovir into a nucleoside analog, which is then incorporated into a newly synthesized strand of DNA[4] resulting in cell death in rapidly proliferating cells, which are also targeted with radiotherapy. A supra-additive cytotoxic effect can thus be

expected when this approach is combined with radiotherapy. Furthermore, these nucleoside analogs increase radiation induced DNA breaks and interfere with DNA repair mechanisms.[4] A similar GDEPT system, efficient in radiosensitizing tumor cells in oxic, as well as hypoxic conditions, consists of the plant enzyme horseradish peroxidase (HRP) and the non-toxic plant hormone indol-3-acetic acid (IAA).[17] The next GDEPT strategy to be also tested with concomitant radiotherapy was the combination of bacterial or yeast cytosine deaminase (CD) and pro-drug 5-fluorocytosine (5-FC). CD converts 5-FC to 5-fluorouracil (5-FU), which is a widely used cancer chemotherapy agent with well-known radiosensitizing effects.[18] These strategies were tested in preclinical studies, which showed added benefits of the combined radio-GDEPT compared with either therapy alone. Both enhanced local tumor growth control and systemic effects were observed. Preclinical studies led to clinical trials, which all involve the *HSV-tk*/GCV combination. Early results from the phase I-II clinical trial using *HSV-tk*/GCV gene therapy combined with radiotherapy for the treatment of previously untreated prostate cancer confirm the safety and feasibility of this approach. In the following clinical trials, this combined therapy proved to be safe, but no significant tumor growth control was detected. [4,5,19]

In order to further increase the therapeutic index, chimeric fusion genes combining *CD* and *HSV-tk*, were designed.[20] This so called double suicide gene therapy approach has been evaluated in combination with radiotherapy in preclinical and also phase I clinical studies.[20-23] Yeast *CD/HSV-tk* fusion gene was tested in combination with radiation and pro-drugs in CNE-2 nasopharyngeal carcinoma xenografts model, demonstrating a synergistic anti-tumor effect.[21] In another study, using the bacterial *CDglyTK* fusion gene, the addition of pro-drugs 5-FC and gancyclovir increased the radiosensitivity of prostate cancer and glioma cells *in vitro* and showed a significant anti-tumor effect in a preclinical model of prostate cancer.[22] Results from phase I clinical trial in patients with locally recurrent prostate cancer, indicated that this double suicide therapy is a relatively safe and effective method for increasing the therapeutic index of radiation.[23]

Endogenous precursors. Next therapeutic gene, attractive for use in combined radiogene therapy, is *inducible nitric oxide synthase iNOS*. iNOS is an enzyme that generates nitric oxide (NO), which has many anticancer properties, including cytotoxicity in hypoxic conditions, anti-angiogenic effects and radiosensitization.[24-26] Although NO is a potent chemical radiosensitizer, its clinical use was limited by systemic side effects.[27] By means of gene therapy NO production can be activated at the site of transfection with *iNOS* gene, where it can synergize with radiation.[28] An additional advantage of *iNOS* gene therapy is pronounced bystander effect: namely, because NO is an easily diffusible gas it can exert its effects deep within the tumor mass, resulting in large tumoricidal effects even when only a small portion of the tumor cells are transfected with the *iNOS* gene.[29] The first *in vitro* study using a gene transfer strategy in a murine sarcoma model demonstrated that genetically produced iNOS can increase the radiosensitivity of hypoxic tumor cells.[28] In a subsequent study, evident tumor growth delay was reported after combined radio-gene therapy with *iNOS* in a human colorectal cancer model.[30] The efficacy and safety of this approach has been confirmed in other studies, which are so far still at the preclinical stage.[31,32]

Gene based immunotherapy

Immunotherapy is a promising strategy for cancer treatment because it has the poten-

tial to fight both the primary tumors and metastases[33], which are the major cause of treatment failure in most cancer types. Recent advances in immunology and radiobiology indicate that radiation can modify the tumor microenvironment and generate an antigen specific immune response.[34] Radiation creates inflammation by the induction of cell death and upregulation of immunomodulatory cell surface molecules and secretory molecules in tumor, stromal and vascular endothelial cells. This radiation induced "danger" microenvironment can then lead to breaking of the tolerance to otherwise weakly immunogenic tumor antigens and the generation of an antigen specific cell-mediated antitumor immune response.

These newly discovered immunomodulatory properties of ionizing radiation have given rise to the idea of combining immunotherapy with radiation therapy.[35] The main gain of combining immunotherapy with local radiotherapy could be the elimination of the radio-resistant fraction of cells in the primary tumor and the prevention of shedding of metastatic cells from the tumor. Results from preclinical studies using different non-gene based immunotherapeutic strategies have shown synergistic effects when combined with radiotherapy. The most promising of these combined strategies are now being tested in clinical trials.[35]

An alternative method for execution of immunotherapy is gene therapy.[36,37] There are two major forms of gene based immunotherapy: genetic vaccination and gene-based immunomodulation.

Genetic vaccination. The first form of gene based immunotherapy for the treatment of cancer involves transfection or vaccination with recombinant viruses expressing tumor associated antigens and usually also costimmulatory molecules (CD80, CD54, and CD58, cytokines).[38-40] An improved therapeutic efficacy of combined vaccination and radiotherapy was reported in a mouse adenocarcinoma tumor model.[41] Vaccines were able to induce an anti-tumor immune response and act synergistically with local tumor irradiation. Furthermore, the development of T cells directed against tumor associated antigens, which were not present in the vaccine, was observed, resulting in broadening of the immune response. This phenomenon, also called antigen spread or antigen cascade, was also indicated in a phase II clinical study in patients with localized prostate cancer.[42] In this study, vaccination with poxvirus encoding prostate specific antigen (PSA) combined with standard external beam radiotherapy was well tolerated and induced a PSA-specific immune response to vaccine in the majority of patients.

Gene-based immunomodulation. In contrast to vaccination, gene-based immunomodulation or cytokine gene therapy is a form of nonspecific immunotherapy. In this strategy, immunostimulatory genes such as cytokines are utilized to boost the immune system.[36,43] Early treatment strategies using systemic administration of recombinant immunostimulatory cytokines were associated with dose limiting normal tissue toxicities.[44,45] Gene therapy approaches significantly improved the prospects for the use of cytokine cancer therapy.[37] Two important cytokine genes, which have been tested in combination with radiotherapy, are *interleukin 12 (IL-12)* and *tumor necrosis factor-α (TNF-α).*

IL-12 is a heterodimeric pro-inflammatory cytokine with multiple functions, including the induction of interferon-γ (IFN-γ), activation of T helper and NK cells[46,47] and anti-angiogenic activity.[48,49] IL-12 has been proved to have potent antitumor and antimetastatic effects against murine tumors.[50] A combination of genetically produced IL-12 and local radia-

tion was tested in a mouse fibrosarcoma model.[51] Intratumoral injection of adenoviral vector with *IL-12* combined with radiotherapy improved both local and systemic tumor control compared to either treatment alone. Enhanced local tumor control could be partially attributed to the anti-angiogenic effects of IL-12, while the systemic, anti-metastatic effect on microscopic metastases distant from the primary irradiated site was clearly due to an IL-12 induced anti-tumor immune response. In another *in vivo* study, adenovirus mediated local *B7/IL-12* immunotherapy combined with radiotherapy was tested in two murine tumor models.[52] In both tumors, growth delay was significantly longer when radiotherapy was combined with immunotherapy. The therapeutic effect was explained by IL-12 mediated activation of T- and NK-cells and inhibition of angiogenesis. Similar results were obtained in subsequent studies combining IL-12 gene therapy and radiotherapy.[53-55]

TNF-α is another attractive candidate for cancer gene therapy since it encodes for secretory protein with a broad range of potent anti-tumor properties, which include induction of the immune system, enhancement of radiosensitivity, direct cytotoxicity and disruption of the tumor vasculature.[56] An additive killing effect of systemic recombinant TNF-α administration and radiation was reported in a MCA-K mouse tumor model.[57] A phase I trial combining systemic TNF-α administration and radiation demonstrated that the systemic toxicities from TNF-α limit the efficacy of treatment.[45] To overcome this problem and at the same time preserve the potent anticancer activity of TNF-α, a new form of gene therapy was designed, in which the *TNF-α* gene is placed under the control of radiation inducible promoter.[10] This form of gene therapy is called transcriptional targeting and will be discussed in more detail later in this article. Briefly, in pre-clinical tests treatment with genetically produced TNF-α was shown to synergize with local radiation to produce an increased anti-tumor effect and was not associated with increased local and systemic toxicity.[13] The same approach was further developed for clinical studies as TNFerade and is now in phase II/III clinical trials.[58] A combination of intra-tumoral injections of TNFerade and concomitant radiation was well tolerated in clinical trials. In addition, substantial anti-tumor responses were reported. However, no systemic effects were observed although TNF-α has the potential to induce an immune response. It seems that the therapeutic effectiveness of this combination cannot be attributed to the immune system, and that some other mechanisms are involved. Most probably antitumor effect of TNF-α is mediated by direct cytotoxicity on the tumor vessels.[59] TNF-α should perhaps therefore be placed in the next group of therapeutic genes, classified as vascular-targeted therapies, which will be discussed in the next chapter.

Vascular-targeted gene therapy

Growth of new blood vessels from pre-existing vessels or angiogenesis is necessary for solid tumor progression and metastasis[60] and is thought to be one of six hallmarks of cancer;[61] it was therefore proposed as a new target in cancer treatment.[62] According to the angiogenic "switch" hypothesis, a shift in the balance between pro- and anti-angiogenic factors toward pro-angiogenic allows the tumor to expand.[63-65] Among many pro-angiogenic factors, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are widely considered to be the most important in the angiogenic process. They are normally opposed by endogenous inhibitors of angiogenesis such as thrombospondin-1 (TSP-1), platelet factor 4 (PF4), soluble Flt-1 (sFlt-1), angiostatin, and endostatin. Vascular-targeted therapies

target different parts of the angiogenesis process, from inhibition of pro-angiogenic factors, or augmentation of anti-angiogenic factors, to direct targeting of tumor endothelial cells. There are several advantages of vascular-targeted therapies over other cancer therapies: the first is that they target endothelial cells, which are more easily accessed and are considered to be relatively genetically stable compared to tumor cells, therefore lower risk of acquired drug resistance is expected;[66] secondly, angiogenesis is very limited in normal physiology, so normal tissue toxicities can be mostly avoided;[67] and lastly, since a large number of cancer cells depend on a small number of endothelial cells for their metabolic supplies, targeting the tumor vasculature should lead to an enhanced antitumor effect.[62,68,69] Vascular targeted therapy can be differentiated into two groups: anti-angiogenic and vascular-disrupting approaches.[70,71] Anti-angiogenic agents inhibit the formation of new blood vessels, consequently they often require chronic administration, and are predominantly beneficial for the treatment of early-stage or metastatic cancers. Vascular disrupting agents, in contrast, destroy the existing tumor vasculature and are thus suitable for acute treatment of advanced disease. In reality, the boundaries between anti-angiogenic and vascular-disrupting agents are not so evident and many vascular targeted agents may exhibit both an anti-angiogenic and a vascular-disrupting action.

A range of vascular targeted approaches, including recombinant proteins, monoclonal antibodies and small molecules, has already been tested for their anti-tumor properties; many are already in clinical trials.[72] Although most proved to be safe and effective in suppressing tumor growth, they were not tumoricidal,[73] indicating the need for prolonged administration to maintain tumor suppression.[72] That is especially true for the anti-angiogenic group of agents. Gene therapy was therefore adopted for the delivery of these agents.[74,72] In addition to being more efficient in the persistent production of therapeutic proteins at therapeutic levels, a further advantage supporting this form of delivery, is the selective expression of the vascular targeting gene only in targeted organs containing tumors, minimizing systemic toxicity, which could become a problem after prolonged treatment. Anti-angiogenic gene therapy strategies can be categorized into those that suppress the pro-angiogenic factors, either by inhibiting the expression of angiogenic genes (antisense and siRNA against *VEGF*) or interfering with angiogenic signaling pathways using decoy receptors (*e.g.* soluble Flt-1 that can sequester VEGF or inactivates its receptors), and those that enhance the inhibition of angiogenesis using genes encoding endogenous angiogenesis inhibitors (*e.g.* endostatin, angiostatin).[72,75] Preclinical studies demonstrate that this type of gene therapy can be effective in controlling or even eradicating tumor growth in animal models, but vascular targeting strategies in the form of gene therapy remain for the moment at the preclinical stage.

Currently, therapies combining vascular targeting strategies with conventional therapies like radiotherapy are receiving great attention.[70,76] There are many possible mechanisms for enhanced tumor response to radiation with anti-angiogenic and vascular disrupting therapies.[68] The original justification for a combined therapy was that it targets two separate cell populations: endothelial cells and cancer cells.[77] Although there was initially some concern that vascular targeting agents would increase tumor hypoxia, and thus limit the effectiveness of radiotherapy, there is accumulating experimental evidence suggesting that these agents actually improve tumor oxygenation, leading to radio-sensitization.[68] This apparently paradoxical evidence could be

explained if the anti-angiogenic therapy was to cause normalization of the otherwise structurally and functionally abnormal tumor vasculature before its destruction.[77] During this brief normalization, the tumor oxygenation status would be improved, leading to an enhanced radiotherapy effect. Improved tumor oxygenation could also be the result of a reduced number of oxygen-consuming tumor and endothelial cells, caused by anti-angiogenic therapies.[68] In the case of vascular disrupting agents, an improved tumor response to radiation is probably the result of additive killing of two micro-regionally different populations of tumor cells. Namely, vascular disrupting agents selectively destroy the tumor vasculature, leading to centralized necrosis within the tumor, whereas the peripheral rim of tumor cells remains viable, probably because those areas are perfused by normal tissue vessels, which are not targeted by vascular disrupting genes. These remaining tumor cells are therefore well-oxygenated and, as such, present an excellent target for radiation therapy.[78] Genes used in combination with radiotherapy because of their vascular targeting properties include *angiostatin, endostatin, IL-12* and *TNF-α.*

Angiostatin and endostatin are both endogenous inhibitors of angiogenesis with confirmed anti-tumor and anti-metastatic activity in preclinical tumor models.[79,80] The anti-tumor efficacy of endostatin gene therapy with radiotherapy was evaluated in a human colorectal tumor model HT29. Intramuscular injection of virus vector expressing endostatin led to sustained endostatin serum levels and enhanced tumor growth delay of HT29 xenografts.[81] An enhanced anti-tumor efficacy of radiation therapy after intratumoral injections of liposome-endostatin complex was also demonstrated in human liver carcinoma BEL7402 xenograft models.[82] In a Lewis lung carcinoma (LLC) mouse tumor model, naked plasmid DNA encoding mouse endostatin gene was injected intratumorally as an adjuvant to radiation.[83] The anti-tumor efficacy of radiotherapy was significantly enhanced with the anti-tumor effect in the combination treatment being at least additive compared with either treatment alone. Gene therapy delivery of angiostatin was also shown to enhance the treatment efficacy of radiotherapy. Using adenovirus expressing a secretable angiostatin-like molecule (AdK3) in combination with radiotherapy in rat C6 gliomas subcutaneously pre-established into athymic mice, significantly higher and possibly synergistic, anti-tumor effects were observed that tightly correlated with an obvious decrease in vascularization of the tumor.[84]

TNF-α and IL-12 are two multifunctional proteins with vascular-disrupting and anti-angiogenic effects. The mechanism of the IL-12 anti-vascular effect is complex, including the induction of secondary cytokines such as IFN-γ or chemokines such as interferon-inducible protein 10 (IP-10), which may have direct cytotoxic and/or anti-angiogenic effects on tumor and endothelial cells.[48,49] The effect of TNF-α is even more complex; its anti-vascular effect can be either stimulatory or inhibitory depending on the amount, the site, the microenvironment, and the presence of other cytokines.[56,85] The anti-vascular effect of TNF-α is considered to be primarily vascular disrupting and not anti-angiogenic.[59,86] Studies involving IL-12 and TNF-α in combination with radiation have already been discussed in the previous chapter.[51-59]

Targeted expression of therapeutic genes

As with other cancer therapies, the major problem of gene therapy is poor therapeutic index caused by uncontrolled gene

expression, which can lead to normal tissue toxicity.[10,87,88] A tightly controlled regulation of transgene expression is required to increase the efficiency and safety of gene therapy. For the clinical success of gene therapy, gene regulation systems are especially desired, not only to maintain the therapeutic level of the transgene product without systemic toxicity but also to be able to adjust transgene expression in response to disease progression. Several strategies have been explored to control gene expression. These involve restricted or targeted vector delivery and transcriptional targeting with the use of tumor and tissue specific promoters and inducible promoter systems.[89,90] The latter are the most important for combination with radiotherapy and will therefore be discussed in more detail in the following chapters.

Inducible promoter systems

Expression of therapeutic genes can be controlled by locating them downstream of promoter regions that are induced in response to various signals.[88] The advantage of this kind of inducible promoter system is that not just the location, but also the level, timing and duration of transgene expression can be modulated. Optimal inducible promoters should have low basal activity and high inducibility, with a so-called "on switch". They should be dose dependent, safe, and reversible (off switch).

Several inducible promoters have already been utilized for use in cancer gene therapy. They can be controlled either by internal (endogenous) or external (exogenous) signals.[91] Internally controlled promoters take advantage of a tumor associated microenvironment such as hypoxia. Externally controlled promoters, on the other hand, can be induced by chemical signals (Tet-On, Tet-Off inducible systems),[92] heat *(heat shock protein 70* promoter*)*,[93] controlled electric stimuli such as administrated in electroporation protocols (*metallothionein* promoter)[94] and, most importantly, ionizing radiation (*Egr-1*, *p21*).[10]

Radio-inducible promoters. It is well known that exposure of cells to ionizing radiation induces DNA damage by direct interaction with DNA and through the generation of reactive oxygen species (ROS), which results in transcriptional activation of a variety of genes, leading to changes in their expression.[13,95] The initial signal for transcriptional activation of these genes is probably generation of ROS by radiation, rather than direct damage to DNA.

Numerous genes that are activated by radiation have so far been identified (*Egr-1*, multiple members of the *jun/fos* family, *NFκB, p21(WAF-1/Cip-1*), bacterial *RecA* gene...). Promoters of these radiation inducible genes can be exploited to drive the expression of therapeutic genes.[10,96] With the use of the excellent targeting properties of new stereotactic radiation techniques, the expression of downstream genes can be spatially and temporally controlled within the irradiated tumor tissue (Figure 2).

By far the most widely used and well characterized promoter for this purpose is that of the *Egr-1* gene, next in line is the promoter of the *p21* gene.

Egr-1 promoter. *Early growth response-1* gene (*Egr-1*) is a transcription factor for some cytokines and growth factors (TNF-α, IL-1, PDGF-β, bFGF) involved in repair or death of tissue after various kinds of stress, including irradiation. The radiation induced expression of *Egr-1* accurs in different cell types and is fast and transient.[97] Sequences responsive for radiation inducibility consist of 425 bp located upstream of the transcription start site of the *Egr-1* gene and contain six consensus motifs CC(A+T rich)GG, known as CArG elements. Their response is mediated by intracellular free radical formation caused by ionizing radiation.[97]

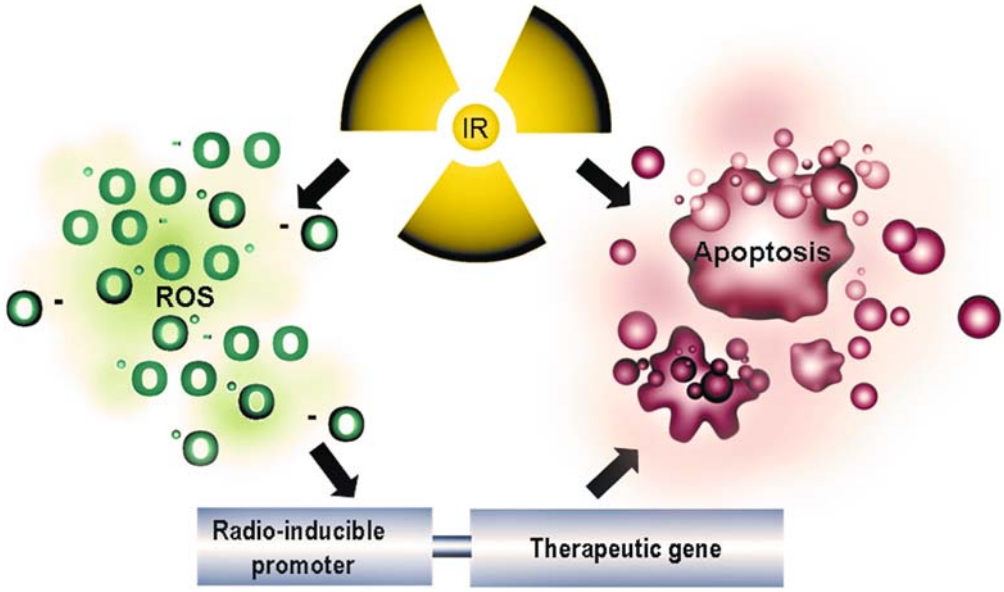

Figure 2. Ionizing radiation induces ROS generation, which causes transcriptional activation of radioinducible promoter leading to increased expression of the therapeutic gene. The combination of the damaging effects of irradiation and increased level of therapeutic protein results in increased tumor cell apoptosis and improved anti-tumor activity.

To check the potential of these radio-inducible sequences for use in radiation inducible gene therapy, *Egr-1* promoter was ligated upstream of the cDNA encoding the *TNF-α* gene. In the initial study, the *Egr-TNF-α* construct was transfected into human leukemia cell line HL525.[98] Induction of *TNF-α* expression was observed when cells were exposed to radiation. Stably transfected cells were then injected into human xenografts of the radio-resistant squamous carcinoma cell line SQ-20B. When animals were treated with radiation, increased TNF-α protein levels were detected in tumors and an increased anti-tumor effect was observed.

The *Egr-TNF-α* chimeric construct was later cloned into a replication-deficient adenoviral vector and termed Ad.Egr.TNF-α. Preclinical studies on human carcinoma, prostate and glioma xenografts showed that tumors transfected with Ad.Egr.TNF-α responded to radiation with 7-8 fold induction of *TNF-α* expression within the irradiated field and substantially increased tumor growth inhibition, compared to tumors treated with radiation alone.[59,99-102] Importantly, the combined treatment was not associated with increased systemic toxicity and also no TNF-α could be detected in the circulation of the experimental animals.

For clinical studies, the same construct containing human *TNF-α* and *Egr-1* radio-inducible promoter was incorporated into a second generation adenovector and called TNFerade.[103-105] Toxicology studies with TNFerade on nude mice showed that combined therapy with radiation is well tolerated and also associated with sub-

stantial anti-tumor activity. There was no systemic toxicity and serum TNF-α levels were not significantly increased.[103] In the first clinical study on patients with a range of advanced, treatment refractory solid tumors, TNFerade was administrated by intratumoral injection with concomitant radiotherapy.[104] The combined treatment was well tolerated and serum TNF-α levels were not significantly increased during the treatment. In addition, no adenovirus was detectable in patients' blood, urine and sputum samples. In the following phase I/II clinical trial on soft tissue sarcoma of the extremity, no dose limiting toxicities were observed, treatment was well tolerated and substantial responses, even in large tissue sarcomas, were reported, and therefore provide support for further evaluation of TNFerade.[13] Therapy has now successfully reached phase II/III clinical trials for treatment of pancreatic and esophageal carcinomas, rectal carcinomas, metastatic melanomas, soft tissue sarcoma and head and neck cancer.[58]

The radio-inducibility of CArG elements has also been demonstrated using reporter genes *β-galactosidase* (*β-gal*) and *green fluorescent protein* (*GFP*) inserted downstream of the *Egr-1* promoter. Expression of *β-gal* under the control of *Egr-1* promoter was enhanced 3-fold after irradiation with 2 Gy in glioma cells.[106] In another study, a construct with *GFP* responded to 5 Gy irradiation with increased GFP expression.[107]

For the use in radiation targeted suicide gene therapy, *Egr-1* promoter was inserted upstream to the *herpes simplex virus thymidine kinase (HSV-tk)* gene. Transfection of *Egr-HSV-tk* constructs into different tumor cells produced enhanced tumor cell killing in the presence of the prodrug gancyclovir following radiation treatment.[107-110]

After the success of *TNF-α* and *HSV-tk* radio-inducible constructs, a number of preclinical studies have successfully used CArG elements to drive the expression of several other cytotoxic or immune-modulatory therapeutic genes such as *IFN-γ,*[111] *iNOS,*[31,112] *mIL-12,*[55] *mIL-18,*[113] etc. using different gene delivery systems (liposomes, adenoviruses, naked DNA injection, cell carrier).

In order to further improve the performance of wild-type *Egr-1* promoter, CArG elements were isolated from *Egr-1* promoter and integrated into synthetic promoters.[107] These new promoters demonstrated greater inducibility and lower basal activity than the wild-type *Egr-1,* despite containing the same number of CArG elements. Furthermore, a cumulative effect was observed after fractionation, with five times 1 Gy doses being as effective as a 5 Gy dose. By increasing the number of CArG elements from four to nine, induction with clinically relevant doses (2-3 Gy) was further improved and a lower basal activity was achieved. Further *in vitro* studies showed that specific alternations of the core A/T sequence in the CArG elements caused an even greater induction after irradiation, while the spacing between the elements had no effect.[114] Using an *HSV-tk*/GCV system, synthetic CArG promoters were also shown to work *in vitro* and *in vivo,* with significant radio-sensitizing and anti-tumor effects.[114]

p21 promoter. Studies with *Egr-1* promoter have led to the investigation of other radiation-inducible promoters, such as the promoter of the *cyclin dependent kinase inhibitor p12*, also known as *WAF1* or *CIP-1.* Gene *p21* is an immediate-early response gene, mediating cell cycle G1 phase arrest in response to a variety of stresses.[95,115] Its expression is regulated mostly by tumor suppressor protein p53,[116] which is activated by DNA damage caused by irradiation and genotoxic agents. The *p21* promoter region contains at least two binding sites for the p53 transcriptional factor, and also specific DNA motifs responsive to a wide range of

other cell growth regulatory signals, indicating that p53- independent pathways for the *p21* gene transcriptional activation also exist.

The promoter of the *p21* gene has predominantly been studied in the context of suicide gene therapy with the *iNOS* gene. First, the response of the *p21* promoter to radiation was tested using a *p21/GFP* reporter gene construct in an *in vitro* model with human endothelial cells HMEC-1 and in an *ex vivo* rat tail arterial segment model.[117] Transfection of both models followed by irradiation with 4 Gy resulted in a significant increase (9.5 and 4.5-fold, respectively) in *GFP* expression. Similarly, when *p21* promoter was used to control expression of the therapeutic gene *iNOS*, a five-fold induction of *iNOS* gene was obtained after 4 Gy radiation in a rat tail arterial segment model. The radio-sensitizing properties of the *p21/iNOS* construct were next tested in murine fibrosarcoma cells RIF-1. After a large single dose of radiation, tumor cell radio-sensitization *in vitro* and tumor growth delay *in vivo* was achieved.[28]

In order to optimize the synergistic interaction between radiation and the transgene product, induction of transgene expression and radiation therapy should be temporally adjusted. An alternative therapeutic regime was therefore proposed using an initial priming dose of 4 Gy to induce transgene expression, followed by a subsequent treatment dose. This approach was tested *in vivo* on *p53* wild-type RIF-1 tumors and *p53* mutant HT29 human colorectal tumor xenografts.[30] Intra-tumoral injection of *p21/iNOS* construct, followed 16 h later by a 4 Gy priming dose and then, 8 h later, by treatment doses of 10 or 20 Gy, resulted in significant radio-sensitization in both tumor types, compared with radiation treatment alone. Furthermore, western blot analysis revealed that transgene protein levels were significantly increased only in tissue within the irradiated volume, even though vector sequences were detected in all the main organs tested, indicating that effective transcriptional targeting had been achieved. A similar approach with a priming radiation dose was later tested on the same tumor models using fractionated radiation schedules at clinically relevant doses per fraction.[118] Again, significant radio-sensitization was demonstrated for both *p35* normal and *p53* mutated tumor models.

Another study focused on the importance of integration of the *p21* promoter into chromatin[119], since there had been reports that the binding of *p53* to its recognition sequence in *p21* promoter depends on the chromatin structure.[120] Indeed, *p21* promoter transduced by recombinant adeno-associated virus vector, which can stably integrate transgenes into chromosomes, proved to be more responsive to low dose radiation than transiently transfected by electroporation. Significant induction of *p21* promoter by radiation doses as low as 0.2 Gy was demonstrated using *luciferase* reporter gene. Induction after 5 Gy reached a 6-fold induction, which was significantly higher than in transiently transfected cells (1.9-fold). Also when cells were stably transduced with suicide gene *HSV-tk* under regulation of the *p21* gene promoter, they were sensitized to repetitive treatment with low dose radiation (1 Gy).

Although p53 was shown to be important for the radiation inducibility of *p21* in some cases, there is plenty of evidence that *p21* can also be activated independently of p53. For instance, as mentioned before, *p21/iNOS* gene therapy was effective in radio-sensitizing both *p53*-wild type and mutant tumors to radiotherapy.[30,118] Characterization of the *p21* promoter in a range of normal and tumor cell lines with different *p53* status using the *GFP* reporter gene revealed that induction by radiation is independent of *p53* status.[118] In addition,

basal level activity of *p21* promoter proved to be high in tumor cells, but low in normal cells. So *p21* promoter is not only inducible by radiation but is also selectively inducible within the tumor environment and, as we will see in the next chapter, can also be induced in response to hypoxic conditions. All these characteristics make *p21* promoter a good candidate for use in cancer gene therapy, especially for the systemic treatment of disseminated disease. Systemic delivery could be used to target metastatic deposits, where tumor and hypoxia specific expression of the transgene would be attained in the absence of radiation.

Chimeric radiation and hypoxia inducible promoters. Hypoxia is a physiological feature of solid tumors that is a major hindrance to radiotherapy[121], since hypoxia leads to radiation resistance because of lack of oxygen to facilitate DNA damage by radiation-induced ROS.[122] Hypoxic conditions also create a microenvironment in which tumor cells become less angiogenesis dependent, more apoptosis resistant, and more malignant.[122,123] The presence of this physiological difference can, on the other hand, be exploited for selective cancer treatment.[124] One way to do that is by using hypoxia inducible promoters to drive the expression of therapeutic genes.[125-127] Namely, similar as radiation, hypoxia can activate the expression of numerous genes, important for angiogenesis, cell metabolism and cell growth. Their response to hypoxia is, in most cases, mediated by binding of hypoxia-inducible factor-1 (HIF-1) to specific hypoxia response elements (HREs) containing the consensus sequence (A/G)CGT(G/C)(G/C) within the promoter regions of these genes.[124]

To date, HRE derived from several hypoxia responsive genes, including *phosphoglycerate kinase 1 (PGK1)*, *vascular endothelial growth factor (VEGF)*, *erythropoietin (Epo)* and *lactate dehydrogenase A (LDH A)* have been successfully used for hypoxia specific targeting of gene expression.[128] Similarly to radio-inducible CArG elements, HREs have also been incorporated into synthetic promoters, tested for inducibility in hypoxic conditions using reporter genes and then used in experimental gene therapy with suicide therapeutic genes such as *HSP, HSV-tk* and *CD*.[128-130]

The oxygenation status of tumor tissue is highly heterogeneous, with areas of low and high oxygen levels indistinctly mixed together. Since hypoxia inducible gene therapy relies on a lack of oxygen and radio-inducible gene therapy needs the production of oxygen derived free radicals, neither approach is adequate for the treatment of an entire tumor. Vectors containing chimeric promoters responsive to both stimuli have therefore been developed.[131]

Chimeric promoters containing HREs derived from *Epo*, *PGK1* and *VEGF* genes and radio-inducible CArG elements were tested using *GFP* reporter assay on human T24 bladder and MCF-7 mammary carcinoma cells.[17] Treatment with 5 Gy irradiation under a 0.1% oxygen concentration resulted in the induction of all promoters, with the *Epo* HRE/CArG promoter being most responsive and robust. Subsequent promoter induction tests in a range of physiological oxygen concentrations characteristic of solid tumors showed that the *Epo* HRE/CArG promoter is most responsive in the radio-biologically significant levels of 0.1-0.5% O_2. *Epo* HRE/CArG promoter was next successfully used to control a *HRP* mediated GDEPT strategy following irradiation under hypoxic conditions *in vitro* and *in vivo*.[114] Similar results were reported when chimeric HRE/CArG promoter was used to control *HSV-tk* expression in human lung carcinoma A549 xenografts.[26] In another study, *Epo* HREs were ligated upstream of the *Egr-TNF-α* construct.[132] Combined treatment with *Epo-Egr-TNF-α* plasmid and radiation resulted in significant tumor growth delay in

human colon adenocarcinoma WIDR xenografts.

Another promoter that can be induced by both radiation and hypoxia is *p21* mentioned earlier.[118] This promoter lacks HRE so induction by hypoxia occurs by a novel mechanism involving the Myc transcriptional factor. In the already mentioned *in vitro* study, *p21* promoter was activated in hypoxic conditions by a factor of 5.4 in the RIF-1 cell line and 4.3 in the HT29 cell line.[118] These findings were extended to other cell lines with different *p53* status. Following exposure to hypoxia, all cell lines showed elevated levels of GFP compared to normoxic cells.

Cre/loxP molecular amplification switch. One problem associated with inducible promoters is that, ones they are induced, they are relatively weak compared to strong constitutive promoters. In addition, transgene expression is restricted only to the period of the associated stimulation. In order to generate sufficient concentrations of transgene product without compromising the specificity of the inducible promoters, the expression should be amplified and sustained. For this purpose, an inducible molecular switch was devised based on the Cre/loxP site specific recombination system of the P1 bacteriophage.[133] In this new approach, the inducible promoter controls the expression of *Cre recombinase* instead of the therapeutic transgene, which is transcriptionally silenced by the loxP "stop" cassette incorporated between the gene and the constitutive promoter (Figure 3).

In the evolution of this molecular switching device, the expression of *Cre recombinase* was first controlled by a radio-inducible promoter and two vectors were required: one containing *Cre recombinase* with an inducible promoter and the other with the therapeutic gene and constitutive promoter. The active components were next incorporated into a single vector and the radio-inducible promoter was replaced by chimeric radiation and a hypoxia inducible promoter.[131]

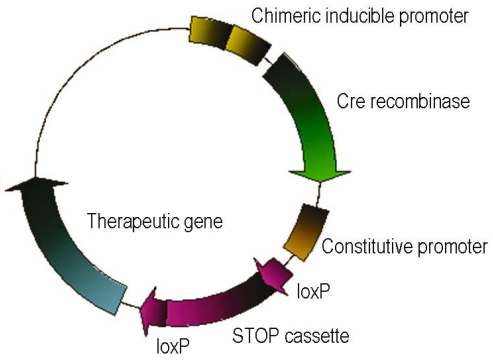

Figure 3. Radiation and/or hypoxia stimulation activates the chimeric promoter on the vector, leading to expression of the *Cre* gene. The produced Cre recombinase then recognizes the loxP sites and cuts out the "stop" cassette, bringing the therapeutic transgene under the control of the strong constitutive promoter.

The efficacy of the two vector system with radio-inducible promoter was tested on a MCF-7 breast cancer cell model using the *GFP* reporter gene and *HSV-tk*/GCV mediated tumor cell killing assay.[133] An increase in *GFP* expression and tumor cell killing was achieved following clinically relevant doses: a 3 Gy dose induced a 40-fold increase in radiation activated *GFP* expression, compared to a two- to three-fold increase when the reporter was controlled directly by the same promoter. Tumor cell growth inhibition equivalent to that of 3 Gy without the switch was achieved by the switch system after a single dose as low as 1 Gy. For testing of a single vector system containing chimeric promoter inducible by hypoxia and radiation, human mammary adenocarcinoma MCF-7 and glioma cells U87-MG, U373-MG were used. *In vitro* higher and more selective tumor cell killing was achieved using switch controlled *HSV-tk*/GCV GDEPT. The single vector switch was also tested in nude mouse xenograft models, in which it

induced significant growth delay and tumor eradication.[134]

Targeted gene therapy in radiotherapy, conclusions

The ultimate aim of gene therapy, common to all cancer therapies, is to selectively target tumor cells while minimizing normal tissue toxicity. Although gene therapy has the potential to provide sustained, high local concentrations of the therapeutic gene, poor tumor specificity is a major problem. Inducible promoters activated by ionizing radiation have the potential to limit gene expression to the irradiated tumor volume. To date, the only radiation-inducible promoter used in clinical trials is the *Egr-1* promoter. Considerable problems remain to be overcome for this radio-gene combined modality to achieve wider clinical application. Each modality of combined treatment has its own drawbacks. For instance, gene therapy still lacks safe and efficient delivery systems. Bystander cell killing can partially improve the efficiency of gene therapy but the quest to find a better delivery system continues. Two major problems of radiotherapy are metastases and radio-resistance. Using radiation induced transcriptional targeting, a high level of local control is achieved at the expense of poor systemic control. One way to solve this problem is to choose a secretory therapeutic gene that has local radio-sensitizing activity and can also induce an effective systemic immune response against tumor antigens or inhibit angiogenesis of metastases (*IL-12*). Another solution is to use a promoter or a combination of promoters that can be induced by radiation, to target the primary tumor, and tumor specific conditions like hypoxia to target the metastases (*p21* promoter, chimeric promoters). An additional advantage of hypoxia inducible promoters is that they target exactly the cell population resistant to radiotherapy. Since a transcriptional targeting approach allows for any therapeutic gene with radio-sensitizing properties to be chosen, careful selection of the best combination of inducible promoters and therapeutic genes is important for translation of this approach to the clinic.

Acknowledgement

The authors acknowledge the financial support of the state budget through the Slovenian Research Agency (Program No. P3-0003 and Projects No.-L3-3375 and J3-9580). We thank Gregor Tevz, Dr. Simona Kranjc, Suzana Mesojednik and Dr. Maja Cemazar for fruitful discussions and critical reading of the manuscript.

References

1 Bernier J, Hall EJ, Giaccia A. Radiation oncology: a century of achievements. *Nat Rev Cancer* 2004; **4(9):** 737-47.

2 Tubiana M, Eschwege F. Conformal radiotherapy and intensity-modulated radiotherapy--clinical data. *Acta Oncol* 2000; **39(5):** 555-67.

3 Shinohara ET, Lu B, Hallahan DE. The use of gene therapy in cancer research and treatment. *Technol Cancer Res Treat* 2004; **3(5):** 479-90.

4 Gridley DS, Slater JM. Combining gene therapy and radiation against cancer. *Curr Gene Ther* 2004; **4(3):** 231-48.

5 Robson T, Worthington J, McKeown SR, Hirst DG. Radiogenic therapy: novel approaches for enhancing tumor radiosensitivity. *Technol Cancer Res Treat* 2005; **4(4):** 343-61.

6 Lumniczky K, Safrany G. Cancer gene therapy: combination with radiation therapy and the role of bystander cell killing in the anti-tumor effect. *Pathol Oncol Res* 2006; **12(2):** 118-24.

7 Stevens CW, Zeng M, Cerniglia GJ. Ionizing radiation greatly improves gene transfer efficiency in mammalian cells. *Hum Gene Ther* 1996; **7(14):** 1727-34.

8 Zeng M, Cerniglia GJ, Eck SL, Stevens CW. High-efficiency stable gene transfer of adenovirus into mammalian cells using ionizing radiation. *Hum Gene Ther* 1997; **8(9):** 1025-32.

9 Sonveaux P, Dessy C, Brouet A, Jordan BF, Gregoire V, Gallez B, Balligand JL, Feron O. Modulation of the tumor vasculature functionality by ionizing radiation accounts for tumor radiosensitization and promotes gene delivery. *FASEB J* 2002; **16(14):** 1979-81.

10 Weichselbaum RR, Hallahan DE, Sukhatme VP, Kufe DW. Gene therapy targeted by ionizing radiation. *Int J Radiat Oncol Biol Phys* 1992; **24(3):** 565-7.

11 Sersa G, Miklavcic D, Rudolf Z, Cemazar M, Pucihar G, Snoj M . Electrochemotherapy in treatment of tumours. *EJSO* 2008; **34(2**): 232-240.

12 Wardman P. Chemical radiosensitizers for use in radiotherapy. *Clin Oncol (R Coll Radiol)* 2007; **19(6):** 397-417.

13 Kufe D, Weichselbaum R. Radiation therapy: activation for gene transcription and the development of genetic radiotherapy-therapeutic strategies in oncology. *Cancer Biol Ther* 2003; **2(4):** 326-9.

14 Greco O, Dachs GU. Gene directed enzyme/prodrug therapy of cancer: historical appraisal and future prospectives. *J Cell Physiol* 2001; **187(1):** 22-36.

15 Dachs GU, Tupper J, Tozer GM. From bench to bedside for gene-directed enzyme prodrug therapy of cancer. *Anticancer Drugs* 2005; **16(4):** 349-59.

16 McKeown SR, Ward C, Robson T. Gene-directed enzyme prodrug therapy: a current assessment. *Curr Opin Mol Ther* 2004; **6(4):** 421-35.

17 Greco O, Marples B, Dachs GU, Williams KJ, Patterson AV, Scott SD. Novel chimeric gene promoters responsive to hypoxia and ionizing radiation. *Gene Ther* 2002; **9(20):** 1403-11.

18 Khil MS, Kim JH, Mullen CA, Kim SH, Freytag SO. Radiosensitization by 5-fluorocytosine of human colorectal carcinoma cells in culture transduced with cytosine deaminase gene. *Clin Cancer Res* 1996; **2(1):** 53-7.

19 Teh BS, guilar-Cordova E, Vlachaki MT, Aguilar L, Mai WY, Caillouet J, Davis M, Miles B, Kadmon D, Ayala G, Lu HH, Chiu JK, Carpenter LS, Woo SY, Grant WH, III, Wheeler T, Thompson TC, Butler EB. Combining radiotherapy with gene therapy (from the bench to the bedside): a novel treatment strategy for prostate cancer. *Oncologist* 2002; **7(5):** 458-66.

20 Rogulski KR, Zhang K, Kolozsvary A, Kim JH, Freytag SO. Pronounced antitumor effects and tumor radiosensitization of double suicide gene therapy. *Clin Cancer Res* 1997; **3(11):** 2081-8.

21 Xia K, Liang D, Tang A, Feng Y, Zhang J, Pan Q, Long Z, Dai H, Cai F, Wu L, Zhao S, Chen Z, Xia J. A novel fusion suicide gene yeast CDglyTK plays a role in radio-gene therapy of nasopharyngeal carcinoma. *Cancer Gene Ther* 2004; **11(12):** 790-6.

22 Freytag SO, Paielli D, Wing M, Rogulski K, Brown S, Kolozsvary A, Seely J, Barton K, Dragovic A, Kim JH. Efficacy and toxicity of replication-competent adenovirus-mediated double suicide gene therapy in combination with radiation therapy in an orthotopic mouse prostate cancer model. *Int J Radiat Oncol Biol Phys* 2002; **54(3):** 873-85.

23 Freytag SO, Khil M, Stricker H, Peabody J, Menon M, Peralta-Venturina M, Nafziger D, Pegg J, Paielli D, Brown S, Barton K, Lu M, guilar-Cordova E, Kim JH. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res* 2002; **62(17):** 4968-76.

24 Mitchell JB, Wink DA, DeGraff W, Gamson J, Keefer LK, Krishna MC. Hypoxic mammalian cell radiosensitization by nitric oxide. *Cancer Res* 1993; **53(24):** 5845-8.

25 Kurimoto M, Endo S, Hirashima Y, Hamada H, Ogiichi T, Takaku A. Growth inhibition and radiosensitization of cultured glioma cells by nitric oxide generating agents. *J Neurooncol* 1999; **42(1):** 35-44.

26 Wang Z, Cook T, Alber S, Liu K, Kovesdi I, Watkins SK, Vodovotz Y, Billiar TR, Blumberg D. Adenoviral gene transfer of the human inducible nitric oxide synthase gene enhances the radiation response of human colorectal cancer associated with alterations in tumor vascularity. *Cancer Res* 2004; **64(4):** 1386-95.

27 Jordan BF, Misson P, Demeure R, Baudelet C, Beghein N, Gallez B. Changes in tumor oxygenation/perfusion induced by the no donor, isosorbide dinitrate, in comparison with carbogen: monitoring by EPR and MRI. *Int J Radiat Oncol Biol Phys* 2000; **48(2):** 565-70.

28 Worthington J, Robson T, O'Keeffe M, Hirst DG. Tumour cell radiosensitization using constitutive (CMV) and radiation inducible (WAF1) promoters to drive the iNOS gene: a novel suicide gene therapy. *Gene Ther* 2002; **9(4):** 263-9.

29 Xie K, Huang S, Dong Z, Juang SH, Gutman M, Xie QW, Nathan C, Fidler IJ. Transfection with the inducible nitric oxide synthase gene suppresses tumorigenicity and abrogates metastasis by K-1735 murine melanoma cells. *J Exp Med* 1995; **181(4):** 1333-43.

30 Worthington J, McCarthy HO, Barrett E, Adams C, Robson T, Hirst DG. Use of the radiation-inducible WAF1 promoter to drive iNOS gene therapy as a novel anti-cancer treatment. *J Gene Med* 2004; **6(6):** 673-80.

31 Worthington J, Robson T, Scott S, Hirst D. Evaluation of a synthetic CArG promoter for nitric oxide synthase gene therapy of cancer. *Gene Ther* 2005; **12(19):** 1417-23.

32 Cook T, Wang Z, Alber S, Liu K, Watkins SC, Vodovotz Y, Billiar TR, Blumberg D. Nitric oxide and ionizing radiation synergistically promote apoptosis and growth inhibition of cancer by activating p53. *Cancer Res* 2004; **64(21):** 8015-21.

33 Tangney M, Casey G, Larkin JO, Collins CG, Soden D, Cashman J, Whelan MC, O'Sullivan GC. Non-viral in vivo immune gene therapy of cancer: combined strategies for treatment of systemic disease. *Cancer Immunol Immunother* 2006; **55(11):** 1443-50.

34 Friedman EJ. Immune modulation by ionizing radiation and its implications for cancer immunotherapy. *Curr Pharm Des* 2002; **8(19):** 1765-80.

35 Demaria S, Bhardwaj N, McBride WH, Formenti SC. Combining radiotherapy and immunotherapy: a revived partnership. *Int J Radiat Oncol Biol Phys* 2005; **63(3):** 655-66.

36 Tuting T, Storkus WJ, Lotze MT. Gene-based strategies for the immunotherapy of cancer. *J Mol Med* 1997; **75(7):** 478-91.

37 Li CY, Huang Q, Kung HF. Cytokine and immuno-gene therapy for solid tumors. *Cell Mol Immunol* 2005; **2(2):** 81-91.

38 Ulmer JB, Donnelly JJ, Liu MA. Toward the development of DNA vaccines. *Curr Opin Biotechnol* 1996; **7(6):** 653-8.

39 Stevenson FK, Ottensmeier CH, Johnson P, Zhu D, Buchan SL, McCann KJ, Roddick JS, King AT, McNicholl F, Savelyeva N, Rice J. DNA vaccines to attack cancer. *Proc Natl Acad Sci U S A* 2004; **101(2):** 14646-52.

40 Hodge JW, Greiner JW, Tsang KY, Sabzevari H, Kudo-Saito C, Grosenbach DW, Gulley JL, Arlen PM, Marshall JL, Panicali D, Schlom J. Costimulatory molecules as adjuvants for immunotherapy. *Front Biosci* 2006; **11:** 788-803.

41 Chakraborty M, Abrams SI, Coleman CN, Camphausen K, Schlom J, Hodge JW. External beam radiation of tumors alters phenotype of tumor cells to render them susceptible to vaccine-mediated T-cell killing. *Cancer Res* 2004; **64(12):** 4328-37.

42 Gulley JL, Arlen PM, Bastian A, Morin S, Marte J, Beetham P, Tsang KY, Yokokawa J, Hodge JW, Menard C, Camphausen K, Coleman CN, Sullivan F, Steinberg SM, Schlom J, Dahut W. Combining a recombinant cancer vaccine with standard definitive radiotherapy in patients with localized prostate cancer. *Clin Cancer Res* 2005; **11(9):** 3353-62.

43 Miller AR, McBride WH, Hunt K, Economou JS. Cytokine-mediated gene therapy for cancer. *Ann Surg Oncol* 1994; **1(5):** 436-50.

44 Kemeny N, Childs B, Larchian W, Rosado K, Kelsen D. A phase II trial of recombinant tumor necrosis factor in patients with advanced colorectal carcinoma. *Cancer* 1990; **66(4):** 659-63.

45 Hallahan DE, Vokes EE, Rubin SJ, O'Brien S, Samuels B, Vijaykumar S, Kufe DW, Phillips R, Weichselbaum RR. Phase I dose-escalation study of tumor necrosis factor-alpha and concomitant radiation therapy. *Cancer J Sci Am* 1995; **1(3):** 204-9.

46 Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 1995; **13:** 251-76.

47 Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003; **3(2):** 133-46.

48 Voest EE, Kenyon BM, O'Reilly MS, Truitt G, D'Amato RJ, Folkman J. Inhibition of angiogenesis in vivo by interleukin 12. *J Natl Cancer Inst* 1995; **87(8):** 581-6.

49 Ogawa M, Yu WG, Umehara K, Iwasaki M, Wijesuriya R, Tsujimura T, Kubo T, Fujiwara H, Hamaoka T. Multiple roles of interferon-gamma in the mediation of interleukin 12-induced tumor regression. *Cancer Res* 1998; **58(11):** 2426-32.

50 Brunda MJ, Luistro L, Warrier RR, Wright RB, Hubbard BR, Murphy M, Wolf SF, Gately MK. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J Exp Med* 1993; **178(4):** 1223-30.

51 Seetharam S, Staba MJ, Schumm LP, Schreiber K, Schreiber H, Kufe DW, Weichselbaum RR. Enhanced eradication of local and distant tumors by genetically produced interleukin-12 and radiation. *Int J Oncol* 1999; **15(4):** 769-73.

52 Lohr F, Hu K, Haroon Z, Samulski TV, Huang Q, Beaty J, Dewhirst MW, Li CY. Combination treatment of murine tumors by adenovirus-mediated local B7/IL12 immunotherapy and radiotherapy. *Mol Ther* 2000; **2(3):** 195-203.

53 Xian J, Yang H, Lin Y, Liu S. Combination nonviral murine interleukin 2 and interleukin 12 gene therapy and radiotherapy for head and neck squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg* 2005; **131(12):** 1079-85.

54 Fujita T, Timme TL, Tabata K, Naruishi K, Kusaka N, Watanabe M, Abdelfattah E, Zhu JX, Ren C, Ren C, Yang G, Goltsov A, Wang H, Vlachaki MT, Teh BS, Butler EB, Thompson TC. Cooperative effects of adenoviral vector-mediated interleukin 12 gene therapy with radiotherapy in a preclinical model of metastatic prostate cancer. *Gene Ther* 2007; **14(3):** 227-36.

55 Yang Y, Liu SZ, Fu SB. Anti-tumor effects of pNEgr-mIL-12 recombinant plasmid induced by X-irradiation and its mechanisms. *Biomed Environ Sci* 2004; **17(2):** 135-43.

56 van HR, ten Hagen TL, Eggermont AM. TNF-alpha in cancer treatment: molecular insights, antitumor effects, and clinical utility. *Oncologist* 2006; **11(4):** 397-408.

57 Sersa G, Willingham V, Milas L. Anti-tumor effects of tumor necrosis factor alone or combined with radiotherapy. *Int J Cancer* 1988; **42(1):** 129-34.

58 U.S. National Institutes of Health (Internet). Retrieved March 2008, from: http://ClinicalTrials.gov

59 Mauceri HJ, Hanna NN, Wayne JD, Hallahan DE, Hellman S, Weichselbaum RR. Tumor necrosis factor alpha (TNF-alpha) gene therapy targeted by ionizing radiation selectively damages tumor vasculature. *Cancer Res* 1996; **56(19):** 4311-4.

60 Brem S, Brem H, Folkman J, Finkelstein D, Patz A. Prolonged tumor dormancy by prevention of neovascularization in the vitreous. *Cancer Res* 1976; **36(8):** 2807-12.

61 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100(1):** 57-70.

62 Denekamp J. Review article: angiogenesis, neovascular proliferation and vascular pathophysiology as targets for cancer therapy. *Br J Radiol* 1993; **66(783):** 181-96.

63 Folkman J, Hanahan D. Switch to the angiogenic phenotype during tumorigenesis. *Princess Takamatsu Symp* 1991; **22:** 339-47.

64 Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996; **86(3):** 353-64.

65 Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 2003; **3(6):** 401-10.

66 Boehm T, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature* 1997; **390(6658):** 404-7.

67 Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995; **1(1):** 27-31.

68 Wachsberger P, Burd R, Dicker AP. Tumor response to ionizing radiation combined with antiangiogenesis or vascular targeting agents: exploring mechanisms of interaction. *Clin Cancer Res* 2003; **9(6):** 1957-71.

69 Scappaticci FA. Mechanisms and future directions for angiogenesis-based cancer therapies. *J Clin Oncol* 2002; **20(18):** 3906-27.

70 Siemann DW, Warrington KH, Horsman MR. Targeting tumor blood vessels: an adjuvant strategy for radiation therapy. *Radiother Oncol* 2000; **57(1):** 5-12.

71 Siemann DW, Bibby MC, Dark GG, Dicker AP, Eskens FA, Horsman MR, Marme D, Lorusso PM. Differentiation and definition of vascular-targeted therapies. *Clin Cancer Res* 2005; **11(2):** 416-20.

72 Kong HL, Crystal RG. Gene therapy strategies for tumor antiangiogenesis. *J Natl Cancer Inst* 1998; **90(4):** 273-86.

73 O'Reilly MS. Angiostatin: an endogenous inhibitor of angiogenesis and of tumor growth. *EXS* 1997; **79:** 273-94.

74 Folkman J. Antiangiogenic gene therapy. *Proc Natl Acad Sci U S A* 1998; **95(16):** 9064-6.

75 Tandle A, Blazer DG, III, Libutti SK. Antiangiogenic gene therapy of cancer: recent developments. *J Transl Med* 2004; **2(1):** 22.

76 O'Reilly MS. Radiation combined with antiangiogenic and antivascular agents. *Semin Radiat Oncol* 2006; **16(1):** 45-50.

77 Jain RK. Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. *Nat Med* 2001; **7(9):** 987-9.

78 Siemann DW, Chaplin DJ, Horsman MR. Vascular-targeting therapies for treatment of malignant disease. *Cancer* 2004; **100(12):** 2491-9.

79 Wahl ML, Moser TL, Pizzo SV. Angiostatin and anti-angiogenic therapy in human disease. *Recent Prog Horm Res* 2004; **59(**73-104.

80 Folkman J. Antiangiogenesis in cancer therapy--endostatin and its mechanisms of action. *Exp Cell Res* 2006; **312(5):** 594-607.

81 Shi W, Teschendorf C, Muzyczka N, Siemann DW. Gene therapy delivery of endostatin enhances the treatment efficacy of radiation. *Radiother Oncol* 2003; **66(1):** 1-9.

82 Zheng AQ, Song XR, Yu JM, Wei L, Wang XW. Liposome transfected to plasmid-encoding endostatin gene combined with radiotherapy inhibits liver cancer growth in nude mice. *World J Gastroenterol* 2005; **11(28):** 4439-42.

83 Luo X, Slater JM, Gridley DS. Enhancement of radiation effects by pXLG-mEndo in a lung carcinoma model. *Int J Radiat Oncol Biol Phys* 2005; **63(2):** 553-64.

84 Griscelli F, Li H, Cheong C, Opolon P, naceur-Griscelli A, Vassal G, Soria J, Soria C, Lu H, Perricaudet M, Yeh P. Combined effects of radiotherapy and angiostatin gene therapy in glioma tumor model. *Proc Natl Acad Sci U S A* 2000; **97(12):** 6698-703.

85 ten Hagen TL, Eggermont AM. Solid tumor therapy: manipulation of the vasculature with TNF. *Technol Cancer Res Treat* 2003; **2(3):** 195-203.

86 Menon C, Ghartey A, Canter R, Feldman M, Fraker DL. Tumor necrosis factor-alpha damages tumor blood vessel integrity by targeting VE-cadherin. *Ann Surg* 2006; **244(5):** 781-91.

87 Weichselbaum RR, Kufe DW, Advani SJ, Roizman B. Molecular targeting of gene therapy and radiotherapy. *Acta Oncol* 2001; **40(6):** 735-8.

88 Robson T, Hirst DG. Transcriptional Targeting in Cancer Gene Therapy. *J Biomed Biotechnol* 2003; **2003(2):** 110-37.

89 Dachs GU, Dougherty GJ, Stratford IJ, Chaplin DJ. Targeting gene therapy to cancer: a review. *Oncol Res* 1997; **9(6-7):** 313-25.

90 Goverdhana S, Puntel M, Xiong W, Zirger JM, Barcia C, Curtin JF, Soffer EB, Mondkar S, King GD, Hu J, Sciascia SA, Candolfi M, Greengold DS, Lowenstein PR, Castro MG. Regulatable gene expression systems for gene therapy applications: Progress and future challenges. *Molecular Therapy* 2005; **12(2):** 189-211.

91 Haviv YS, Curiel DT. Conditional gene targeting for cancer gene therapy. *Adv Drug Deliv Rev* 2001; **53(2):** 135-54.

92 Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* 1992; **89(12):** 5547-51.

93 Huang Q, Hu JK, Lohr F, Zhang L, Braun R, Lanzen J, Little JB, Dewhirst MW, Li CY. Heat-induced gene expression as a novel targeted cancer gene therapy strategy. *Cancer Res* 2000; **60(13):** 3435-9.

94 Rubenstrunk A, Trollet C, Orsini C, Scherman D. Positive in vivo heterologous gene regulation by electric pulses delivery with metallothionein I gene promoter. *J Gene Med* 2005; **7(12):** 1565-72.

95 Chastel C, Jiricny J, Jaussi R. Activation of stress-responsive promoters by ionizing radiation for deployment in targeted gene therapy. *DNA Repair (Amst)* 2004; **3(3):** 201-15.

96 Nuyts S, Van ML, Barbe S, Lammertyn E, Theys J, Landuyt W, Bosmans E, Lambin P, Anne J. Insertion or deletion of the Cheo box modifies radiation inducibility of Clostridium promoters. *Appl Environ Microbiol* 2001; **67(10):** 4464-70.

97 Datta R, Taneja N, Sukhatme VP, Qureshi SA, Weichselbaum R, Kufe DW. Reactive oxygen intermediates target CC(A/T)6GG sequences to mediate activation of the early growth response 1 transcription factor gene by ionizing radiation. *Proc Natl Acad Sci U S A* 1993; **90(6):** 2419-22.

98 Weichselbaum RR, Hallahan DE, Beckett MA, Mauceri HJ, Lee H, Sukhatme VP, Kufe DW. Gene therapy targeted by radiation preferentially radiosensitizes tumor cells. *Cancer Res* 1994; **54(16):** 4266-9.

99 Hallahan DE, Mauceri HJ, Seung LP, Dunphy EJ, Wayne JD, Hanna NN, Toledano A, Hellman S, Kufe DW, Weichselbaum RR. Spatial and temporal control of gene therapy using ionizing radiation. *Nat Med* 1995; **1(8):** 786-91.

100 Chung TD, Mauceri HJ, Hallahan DE, Yu JJ, Chung S, Grdina WL, Yajnik S, Kufe DW, Weichselbaum RR. Tumor necrosis factor-alpha-based gene therapy enhances radiation cytotoxicity in human prostate cancer. *Cancer Gene Ther* 1998; **5(6):** 344-9.

101 Gupta VK, Park JO, Jaskowiak NT, Mauceri HJ, Seetharam S, Weichselbaum RR, Posner MC. Combined gene therapy and ionizing radiation is a novel approach to treat human esophageal adenocarcinoma. *Ann Surg Oncol* 2002; **9(5):** 500-4.

102 Weichselbaum RR, Kufe DW, Hellman S, Rasmussen HS, King CR, Fischer PH, Mauceri HJ. Radiation-induced tumour necrosis factor-alpha expression: clinical application of transcriptional and physical targeting of gene therapy. *Lancet Oncol* 2002; **3(11):** 665-71.

103 Rasmussen H, Rasmussen C, Lempicki M, Durham R, Brough D, King CR, Weichselbaum R. TNFerade Biologic: preclinical toxicology of a novel adenovector with a radiation-inducible promoter, carrying the human tumor necrosis factor alpha gene. *Cancer Gene Ther* 2002; **9(11):** 951-7.

104 Senzer N, Mani S, Rosemurgy A, Nemunaitis J, Cunningham C, Guha C, Bayol N, Gillen M, Chu K, Rasmussen C, Rasmussen H, Kufe D, Weichselbaum R, Hanna N. TNFerade biologic, an adenovector with a radiation-inducible promoter, carrying the human tumor necrosis factor alpha gene: a phase I study in patients with solid tumors. *J Clin Oncol* 2004; **22(4):** 592-601.

105 Mundt AJ, Vijayakumar S, Nemunaitis J, Sandler A, Schwartz H, Hanna N, Peabody T, Senzer N, Chu K, Rasmussen CS, Kessler PD, Rasmussen HS, Warso M, Kufe DW, Gupta TD, Weichselbaum RR. A Phase I trial of TNFerade biologic in patients with soft tissue sarcoma in the extremities. *Clin Cancer Res* 2004; **10(17):** 5747-53.

106 Manome Y, Kunieda T, Wen PY, Koga T, Kufe DW, Ohno T. Transgene expression in malignant glioma using a replication-defective adenoviral vector containing the Egr-1 promoter: activation by ionizing radiation or uptake of radioactive iododeoxyuridine. *Hum Gene Ther* 1998; **9(10):** 1409-17.

107 Marples B, Greco O, Joiner MC, Scott SD. Radiogenetic therapy: strategies to overcome tumor resistance. *Curr Pharm Des* 2003; **9(26):** 2105-12.

108 Joki T, Nakamura M, Ohno T. Activation of the radiosensitive EGR-1 promoter induces expression of the herpes simplex virus thymidine kinase gene and sensitivity of human glioma cells to ganciclovir. *Hum Gene Ther* 1995; **6(12):** 1507-13.

109 Takahashi T, Namiki Y, Ohno T. Induction of the suicide HSV-TK gene by activation of the Egr-1 promoter with radioisotopes. *Hum Gene Ther* 1997; **8(7):** 827-33.

110 Wang WD, Chen ZT, Li DZ, Duan YZ, Cao ZH. [Experimental study on lung carcinoma-targeted suicide gene therapy induced by irradiation]. *Zhonghua Jie He He Hu Xi Za Zhi* 2003; **26(2):** 84-7.

111 Wu CM, Li XY, Huang TH. Anti-tumor effect of pEgr-IFNgamma gene-radiotherapy in B16 melanoma-bearing mice. *World J Gastroenterol* 2004; **10(20):** 3011-5.

112 Coulter JA, McCarthy HO, Worthington J, Robson T, Scott S, Hirst DG. The radiation-inducible pE9 promoter driving inducible nitric oxide synthase radiosensitizes hypoxic tumour cells to radiation. *Gene Ther* 2008; **15(7):** 495-503.

113 Jin GH, Jin SZ, Liu Y, Xu RM, Yang JZ, Pan XN, Liu SZ. Therapeutic effect of gene-therapy in combination with local X-irradiation in a mouse malignant melanoma model. *Biochem Biophys Res Commun* 2005; **330(3):** 975-81.

114 Scott SD, Joiner MC, Marples B. Optimizing radiation-responsive gene promoters for radiogenetic cancer therapy. *Gene Ther* 2002; **9(20):** 1396-402.

115 Harada K, Ogden GR. An overview of the cell cycle arrest protein, p21(WAF1). *Oral Oncol* 2000; **36(1):** 3-7.

116 El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993; **75(4):** 817-25.

117 Worthington J, Robson T, Murray M, O'Rourke M, Keilty G, Hirst DG. Modification of vascular tone using iNOS under the control of a radiation-inducible promoter. *Gene Ther* 2000; **7(13):** 1126-31.

118 McCarthy HO, Worthington J, Barrett E, Cosimo E, Boyd M, Mairs RJ, Ward C, McKeown SR, Hirst DG, Robson T. p21((WAF1))-mediated transcriptional targeting of inducible nitric oxide synthase gene therapy sensitizes tumours to fractionated radiotherapy. *Gene Ther* 2007; **14(3):** 246-55.

119 Espinosa JM, Emerson BM. Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. *Mol Cell* 2001; **8(1):** 57-69.

120 Nenoi M, Daino K, Ichimura S, Takahash S, Akuta T. Low-dose radiation response of the p21WAF1/CIP1 gene promoter transduced by adeno-associated virus vector. *Exp Mol Med* 2006; **38(5):** 553-64.

121 Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev* 2007; **26(2):** 225-39.

122 Brown JM, Wilson WR. Exploiting tumour hypoxia in cancer treatment. *Nat Rev Cancer* 2004; **4(6):** 437-47.

123 Brown JM. Tumor hypoxia in cancer therapy. *Methods Enzymol* 2007; **435(**297-321.

124 Kizaka-Kondoh S, Inoue M, Harada H, Hiraoka M. Tumor hypoxia: a target for selective cancer therapy. *Cancer Sci* 2003; **94(12):** 1021-8.

125 Dachs GU, Stratford IJ. The molecular response of mammalian cells to hypoxia and the potential for exploitation in cancer therapy. *Br J Cancer Suppl* 1996; **27:** 126-132.

126 Greco O, Patterson AV, Dachs GU. Can gene therapy overcome the problem of hypoxia in radiotherapy? *J Radiat Res (Tokyo)* 2000; **41(3):** 201-12.

127 Ruan H, Deen DF. Use of hypoxia-regulated gene expression in tumor-specific gene therapy. *Curr Opin Investig Drugs* 2001; **2(6):** 839-43.

128 Greco O, Marples B, Joiner MC, Scott SD. How to overcome (and exploit) tumor hypoxia for targeted gene therapy. *J Cell Physiol* 2003; **197(3):** 312-25.

129 Shibata T, Giaccia AJ, Brown JM. Development of a hypoxia-responsive vector for tumor-specific gene therapy. *Gene Ther* 2000; **7(6):** 493-8.

130 Patterson AV, Williams KJ, Cowen RL, Jaffar M, Telfer BA, Saunders M, Airley R, Honess D, van der Kogel AJ, Wolf CR, Stratford IJ. Oxygen-sensitive enzyme-prodrug gene therapy for the eradication of radiation-resistant solid tumours. *Gene Ther* 2002; **9(14):** 946-54.

131 Scott SD, Greco O. Radiation and hypoxia inducible gene therapy systems. *Cancer Metastasis Rev* 2004; **23(3-4):** 269-76.

132 Salloum RM, Saunders MP, Mauceri HJ, Hanna NN, Gorski DH, Posner MC, Stratford IJ, Weichselbaum RR. Dual induction of the Epo-Egr-TNF-alpha- plasmid in hypoxic human colon adenocarcinoma produces tumor growth delay. *Am Surg* 2003; **69(1):** 24-7.

133 Scott SD, Marples B, Hendry JH, Lashford LS, Embleton MJ, Hunter RD, Howell A, Margison GP. A radiation-controlled molecular switch for use in gene therapy of cancer. *Gene Ther* 2000; **7(13):** 1121-5.

134 Greco O, Joiner MC, Doleh A, Powell AD, Hillman GG, Scott SD. Hypoxia- and radiation-activated Cre/loxP 'molecular switch' vectors for gene therapy of cancer. *Gene Ther* 2006; **13(3):** 206-15.