Chapter

General introduction and scope of this thesis
Cancer gene therapy

In the year 2000 alone, worldwide 6.2 million persons died from cancer. Moreover, the number of new cases is expected to increase by 50% over the next 20 years, reaching a total of 15 million new patients in the year 2020.¹ These devastating numbers emphasize the need for other treatment methods. A promising method is cancer gene therapy, which can be defined as the delivery of genetic material into cells to accomplish an anti-cancer effect.

At this moment, two anti-cancer gene therapy products are commercially available. The first approved product was Gendicine (Shenzhen SiBiono GeneTech), which is available in China only. This product consists of a modified adenoviral vector engineered to express p53, and infection with this virus results in the death of tumor cells. Cancer patients from all over the world are now travelling to China for treatment with this virus.² The second commercially available gene therapy vector is the oncolytic adenovirus H101 (Shanghai Sunway Biotech), which causes specific oncolysis of tumor cells. Also this product is only available in China.³

Cancer gene therapy comprises a very broad research area, with many different approaches for the treatment of cancer. Currently, the major drawbacks of cancer gene therapy are a lack of specificity and a lack of efficacy. In this thesis, two different cancer gene therapeutic approaches are addressed: Exogenous as well as endogenous gene regulatory mechanisms are used to achieve more specific and efficient cancer gene therapy. Both approaches utilize the characteristics of the epithelial glycoprotein-2 (EGP-2).

The epithelial glycoprotein-2 (EGP-2)

EGP-2 is also known as EpCAM (epithelial cell adhesion molecule) or 17-1A, and is encoded by the GA733-2 gene. This protein is involved in homotypic calcium-independent cell-cell adhesion.⁴ Most importantly, EGP-2 is highly expressed in the majority of cancer types and shows only limited expression in healthy tissues.⁵,⁶ This expression pattern makes EGP-2 a promising candidate for specific retargeting towards the tumor tissue. In this respect, monoclonal antibodies directed against EGP-2 are currently tested in phase III clinical trials,⁴ indicating the potency of EGP-2 as a specific target for anti-cancer therapy.

Recently, two independent research groups have demonstrated that EGP-2 has an effect on the oncogenic potential of tumor cells.⁷,⁸ Down-regulation of the endogenous EGP-2 expression can therefore result in an anti-cancer treatment.

Using the EGP-2 promoter for specific retargeting of adenoviral vectors

For the delivery of genetic material to tumor cells, a wide variety of vectors can be employed. One of the most efficient gene transfer vectors currently available is the adenovirus type 5.⁹ The adenovirus can infect dividing as well as non-dividing cells, is easy to manipulate, and can be produced in high titers. At this moment, adenoviral vectors are the most commonly used vectors in gene therapy clinical trials world wide (26%, 301 clinical trials in 2006) (www.wiley.co.uk/genmed/clinical).

The natural infection spectrum of adenoviral vectors type 5 is not confined to the tumor tissue, which is a complication for achieving a specific anti-cancer gene therapy. More precisely, after systemic administration over 90% of the adenovirus homes directly to the liver.¹⁰,¹¹ To increase the specificity of adenoviral cancer gene therapy towards the
In this respect, there are several approaches to increase the specificity of adenoviral vectors towards the tumor tissue, which are outlined in Chapter 2.

A promising approach to increase the specificity of gene transfer is transcriptional retargeting, in which a particular promoter controls the expression of a transgene. For the treatment of cancer, the promoter activity should be confined to the tumor tissue. EGP-2 is expressed in the majority of carcinomas, including lung, breast, and prostate cancer, which are the most deadly types of cancer in The Netherlands. Most importantly, this protein demonstrates only limited expression in healthy tissues and is not expressed in mature hepatocytes. This selective activity pattern of the EGP-2 promoter is therefore extremely suitable for transcriptional retargeting approaches.

The ultimate goal of cancer gene therapy is the eradication of the tumor tissue. The expressed transgene should therefore induce tumor cell death. These genes include tumor suppressor genes (e.g. p53 used in the gene product Gendicine), cytokines, and so-called suicide genes. Currently, almost 8% of all clinical gene therapy trials worldwide are studies with suicide-genes (www.wiley.co.uk/genmed/clinical). A very potent suicide gene is thymidine kinase (TK). TK phosphorylates the non-toxic pro-drug ganciclovir, ultimately generating a toxic compound that integrates into the DNA and blocks DNA replication. An adenoviral vector expressing thymidine kinase causes severe liver toxicity after systemic injection and administration of ganciclovir in rat, indicating the necessity for retargeting of adenoviral vectors towards the tumor tissue.

In this thesis, we engineered an adenoviral vector containing an expression cassette in which the EGP-2 promoter controlled the expression of a modified form of TK (SR39 TK) (Chapter 3). SR39 TK displays a superior sensitivity compared to native thymidine kinase. We evaluated the specificity and efficiency of this engineered adenoviral vector to eradicate EGP-2 positive (tumor) cells (Figure 1) as a novel anti-cancer treatment.

![Cell death](Figure 1. Confined toxicity to EGP-2 positive cells)

EGP-2 promoter controlled expression results in the production of thymidine kinase in EGP-2 positive cells only. Thymidine kinase can subsequently convert the non-toxic pro-drug ganciclovir (GCV) into a toxic compound.
Using the EGP-2 promoter for efficient and specific retargeting of adenoviral vectors

For the treatment of cancer it is necessary to eradicate all tumor cells, otherwise the malignant tissue will re-occur. This indicates that the toxic agent has to reach every tumor cell present. Although the adenovirus is one of the most efficient gene transfer vectors currently available, it will not penetrate into the tumor mass even after direct intratumoral administration. Therefore, the suicide gene will not be expressed in all tumor cells, and although phosphorylated GCV does have a bystander effect, this will often not be sufficient for the eradication of all malignant cells.

Recombinant adenoviral vectors are deleted for the gene E1, which is essential for the replication of the adenovirus. This results in harmless gene transfer vectors. Replication of the adenovirus results in cell lysis and release of progeny virus. These newly formed viral particles can subsequently infect the surrounding cells, repeating the cycle of replication and cell lysis (Figure 2). In this thesis, we aimed to augment the infection efficiency by inducing adenoviral replication selectively in tumor cells. Tumor-specific adenoviral replication is also the underlying principle of the commercially available gene product H101. This adenovirus has many similarities with the adenovirus Onyx-015, and should replicate in p53-deficient tumor cells only. Noteworthy, the selectivity profile of the Onyx-015 adenoviral replication is currently questioned.

We aimed to increase the efficiency of cancer gene therapy by targeting adenoviral replication towards the EGP-2 positive tumor cells (Chapter 4). For this purpose, the adenoviral replication-essential gene E1 was placed back into the adenoviral genome, but now under the control of the EGP-2 promoter. Confined E1 expression should lead to replication in EGP-2 positive cells only (Figure 2).

Figure 2. Restricted oncolysis of EGP-2 positive tumor cells.
Expression of a gene essential for adenoviral replication under the control of the EGP-2 promoter will result in cell death of EGP-2 positive cells only. Release of viral progeny will subsequently cause infection of neighboring cells, continuing the cycle of replication and cell death.
Increasing the specificity of adenoviral replication

The EGP-2 promoter demonstrates only limited activity in healthy tissues. However, only a modest amount of E1 protein is sufficient to induce replication. Moreover, sequences within the adenoviral genome present upstream of the inserted promoter can act as enhancer elements of gene expression. These enhancer elements can interfere with the activity pattern of the promoter, inducing gene expression and ultimately replication in promoter-negative tissues. Potential adenoviral replication due to E1 expression in normal tissues can result in harmful side-effects.

This expression of E1 and subsequent adenoviral replication in normal tissues signifies the need for more specific control of adenoviral replication. To more pronouncedly restrict adenoviral replication to the malignant tissue, two tumor-specific promoters can be utilized to restrict the expression of two adenoviral genes essential for replication. Consequently, virus progeny production can only occur when both promoters are active. As a first proof of principle, we explored a trans-complementing adenoviral system (Chapter 5). In this concept, two viruses are employed that contain deletions for two replication essential genes in their genome. The first virus contains one replication essential gene under the control of a tumor specific promoter, and the second virus contains the other replication essential gene under the control of a tumor specific promoter. These adenoviruses can only replicate when both replication-essential genes are expressed, and therefore both viruses need to complement each other in the same promoter positive tumor cell.

This approach should dramatically increase the safety profile of replicating adenoviruses. However, because both vectors need to infect the same cell, the efficiency will decline. Subsequently, to retain the increased specificity and re-introduce the efficiency, we placed two tumor-specific promoters controlling the expression of two replication essential genes in the same adenoviral backbone.

Targeting the endogenous EGP-2 promoter to decrease the oncogenic potential of tumor cells

The protein EGP-2 is directly involved in the oncogenic potential of tumor cells, as previous studies demonstrated that down-regulation of EGP-2 expression at the RNA level results in a decrease in the migration, invasion, and proliferation potential of tumor cells in vitro. Since the expression of EGP-2 is up-regulated in most carcinomas, inhibiting this expression provides a novel method for the treatment of a broad spectrum of cancer types.

Currently, the most efficient method of post-transcriptional gene silencing is RNA interference. The introduction of short double-stranded RNA molecules in the cell results in the assembly of the RNA-induced silencing complex (RISC) and base-pairing with complementary target mRNA molecule. Subsequently, the target mRNA strand is cleaved by the enzyme DICER, ultimately resulting in a decreased amount of protein.

Down-regulating gene expression by targeting the mRNA requires the targeting of many molecules. This can be less efficient in comparison to directly targeting the DNA strands and inhibiting the endogenous promoter activity. Moreover, RNA interference might cause off-target gene regulation and the induction of an interferon response. In addition, recently it was shown that introduced short hairpin RNA can compete with endogenous microRNA for nuclear export via exportin-5, resulting in mortality in mice.
These drawbacks of RNA interference indicate the need for novel methods for the modulation of endogenous gene expression.

In this thesis, we aimed to directly target the endogenous EGP-2 promoter to down-regulate the expression of EGP-2. Direct inhibition at the DNA level can be accomplished by employing artificial transcription factors (ATFs). Conventionally, ATFs consist of a DNA-binding domain linked to an effector domain. Several classes of DNA-binding domains are currently explored, including triplex-forming oligonucleotides, poly-amides and zinc finger proteins (ZFPs). In this respect, ZFPs are very well developed and have already proceeded into phase I clinical testing (Sangamo Biosciences). The power of ZFP-TFs to modulate endogenous gene expression is discussed in Chapter 6.

ZFPs consist of modular domains, with each domain recognizing a 3-4 base pair sequence (Figure 3). At this moment these domains can be engineered to target all ANN, GNN, and CNN triplets. The individual domains can be stitched together to recognize an extended sequence, generating ZFPs capable of recognizing a unique sequence within the human genome.

A wide variety of effector domains can be attached to the DNA-binding ZFPs: Domains that inhibit or activate transcription, but also domains that methylate the histones for long-lasting repression of transcription or domains that generate double-stranded breaks in the DNA to stimulate homologous recombination. ZFPs can thus fulfill many different objectives, depending upon the effector domain attached.

In this thesis, we aimed to engineer zinc finger proteins targeting specific sequences within the EGP-2 promoter (Chapter 7). The attachment of a transcription repression domain to the zinc finger protein should subsequently result in specific down-regulation of EGP-2 promoter activity. Ultimately, down-regulation of EGP-2 expression will result in a decreased oncogenic potential of tumor cells, generating a novel anti-tumor therapy for a wide spectrum of tumor types.

Figure 3. Zinc finger domain

The α-helix of each zinc finger domain specifically binds the DNA by interaction between amino acid residues at specific locations within the α-helix and the base-pairs of the DNA strand.

In summary, in this thesis we used the exogenous EGP-2 promoter to achieve specific transgene expression in tumor cells. In addition, we engineered ZFP transcription factors to down-regulate the endogenous EGP-2 promoter to decrease the oncogenic potential of tumor cells. These two approaches therefore present two very different promising approaches for the treatment of cancer. We outline and discuss our most important findings in Chapter 8, followed by an overview in Dutch in Chapter 9.
Scope of this thesis

The aim of this thesis was to improve the efficiency and specificity of cancer gene therapy by targeting EGP-2 positive tumor cells, using two different gene therapeutic methods.

In the first approach, we aimed to achieve selective \textit{exogenous gene expression} by using the tumor-specific activity pattern of the EGP-2 promoter. To accomplish selective tumor cell death by restricted conversion of the pro-drug GCV into a toxic compound, we aimed to restrict the expression of thymidine kinase to EGP-2 positive tumor cells. In order to restrict cell lysis due to the adenoviral replication strictly to EGP-2 positive tumor cells, we aimed to control the expression of an adenoviral replication essential gene by the EGP-2 promoter. Moreover, to further increase the specificity of adenoviral replication, we explored a trans-complementing approach.

In the second approach, we aimed to selectively inhibit \textit{endogenous gene expression} by using engineered transcription factors targeting the endogenous EGP-2 promoter. Down-regulation of EGP-2 expression can ultimately result in a decreased carcinogenesis.

Both approaches can ultimately result in powerful novel therapeutics for the treatment of the most common carcinomas.
References


