Exogenous and endogenous gene regulation for specific and efficient cancer gene therapy
Gommans, Willemijn Maria

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Engineering zinc finger protein transcription factors: The therapeutic relevance of switching endogenous gene expression on or off at command

WM Gommans, HJ Haisma and MG Rots

Department of Therapeutic Gene Modulation, University Center for Pharmacy, University of Groningen, Groningen, The Netherlands

Abstract
Modulating gene expression directly at the DNA level represents a novel approach to control cellular processes. In this respect, zinc finger protein DNA-binding domains can be engineered to target virtually any gene. Coupling of a transcription activation or repression domain to these zinc fingers permits regulating gene expression at will, providing a platform of unlimited therapeutic applications. In this review, steps involved in the engineering of zinc finger protein transcription factors are described. In addition, an overview of endogenous genes successfully targeted for modulating expression by engineered zinc finger protein transcription factors is given. So far, research has mainly focused on targeting genes involved in cancer and angiogenesis, with encouraging evaluation in vivo and progression into a clinical trial. Altogether, engineered zinc finger proteins offer a new and exiting direction in the field of medical research with promising prospects.
Artificial transcription factors

Regulation of endogenous gene expression by specific DNA binding artificial transcription factors offers a new and exiting direction in medical research. Various advantages exist for directly regulating the expression of endogenous genes by targeting the DNA when compared with exogenous gene introduction to upregulate gene expression or when compared with the use of oligodeoxynucleotides (ODNs) or RNA interference (RNAi) approaches to downregulate gene expression. For downregulation of endogenous genes directly at the DNA level, efficiency is likely to be improved as in general only two DNA copies have to be targeted per cell compared to multiple mRNA molecules in RNAi approaches. Moreover, the altered endogenous gene expression profile can be either a temporary modification or a permanent change. In this respect, it has recently been shown that microRNAs might also play a role in direct gene silencing. Direct DNA binding agents do not only provide tools to switch gene expression off, but also allow upregulation of endogenous gene expression.

Generally, induction of gene expression is achieved by exogenous gene introduction. As gene transfer vectors usually have a limited insert size, therapeutic gene introduction is generally confined to the delivery of one splice variant of a gene. However for the proper regulation of cellular processes, expressing all splice variants of a gene in the correct ratio can be of extreme importance. Alternative splicing is a significant phenomenon in nature, and at least one third of the human genes is thought to be alternatively spliced. Induction of endogenous gene expression using artificial transcription factors results in the expression of all splice variants. The importance of the correct stoichiometric expression of all splice variants of a gene was shown for angiogenesis in a mouse model: induction of endogenous gene expression of VEGF-A resulted in more mature vessels when compared with the exogenous introduction of the gene encoding only one splice variant of VEGF-A. Genes encoding such artificial transcription factors are relatively small in size, making it possible to place several of these elements in one vector to achieve modulation of multiple genes and to initiate a whole cascade of gene regulatory events. It is also feasible to target a common DNA sequence for different genes of one gene family. Thus the expression of multiple genes can easily be influenced using either multiple engineered transcription factors or only one.

One of the first steps in regulating endogenous gene expression is the selective targeting of the specific DNA. There are several approaches to target specific DNA-sequences, as reviewed previously. Synthetic single-stranded oligonucleotides are capable of binding to specific purine-rich sequences in the major groove of double stranded DNA, thereby forming a triple helix structure. Also, polyamides have been constructed which can bind specifically to the minor groove of double stranded DNA. In addition, there are naturally occurring DNA-binding domains found in transcription factors that can be engineered to recognize and bind double stranded DNA in a sequence specific manner.

Fusion of any of such DNA binding domain with an activation or repression domain generates promising devices for modulating expression of any endogenous gene; thereby creating agents that can be targeted to predetermined DNA sequences and specifically repress or activate gene expression. The most advanced field in construction of gene-specific artificial transcription factors is based upon modification of the natural occurring DNA binding zinc finger domain. A zinc finger domain recognizes 3-4 base pairs and several domains can be stitched together to result in recognition of an extended
sequence. For example, stitching six zinc finger domains together will result in the recognition of 18-19 base pairs, which can be a unique sequence within the human genome. This modular characteristic makes zinc fingers very attractive DNA binding agents for regulating endogenous gene expression. Several research groups have demonstrated that it is feasible to engineer zinc finger domains recognizing specific DNA sequences within the chromatin context. This review describes pre-clinical achievements made utilizing such engineered zinc finger DNA binding motifs as a tool to activate or inhibit endogenous gene expression, presenting zinc finger protein transcription factors as a new class of potent therapeutics.

The modularity of ZFP transcription factors: flexibility both at the level of DNA binding and effector domains

Zinc finger modules

Zinc finger proteins (ZFPs) are one of the most common DNA-binding proteins in eukaryotes. ZFPs can function in binding RNA\textsuperscript{10} and also participate in protein-protein interactions,\textsuperscript{11} but their major role is the recognition of DNA. The motifs are called zinc finger motifs, because of the folding of a short stretch of amino acids around a zinc-atom in the shape of a finger. One very well explored subset of zinc fingers are the Cys\textsubscript{2}/His\textsubscript{2} zinc fingers (Figure 1). These proteins consist of fingers in which two histidine amino acid residues on the α-helix and two cysteine amino acid residues on the β-sheet interact with the zinc atom. Each individual finger is highly conserved in its structure and consists of around 30 amino acids, built as a ββα fold, held together by the zinc atom. The α-helix is formed at the C-terminal part of the finger, the β-sheet at the N-terminal part. The zinc fingers are arranged around the DNA strand in such a way that the α-helix of each finger contacts the DNA, forming a nearly continuous stretch of α-helices around the DNA-molecule. Interestingly, not the shape but a short stretch of amino acids in the α-helix confer binding specificity for 3-4 base pairs. The primary position of the amino acids within the α-helix interacting with the DNA are at –1, 3 and 6 position relatively to the first amino acid residue of the α-helix.\textsuperscript{12,13} Nevertheless, other amino acid positions can also influence binding specificity by assisting amino acids to bind a specific base or by contacting a fourth base in the opposite strand, causing target-site overlap.\textsuperscript{14} In this respect, several amino acid positions

![Figure 1. Schematic structure of a Cys2-His2 type zinc finger.](image-url)

This zinc finger motif consists of a simple ββα-fold. Amino acid residues within the α-helix recognize the target DNA sequence. (Taken from Uil et al.\textsuperscript{7} with permission from Oxford University Press.)
residues at position 2 within the α-helix have been described to play a role in the DNA recognition as well. Strong evidence of target site overlap caused by position 2 in the α-helix appeared when an aspartate was located at this position, forcing the 5’ position of the previous target DNA triplet to be either a guanine or a thymine.

Thus, when altering the amino acid residues present at the DNA recognition sites, different DNA-sequences will be specifically bound. Consequently, (consensus) zinc finger motifs have been used as scaffolds, in which stretches of known DNA-interacting amino acid residues can be grafted. This results in the recognition of new target DNA sequences without altering the shape of the zinc finger motif itself. In this respect, phage display libraries have been constructed which identified α helix sequences to target ANN, GNN, and a few TNN and CNN triplets. The fingers identified through such library screens are able to discriminate between two closely related sequences, such as GGG and GCG. However, from this database no straightforward code could be revealed connecting amino acid residues in the recognition helix to the targeted DNA triplet. Although in this respect it is worth mentioning that Choo et al could demonstrate a relationship between certain amino acid residues within the α-helix and recognized bases within the target DNA sequence.

Indeed, a non-degenerative code has been suggested by which ZFPs can be constructed to bind to specific DNA sequences provided that at least 3 guanine bases at different positions are available for binding in the first 9 base pairs. Except for targeting the thymidine base, the code is in agreement with reported chemical and stereochemical rules for ZFP-DNA interactions. As the field of engineering ZFPs is rapidly progressing, it is to be expected that all possible triplets can be targeted in the nearby future.

Engineering zinc finger proteins to target any DNA of interest

Stitching several zinc finger domains together will result in the recognition of an extended DNA sequence, with each new attached zinc finger domain adding 3-4 base pairs to the recognized sequence. Since the targeted DNA sequence does not have to be symmetric, ZFP-based DNA binding proteins have an advantage over most other DNA binding motifs derived from natural transcription factors, which bind DNA as dimers. This characteristic makes ZFP transcription factors (ZFP-TFs) very suitable for targeting virtually any DNA sequence.

The concept of ZFP engineering resulting in agents capable of binding to any specific endogenous DNA sequence to regulate transcription was first shown in 1994 by the group of Klug. They constructed three-finger proteins using a combination of phage display and rational design, which could bind to the BCR-ABL fusion oncogene region, which is a chromosomal translocation associated with leukaemia. The selected ZFP could discriminate between the BCR-ABL translocation and genomic BCR or ABL sequence. The potency of the constructed ZFP to downregulate the expression of this oncogenic fusion product was tested in a cell line that was rendered IL-3 independent by integration of a plasmid encoding a p190BCR-ABL oncogene. After introducing the plasmid encoding the ZFP into these cells, 90% of the transfected cells died within 24 hrs after IL-3 withdrawal. As the zinc finger bound within the transcribed region of the BCR-ABL oncogene, the inhibition shown in this study was likely due to obstructing progression of the RNA polymerase.
The ZFP used in this study was not coupled to an effector domain, but in the same paper the zinc finger peptide fused to the activation domain VP16 from the Herpes Simplex Virus was used to stimulate expression of a reporter gene contained in a plasmid by a factor thirty-fold above control. In other, more recent studies, ZFPs have been attached to specific domains that modulate transcription and are generally used to direct these domains to specific endogenous promoter regions. Fusion of such DNA binding domains to distinct effector domains allows modularity on top of the modularity of the individual fingers, providing additional flexibility when engineering ZFP based transcription factors. It is feasible to fuse nearly any desired effector domain to ZFPs generating the appropriate ZFP-based transcription factor of choice (Figure 2). It is this modularity that makes ZFP-based artificial transcription factors extremely promising and flexible devices for the targeted regulation of endogenous genes.

Figure 2. Modularity of ZFP-TFs
It is feasible to couple a wide variety of effector domains to the DNA-binding domain because of the modularity of transcription factors. In addition, the individual building blocks of the DNA binding domain, the zinc finger motifs, are modular in nature and can be stitched together to recognize an extended target sequence.

**Effector domains**

The modular characteristic of natural transcription factors allows natural occurring transcriptional effector domains to be stitched to any engineered ZFP. A commonly used repression domain is the human Kruppel-associated box (KRAB) domain of the KOX-1 protein. This domain is divided in A and B boxes and is highly conserved between eukaryotes. A minimal domain of 45 amino acids in the KRAB-A box is sufficient for the repression of transcription. ZFP-TFs have been constructed utilizing either the complete KRAB repression domain or only KRAB-A boxes to achieve gene repression. Also, the ligand dependent thyroid hormone receptor, or its viral relative vErbA which is a
constitutive repressor, and the mSin3 interaction domain (SID) have successfully been used for repression of endogenous gene transcription. Alternatively, permanent repression can be achieved through methylation by the enzyme histone methyl transferase.

There is also a variety of activation domains that can be coupled to ZFPs to upregulate gene expression, such as the highly acidic portion of the herpes simplex virus protein VP16 and VP64 which consists of four minimal VP16 activation subunits. Another activation domain that has been coupled to an engineered zinc finger domain to activate endogenous transcription is the NF-κB transcription factor p65 domain. The p65 domain contains abundant serine, hydrophobic and acidic amino acid residues, which are essential for its transactivation activities.

In addition, other types of effector domains have also been coupled to ZFPs to modify endogenous gene function, such as chimeric endonucleases to create site-specific double stranded breaks in the DNA to improve the efficiency of homologous recombination in gene correction studies and integrases to improve site specific integration approaches. However, engineered ZFPs are usually exploited in transcriptional regulation of endogenous genes, accomplished by attaching either a repressor or an activator domain to the engineered ZFP.

The specificity of ZFPs in targeting genes within the chromatin environment

The potency of engineered ZFP-TFs as therapeutic agents depends on gene specific binding. Several studies have investigated the specificity of engineered ZFP-TFs in living cells. In a study examining the regulation of the oncogene erbB-2 with engineered ZFP-TF, no alteration in the levels of the related genes erbB-1 and erbB-3 were seen, even though the sequence in the erbB-3 5’-UTR differed in only 3 base pairs compared with the 18 bp target sequence targeted in erbB-2. Similarly, a ZFP-TF engineered to modulate transcription of the erbB-3 gene did not significantly affect erbB-1 or erbB-2 expression, again demonstrating specificity.

For a more high throughput screening of specificity, DNA micro-arrays can be used. To test the specificity of a three-finger protein made to alter the expression of the gene encoding Vascular Endothelial Growth Factor-A, nearly 7500 other genes were surveyed for modulations in expression levels. Of these genes, 51 genes showed an altered expression profile. Some of these genes are possible downstream targets of VEGF-A, the regulation of other genes can be explained by the fact that only 9 base-pairs were targeted in this study, which is probably not unique in the human genome.

The effect of a five-finger protein, constructed to inhibit the expression of the multidrug resistance gene MDR1, was analysed by determining the expression of 2059 genes. An altered expression profile was observed for only 7 genes. Three of these genes (mtn1, CaN19, growth-arrest-specific protein) showed a reduction in mRNA level although this was much less prominent (4- to 6-fold) compared to the MDR1 gene (15-fold). Surprisingly four genes (human mitogen-activated protein-kinase kinase 3b, human integrin α-3, semaphorin E, bcl-xL) showed a 3-fold increase in expression, while the designed ZFP transcription factor contains two KRAB repression domains. The promoter regions of any of these genes did not have a significant consensus with the target site of the ZFP, therefore the altered expression profile of these genes is most likely attributed to downstream effects of the altered MDR1 expression.
Recently, the outstanding specificity of engineered ZFPs has been shown convincingly for a six-finger protein. This ZFP-TF, engineered to inhibit checkpoint kinase 2 (CHK2) expression, did not alter the gene expression of 16,000 genes in two different cell lines. Modulation of CHK2 expression does not have any downstream effects in undamaged cells, therefore the overall gene expression should not be altered except for CHK2. This study demonstrates the remarkable gene specific modulation using engineered six-finger proteins.

**Figure 3. Flow scheme for the construction of ZFP-TFs.**

For the construction of ZFP-TFs, a general flow scheme can be followed. **Step 1.** Determination of the target DNA sequence of the specified endogenous gene. This can be done by determining DNase I hypersensitivity sites (DNase HS), the position of nucleosomes, or by in silico analysis of potential binding sites of natural occurring transcription factors (TF). See text for more detail. **Step 2.** Construction of the ZFP DNA binding domain. In general this can be done by sequential or bipartite selection or modular design. See text for more detail. **Step 3.** Validation of the engineered ZFP within in cell-free systems. EMSA, ELISA, CAST assay or surface plasmon resonance (SPR)/BIAcore techniques can be used to validate the affinity and specificity of binding. **Step 4.** Attachment of the chosen effector domain. See text for more detail. **Step 5.** Validation of the constructed ZFP-TF within the cellular environment. Functionality of the engineered protein can be tested after cotransfection with a plasmid containing a reporter gene under the control of the targeted promoter. The binding of the DNA within the chromatin environment can be investigated using DNase I footprinting and chromatin immunoprecipitation (ChIP). To examine the potency of the engineered ZFP-TF to regulate endogenous gene expression, commonly used techniques are western blotting, flow cytometry and real-time PCR (RT-PCR). To determine the specificity by analyzing a large variety of genes, cDNA arrays can be performed. **Step 6.** Therapeutic potency of the engineered ZFP-TF. The therapeutic potency can be evaluated with end point assays and animal experiments. If in step 3, 5, or 6 the engineered ZFP transcription factors do not fulfill their expectations, new ZFPs will be constructed.

**Engineering ZFP-TFs to regulate endogenous gene expression at command**

ZFP-TFs show great potential in regulating endogenous transcription of potentially any gene. Nowadays, it is feasible to engineer these ZFP DNA-binding domains with an affinity for their target sequence comparable with the affinity for the target DNA of a natural zinc finger protein (within the nanomolar range). For the construction and evaluation of ZFP-TFs often the flow scheme as depicted in Figure 3 is followed. First, the DNA target area of the specified endogenous gene is determined (step 1), followed by the construction of zinc finger proteins to specifically bind within this area (step 2).
engineered ZFPs are validated for affinity and specificity in cell-free systems (step 3) and after coupling to the appropriate effector domain (step 4) for specificity and efficiency in living cells (step 5). Finally, the engineered ZFP-TFs will be tested for their therapeutic potency (step 6). If in step 3, 5, or 6 the engineered ZFP-TFs do not fulfill their expectations, new zinc finger proteins will be constructed (step 2). These six essential steps will be described in more detail below.

**Determination of the target DNA area**

It has been demonstrated that activation of extra-chromosomal reporter constructs with engineered ZFP-TFs is not always comparable to activation of the endogenous gene. For example, a ZFP-TF targeted to the VEGF-A gene caused upregulation of a synthetic reporter gene up to 5-fold but the same transcription factor had no effect on the expression of the endogenous gene. In comparison, another ZFP-TF from the same study could not only induce the reporter gene expression about 3-fold, but also induced endogenous VEGF-A expression up to 5-fold. Therefore, it has been recommended to first determine open chromatin regions before designing a ZFP-TF. Generally, this is done with a DNase I hypersensitivity assay. Besides identifying possible accessible regions in the DNA, DNase I hypersensitivity assays also identify potential regions of active regulatory DNA. Due to histone modifications these regions can be more accessible for ZFP binding.

Although the DNase I hypersensitivity assay does give an indication of open chromatin areas, it does not exclude the presence of other ZFP DNA binding sites. In this respect, there was no DNase I cleavage site detected at a functional ZFP binding position in the endogenous erythropoietin transcription unit. However, chromatin immunoprecipitation indicated enrichment for the ZFP-TF at its target site of the endogenous gene. A second analysis of the promoter region was performed with micrococcal nuclease, which is smaller compared to DNase I and can gain excess to the linker DNA between nucleosomes. The binding region of the ZFP-TF appeared to be within a major region of micrococcal nuclease sensitivity. Therefore, besides DNase I hypersensitivity assays it may also be of importance to determine the position of nucleosomes before selecting the target sequence. Ongoing research in the field will determine the importance of determining open chromatin areas for target definition. In that respect, it has to be noted that several other studies have obtained successful regulation of endogenous gene expression with engineered ZFP-TFs without prior assessment of the accessibility of the targeted DNA sequences. Targeting sequences which were selected in silico to be close to natural binding sites for transcription factors, resulted in successful modulation of expression of the gene of interest.

**Construction of artificial ZFPs**

For the construction of novel target sequence-specific ZFPs, there are several approaches, which are described in more detail elsewhere. Briefly, these engineering methods can be categorized in sequential selection, bipartite selection, and modular design (Figure 3). Sequential selection presents a method in which each finger is selected from a library in context with the pre-selected neighboring finger and therefore takes into account the possible target-site overlap between different fingers. This interaction between different fingers to recognize the same base can result in a synergistic effects in terms of affinity and specificity, however the procedure is very laborious. The bipartite selection method also takes into account the possible target site overlap between the different fingers, but is
substantially less laborious when compared with the sequential selection approach. This designing approach combines two one-and-a-half zinc fingers previously selected from a phage display library to recognize a new target DNA sequence.\textsuperscript{46}

Phage display library screenings have resulted in a lexicon describing the amino acid sequences of DNA recognition helixes and their DNA targets.\textsuperscript{18,19} Based on these data, a PCR method has been described for the construction of novel three-finger proteins\textsuperscript{20} using the consensus framework of the protein Sp1 as a scaffold. This so-called modular design method circumvents laborious screening procedures and can account for known target site overlap events. However the approach does not consider the possibility of yet unknown target site overlap or positional effects of the fingers in the protein, although the latter does not seem to interfere with target specificity.\textsuperscript{17}

Although successful, phage display screening to identify fingers binding the 3-4 base pairs of interest is a laborious process. To circumvent multiple enrichment / amplification cycles, a rapid bacterial two-hybrid system has been developed.\textsuperscript{47} In this system, binding of a three-finger fusion protein to its target sequence causes interaction between two yeast protein fragments subsequently resulting in the expression of genes essential for cell survival. This way, a ZFP library can be rapidly screened as the third finger in the context of two fingers with a known DNA binding sequence. The selected fingers showed comparable if not better affinity and specificity compared to fingers selected by phage display. As described above, the selection of a zinc finger is based on its binding affinity to the target sequence. Subsequently, the novel engineered transcription factor is introduced into the cell to examine the regulation of expression of the desired endogenous gene (Figure 3). This process has been reversed by Barbas III and co-workers.\textsuperscript{48} This group made a library of 3 randomly coupled ZFP domains attached to the activation domain VP64. These 3 ZFPs subsequently served as starting material for a library to construct 6 ZFPs. The libraries were introduced into a retroviral vector to infect cells. The cells were subsequently screened with flow cytometry using antibodies against proteins of interest to select ZFP-TFs that could upregulate the desired gene.

Yet another approach to engineer zinc fingers came from the group of Kim.\textsuperscript{33} Using a modified yeast one hybrid system, different human zinc finger domains were screened to determine the corresponding target DNA sequence. Subsequently, new ZFPs were engineered by stitching different human zinc finger domains together to recognize an extended DNA sequence. This approach has been successfully used to target and activate the VEGF-A promoter by coupling the ZFPs to either the p65 or VP16 activation domain. The engineered ZFP-TFs were able to upregulate the VEGF protein production 13 to 21-fold.\textsuperscript{33}

**Validation of engineered ZFPs as DNA binding domains**

Usually, engineered ZFPs are evaluated for their affinity and/or specificity after construction and before the attachment of the effector domain (Figure 3). The affinity of ZFPs for their target sequence can be measured using an Electromobility Shift Assay (EMSA). This assay is based upon the reduced electrophoretic mobility of a DNA-protein complex compared with unbound DNA. Consequently, the equilibrium constant can be determined by altering the ZFP concentration.

The target site specificity of the constructed ZFPs can be examined using an ELISA or cyclical amplification and selection of targets (CAST) assay with
oligonucleotides in which either one or several bases are altered relative to the target sequence. The same principle is used with the surface plasmon resonance (SPR)/BIAcore. In this system, the engineered zinc finger proteins float over target or non-target oligonucleotides and the signal will be modulated by binding of the engineered ZFP.

Using the modular approach, 80 artificial three-finger proteins have been constructed and validation with ELISA showed that 79 bound to their target sequence and only in five cases a high affinity for another sequence was demonstrated. Using the degenerate code, ten three-finger proteins were constructed of which five bound to their predicted target sequence with high affinity, as was determined using EMSA. Interestingly, assembling two non-functional three-finger proteins resulted in binding of their target sequence.

The next step after construction and validation of the desired DNA-binding domain is to attach a nuclear localization signal and the chosen effector domain (Figure 3) and subsequently validate the engineered ZFP-TF.

Validation of engineered ZFP-TFs

The constructed ZFP-TF should subsequently be validated within the cellular environment (Figure 3). To determine the efficiency of gene expression modulation, a plasmid encoding the ZFP-TF can be co-transfected with a plasmid containing the targeted promoter region and a reporter gene. The proper binding of the ZFP-TF within the chromatin environment can be investigated using DNase I footprinting and chromatin immunoprecipitation. To evaluate the efficiency and specificity of the engineered ZFP-TF to target endogenous DNA sequences, commonly used techniques are real-time PCR, flow cytometry, and western blotting. Another, more high through put measurement of the specificity of the engineered ZFP-TF is the DNA micro-array. This assay has clearly demonstrated the outstanding specificity of engineered ZFP-TFs as has been described above. The above-described techniques are performed to detect a-specific and non-functional gene expression modulation. Although the transcription factor binds specific to the target DNA, this might not result in an effect. This has been explained by the context dependency of the effector domain. In this respect, a phage display library of three-ZFPs was screened for binding against a 1.4 kb fragment of the erbB-2 promoter. The bound ZFPs were isolated and subsequently attached to either the activation domain VP64 or the KRAB repression domain. Five of the ten constructed ZFP-TFs could upregulate erbB-2 expression over two-fold and two could downregulate expression over two-fold. Surprisingly, only one ZFP-TF could upregulate as well as downregulate erbB-2 expression depending upon the attached effector domain. This study clearly indicates that the binding position of the zinc finger is important in determining whether the attached effector domain is capable of influencing transcriptional activity. Subsequently, based upon the target-site of the ZFP that could cause up as well as downregulation depending on the attached effector domain, three six-finger proteins were constructed to increase the specificity of binding. Only one of these engineered transcription factors could both down- and upregulate erbB-2 transcription depending on the attached effector domain, resulting in an approximately 10-fold alteration in protein production as measured by flow cytometry.

Comparison of different repressor domains or different activator domains when fused to the same DNA binding domain also demonstrated context dependency. These studies show that in addition to constructing gene-specific ZFP-TFs, the choice of effector
domains might also be critical in determining the outcome of gene expression modulation approaches.

**Therapeutic gene expression modulation**

After validation of the engineered ZFP-TF, the constructed protein is further explored for its therapeutic potency (Figure 3). Interestingly, animal experiments have reported successful therapeutic gene expression modulation in two different animal models. The following sections will describe the findings of regulating endogenous genes in more detail.

**Regulation of genes involved in cancer**

Artificial ZFP-TFs present a novel platform for the treatment of many diseases, such as cancer. Cancer is known to be a pathophysiological condition with many different genes involved. Treating cancer may therefore require the simultaneous regulation of different genes, which can be achieved by using ZFP-TFs: When a cluster of genes has the same transcription factor binding-site, one ZFP-TF can be targeted towards this site. The attached effector domain can subsequently cause either inhibition or activation of transcription. One single ZFP-TF can therefore modulate the expression profile of many different genes, generating a very powerful tool in the fight against cancer. On the other hand, several ZFP-TFs can be delivered simultaneously by placing the small genes in one vector. It appears that modulating the expression of one single gene can also be sufficient to result in a cellular effect as was demonstrated by a hybrid partially engineered transcription factor to upregulate the expression of p21.

In addition, six-finger proteins were engineered to target a DNA sequence in the 5' untranslated region (5'-UTR) of the oncogene erbB-2. Overexpression of this gene is correlated with a poor prognosis in cancer. The ZFP DNA-binding domain was constructed by linking 6 zinc finger domains together. Subsequently, an effector domain was coupled to the designed ZFP, either the activator domain VP64 or the repressor Kox-1 KRAB domain. The ZFP-TFs were introduced into the cell by means of retroviral vectors containing genes encoding the ZFP-TFs. The zinc finger coupled to the VP64 activator domain was able to induce a clear upregulation in erbB-2 expression in 27% of the cells as measured by flow cytometry. When the same ZFP was linked to a KRAB repressor domain, 59% of the infected cells showed a drastic repression in erbB-2 expression rendering these cells essentially erbB-2 negative. Downregulation of overexpressed erbB-2 genes is known to result in inhibition of cell cycle progression. Indeed, therapeutic applicability of downregulation of erbB-2 expression was indicated by an accumulation of infected cells in G1 phase.

Also for the erbB-3 gene, ZFP-TFs were engineered resulting in either up- or down regulation of endogenous gene expression. A six-finger protein recognizing the erbB-3 5'-UTR was coupled to either the activating VP64 domain or the KRAB repressor domain. The zinc fingers were separately introduced into cells using retroviral infection. Flow cytometry revealed that erbB-3 expression was abolished in 74% of the infected cells when the zinc finger was attached to the KRAB domain. On the other hand, when the same ZFP was attached to the VP64 domain a nearly 8-fold increase in erbB-3 expression was noticed in 48% of the infected cells.
As shown in the above-described study, it is possible to alter a disturbed gene expression pattern utilizing ZFP-TFs and thereby providing a new approach to tackle cancer. There are numerous other genes that can also be a target for cancer therapy. In this respect, an engineered ZFP-TF was constructed to alter the expression of the multidrug resistance (MDR1) gene. This gene encodes for the P-glycoprotein, which is capable of transporting anti-cancer drugs out of the cell, causing resistance to chemotherapy. To downregulate endogenous MDR1 expression, a hybrid ZFP was constructed. This ZFP consisted of two zinc finger domains of the human Sp1 transcription factor, one zinc finger domain of murine Zif268 and two newly synthesized domains. This 5-finger protein could bind to the EGR1/SP1/WT1 site present in the MDR1 promoter. However, binding of this ZFP without an attached repressor domain only moderately inhibited the activity of a synthetic MDR1 promoter. To increase the inhibitory effect, this ZFP was linked to two KRAB-A domains resulting in 22-fold repression of a synthetic MDR promoter, as measured by luciferase activity. Moreover, this ZFP-TF was able to inhibit endogenous expression of the P-glycoprotein, as analysed by flow cytometry. However, it did not inhibit the expression of α5β1 integrins (of which the promoters contain SP1 sites), thereby providing a novel specific approach to prevent multidrug resistance.

In a subsequent study, the possible therapeutic effect of inhibiting endogenous expression of the MDR1 gene by this constructed ZFP-TF was evaluated. A breast carcinoma cell line that highly overexpressed P-glycoprotein was stably transfected with DNA encoding the ZFP-TF described above. The expression of this zinc finger transcription factor could be tightly regulated using an inducible promoter. When this five-finger protein was expressed, a significant reduction of 90% or more in P-glycoprotein expression was seen both by northern and western blotting and by flow cytometry. In addition, an increased rate of a P-glycoprotein substrate accumulation inside the cell was demonstrated. Indeed a substantial rise in cytotoxic effects of the anti-tumor drug doxorubicin in a dose response profile was seen, causing a 1-log increase in drug sensitivity after the introduction of the ZFP-TF.

The tumor suppressor gene p53 is an important target in cancer research. This gene is the most frequently mutated gene in cancer cells. One of the many targets of p53 is the gene Bax. The Bax protein can induce the release of cytochrome c from the mitochondria, which will lead to caspase-dependent apoptosis. A ZFP-TF was constructed to upregulate the expression of Bax and therefore indirectly induces apoptosis when p53 is not functional anymore. This engineered ZFP-TF consisted of 5 modular zinc finger domains, three domains from the naturally occurring transcription factor Zif268 and two newly synthesized domains, attached to the activation domain VP16. To investigate if the newly synthesized ZFP-TF was able to selectively activate the Bax gene, the gene expression of another p53 target gene, named p21, was determined as well. An EMSA study showed binding to the Bax promoter region and not to the p21 promoter region. After transiently transfecting cells with a plasmid encoding the designed ZFP-TF, a moderate upregulation in Bax but not in p21 expression was seen, as was detected by western blotting. This upregulation resulted in reduced cell viability in 65% of p53 deficient cells, as was determined by counting the remaining transfected cells.

A cellular regulator of p53 activity is checkpoint kinase 2 (CHK2). This protein plays a role in cell–cycle progression, DNA damage and cell death and is therefore a possible therapeutic target in cancer treatment. Tan et al studied the repression of CHK2
transcription by engineered ZFP-TFs.\textsuperscript{38} The human CHK2 promoter was mapped to find accessible regions in the gene. A six-finger protein that could bind within this region was subsequently coupled to the KRAB repression domain. This transcription factor could generate a 10-fold reduction in CHK2 mRNA levels in two different cell lines, which resulted in barely detectable protein levels as shown by immunoblotting. Also a loss of DNA-damage-induced CHK2 dependent p53 phosphorylation was seen.\textsuperscript{38}

The above-described approach of interfering with the gene expression profile of cancer cells can be extended to include not only tumor cells, but also endothelial cells for ZFP-based therapy. Inhibiting angiogenesis by targeting the tumor endothelium will result in the depletion of nutrients and oxygen for tumor cells. The combination of ZFP-TFs interfering with the gene expression profile of both the tumor cells as well as the tumor endothelium cells can therefore provide a powerful approach for the treatment of cancer.

An important gene for angiogenesis is vascular endothelial growth factor A (VEGF-A). Generally, this gene is upregulated during hypoxia to induce neovascularisation. In cancer patients, upregulation of VEGF-A is associated with a poor prognosis. Down-regulation of VEGF-A in tumor tissue therefore will cause a decrease in tumor vessel growth, which subsequently will result in a depletion of nutrients to the tumor cells.

ZFPs constructed in a study from Liu et al (see below) were used for downregulation of VEGF-A expression.\textsuperscript{2} Each protein consisted of three zinc finger DNA-binding domains coupled to the repressor domain vErbA. In a transient transfection study, each of these ZFP-TFs was capable of inhibiting VEGF-A expression down to 50% of the mRNA amount when compared with mock-transfected cells. To eliminate the influence of untransfected cells, a stable inducible cell line was constructed for one of the ZFP-TFs. After induction, this expressed ZFP-TF could strongly repress VEGF-A protein production for about 80%. Also in a stably transfected highly vascularizing tumorigenic cell line a 20-fold reduction in VEGF-A protein expression was detected after induction of the ZFP-TF. The reduction seen in this cell line brought the VEGF-A protein level back to the level observed in a non-tumorigenic cell line.\textsuperscript{2}

This group also conducted experiments with the same zinc fingers coupled to another repression domain, the minimal catalytic domain of the enzyme histone methyl transferase.\textsuperscript{30} Repression of endogenous VEGF-A expression was accomplished through local methylation of histone H3 and was comparable to repression accomplished by the v-ErbA domain as was measured by quantitative RT-PCR.\textsuperscript{30} The methylation of histone H3 marks heterochromatin-dependent gene silencing. This report therefore presents an alternative approach to repress gene expression in which the transient expression the engineered ZFP-TF can result in a long-term repression of gene transcription due to the induction of gene silencing.

\textit{Regulation of genes playing a role in angiogenesis}

The modularity of ZFP-TFs allows swapping between effector domains and ZFP DNA binding domains. One artificial ZFP can therefore be utilized for either the induction or repression of the same gene depending upon the attached effector domain. In this respect, the process of angiogenesis is a good model to study the therapeutic effects of repression or induction of endogenous gene expression; because apart from inhibiting the development of blood vessels for the treatment of cancer, it is important to stimulate blood vessel formation for the treatment of other diseases, like peripheral vascular disease.
To induce upregulation of endogenous VEGF-A expression, three-finger proteins were engineered to bind to accessible regions in the VEGF-A promoter, which were determined by DNase I hypersensitivity mapping. The constructed zinc-finger DNA-binding domains were coupled to the activation domain VP16 or p65. These ZFP-TFs increased VEGF-A mRNA expression up to 10-fold as was measured by quantitative RT-PCR, resulting in up to 15-fold enhancement in VEGF-A protein expression as was determined by ELISA. This ZFP-TF-induced upregulation exceeded levels induced by hypoxia. Importantly, hybridisation experiments showed that all the functionally different splice variants of the VEGF-A gene proportionally increased after ZFP-TF induced upregulation of VEGF-A expression.

ZFP-TFs developed in the previous study were further utilized to test whether these transcription factors could also induce transcription of the VEGF-A gene in vivo. To achieve this, genes encoding ZFP-TFs fused to a VP16 activation domain were introduced in recombinant adenoviral vectors, which were injected in the quadriceps of mice. A 3-fold increase in the expression of VEGF-A, measured by RT-PCR was reported, resulting in an improved experimental wound healing. In addition, formation of new mature blood vessels was seen in the ears of mice after subcutaneous injection. To assess the specificity of this ZFP-TF construct in vivo, the expression profile of several angiogenesis related genes were determined. A 2-fold increase in mRNA expression was shown for VEGF-D, but not for VEGF-B, C, or angiopoietin. This increase in VEGF-D mRNA may be due to partially overlapping sequences of the VEGF-D promoter with the target sequence, or indirectly caused by the increased VEGF-A expression.

The neovascularization after the activation of endogenous VEGF-A by the zinc finger constructs was compared with the neovascularization after introduction of VEGF-A164 cDNA. Although the mice ears treated with an adenovirus expressing VEGF-A164 showed induced vessel growth, the vessels were hyperpermeable, as was detected by Evans blue dye infusion. On the contrary, the vasculature in the mice ears treated with an adenovirus containing the zinc finger construct was not permeable for Evans blue dye. This suggests that modulating endogenous VEGF-A induces the formation of more mature vessels, showing the importance of inducing all splice variants.

The same ZFP-TF was subsequently investigated in a clinically relevant rabbit hind limb ischemia model. After intramuscular injection of the plasmid encoding the ZFP-TF an increase was seen in the mRNA level of all splice variants compared with animals treated with empty plasmids. Also, the capillary density increased almost 2-fold, and blood flow increased also around 2-fold in animals treated with the VEGF-A ZFP-TF at all measured time points.

Currently, phase II clinical trials are performed with (adenoviral) delivery of a gene encoding one VEGF-A isoform with limited success. The potency of ZFP-TFs to induce all isoforms of VEGF-A resulting in the development of mature vessels as shown in two different animal models provides therefore a promising new therapeutic treatment option. A clinical trial has been approved to investigate an engineered ZFP-TF for the induction of VEGF-A in cardiovascular disease.

Also other genes playing a role in angiogenesis have been targeted with engineered ZFP-TFs such as the vascular endothelial cadherin protein (CDH5). CDH5 plays a role in the permeability of the endothelium and selected ZFP-TFs were able to induce expression up to 80-fold. Subsequently the binding sites of these ZFPs in the
promoter region of the CDH5 gene were determined. Flow cytometry results showed that one three-finger protein was capable of upregulating CDH5 transcription in all 5 different cell lines tested. Similarly, four different six-finger proteins were selected that could alter the expression of the intercellular adhesion molecule (ICAM-1). Of these four, one was further optimized to increase the specificity. When this engineered zinc finger domain was coupled to the VP64 activation domain it could increase ICAM-1 expression up to 135-fold, depending on the cell line tested or repress ICAM-1 expression down to 3-23% when coupled to the repression domain KRAB. Interestingly, the induced expression utilizing this ZFP-TF exceeded the degree of induction of ICAM-1 expression by naturally occurring cytokines. Moreover, when coupled to the repression domain KRAB this ZFP-TF was capable to completely repress ICAM-1 expression after cytokine-induced upregulation. The alterations in ICAM-1 expression was specific, as the expression of 7 other endothelial cell markers determined in two different cell lines were not affected by this ZFP-TF.

**Activation or repression of other genes within the chromatin environment**

Although most research on therapeutic applications for artificial ZFP-based transcription factors is currently focussed on cancer and (cancer related) angiogenesis, this technology is essentially applicable for activation or repression of any gene for the treatment a broad spectrum of diseases. For example, a three-finger protein coupled to the VP16 domain has been engineered to activate the expression of the erythropoietin gene (EPO). This protein controls the biogenesis of erythrocytes. In a stably transformed cell line, expression of the engineered zinc finger resulted in an increase in EPO from 0 mU/ml up to 200 mU/ml as detected by ELISA.

Another ZFP-TF has been utilized to induce expression of γ-globin. The induction of the normally silenced fetal γ-globin can alleviate symptoms of sickle cell disease by forming fetal hemoglobin and therefore compensating for the mutated β-globin gene. The constructed ZFP-TF consisted of six zinc finger domains attached to the activation domain VP64. This transcription factor was able to induce γ-globin expression up to 14-fold after retroviral infection. Interestingly, the same engineered ZFP-TF was able to repress γ-globin expression after attachment of the KRAB repression domain up to 90% after retroviral transduction. This ZFP-TF presents a new and promising approach for the treatment of sickle cell disease. Engineered ZFPs can be utilized for many different applications. Also modulation of the expression of epigenetically silenced genes is feasible, as was demonstrated by Jouvenot et al. Using mouse-human cell hybrids containing one human chromosome of either paternal or maternal origin, RNAse protection assays could detect ZFP-TF-induced upregulation of the paternal repressed H19 gene or the maternal repressed IGF2 gene. Yet again, this shows the broad applications possible for engineered zinc fingers to modify endogenous gene expression.

Besides targeting potential therapeutic endogenous genes, engineered ZFP-TFs can also be powerful tools in fundamental research. In this respect, the regulation of the endogenous gene PPARγ has been investigated. This gene consists of the two splice variants γ1 and γ2 and plays a role in adipogenesis. The amino terminus of splice variant γ2 is regulated from a different promoter. In order to study the regulation in more detail, two 6-ZFPs coupled to the repression domain KRAB were targeted to open areas in this promoter, as determined with DNase I digestion. The first ZFP-TF was capable to almost
completely inhibit the induced upregulation of both splice variants, as shown by western blot and quantitative RT-PCR. In contrast, the second zinc finger was capable to selectively inhibit splice variant γ2 for approximately 50%. In a subsequent experiment, the first ZFP was used to knock down the expression of PPARγ after which either one of the splice variants were reintroduced to determine the role of this splice variant in adipogenesis. This study very elegantly shows the utility of zinc fingers as a tool in fundamental research.

Engineered ZFP-TFs have also been used to control the fate of stem cells. A six-finger protein linked to a KRAB repression domain was engineered to target the promoter of the Oct-4 gene. This gene is known to play a role in stem cell differentiation. By inhibiting the expression up to three fold as was measured by quantitative RT-PCR, it was possible to change the expression profile of downstream genes and alter the morphology of stem cells. These studies indicate the broad applicability of ZFP-TFs in many different cell types.

Concluding remarks

Several classes of DNA-binding agents are exploited for therapeutic gene modulation. The targeting of DNA represents several advantages over targeting of RNA, which can be achieved by promising approaches such as RNAi: Generally, DNA-binding agents need to bind only two copies per cell, can cause repression as well as induction of gene expression and several genes can potentially be modulated. In this respect, engineered ZFP-TFs are currently the most advanced and can be designed to target unique sequences in the human genome. The modularity of ZFP-TFs allows the attachment of a wide variety of effector domains, causing temporary or permanent changes in gene expression, and being constitutively active or inducible.

The DNA-binding specificity of an engineered six-finger protein in a study targeting the CHK2 gene was shown to be outstanding because of the 16,000 genes surveyed, only the gene expression profile of the CHK2 gene was altered. Nevertheless, it has to be noted that other studies demonstrated less specificity, possibly due to downstream effects or because the targeted sequence was not unique within the genome. However, the study targeting the CHK2 gene indicates that engineered ZFP-TFs might be more specific compared to other regulators of gene expression, including RNAi. Because RNAi can induce an interferon response, engineered zinc finger proteins can be a more safe class to regulate endogenous gene expression. The amount of genes that can be regulated by engineered ZFP-TFs seems to be unlimited. Many endogenous genes have already been targeted to achieve selective up- or downregulation and two different animal models have shown convincing therapeutic efficacy.

In addition to the above-described advantages of ZFP-TFs, the size of these artificial transcription factors is very small. This allows the incorporation of multiple genes encoding (different) transcription factors in one gene transfer vector. Generally, the described studies employ both viral and non-viral vectors to deliver the gene encoding the engineered ZFP-TFs into cells. Depending of the therapeutic goal, the appropriate vector can be selected. When a prolonged expression of the engineered ZFP-TF is required for therapeutic effects, a vector can be chosen which causes integration of the ZFP-TF gene into the genome of the host cell. On the other hand, when a temporary effect of the ZFP-TF is sufficient, insertion into the genome of the host cell is not essential. Importantly, besides the delivery of genes encoding ZFP-TFs, the transcription factors can also be efficiently delivered as proteins.
The addition of protein-transduction domains (PTD)\textsuperscript{64} to a designed ZFP-TF facilitates transport over the cell membrane. Without the attached PTD, the ZFP-TF was not capable of crossing the cell membrane. Therefore, protein as well as gene therapeutic approaches are feasible for the delivery of ZFP-TFs.

In conclusion, modulation of endogenous gene expression represents a novel promising approach for the treatment of many different types of pathological conditions. In this respect, ZFP-TFs are flexible tools for the specific regulation of endogenous genes. ZFP-TFs have many possible applications, they can be used for fundamental research, development of bioassays used for drug discovery\textsuperscript{65} and therapeutic applications. This makes these engineered proteins very valuable and promising tools in the field of biomedical research.

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