Epigenetic Editing as a novel approach to modulate expression of key genes in cancer

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Chapter 1

General introduction

Epigenetics

A classic definition of epigenetics is "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" [1]. In 2009, a more complete definition proposed that "An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence"[2]. In fact, all cells of a complex multicellular organism contain the same genetic information but during development, each single cell differentiates into (and remembers) a specific phenotype without any changes in DNA sequence. This feature of epigenetics implies that the accuracy of epigenetic modifications is vital for maintaining the genome integrity and the cell phenotype. Aberrant epigenetic modifications are associated with different heritable (e.g. imprinting disorders and some cancers) and non-heritable diseases (e.g. most cancer types). Indeed, epigenetics contributes to the understanding of mechanisms underlying different diseases for which genetic mutations are not the (only) cause. Among the possible epigenetic modifications, DNA methylation and histone modifications are the most intensively studied by epigenetic researchers for unraveling their role in gene expression regulation and their involvement in diseases.

DNA methylation

Methylation of DNA occurs at the 5'-carbon of a cytosine, commonly (but not exclusively) when preceding guanine (CpG). CpGs as targets of DNA methylation can be clustered in CpG rich regions (CpG islands), be scattered in regions with less condensed CpGs around the CpG islands (CpG shores) or be found as individual CpGs. DNA methylation is catalyzed by DNA methyltransferase enzymes (DNMTs): DNMT1 methylates hemi-methylated DNA (maintenance DNA methylation), DNMT3A and DNMT3B generate new DNA methylation marks and are necessary for DNA methylation patterns in early development (de novo DNA methylation) [3]. DNA methylation is considered as an important factor in the regulation of gene expression and dependent on the location, it was found to be linked to the gene expression regulation [3, 4].

In contrast to what was previously thought, DNA methylation is not a stable epigenetic mark which can only be reversed by inhibiting the maintenance enzyme during cell divisions (passive DNA demethylation): DNA methylation

can be reversed in an active manner (active DNA demethylation). In fact, DNA demethylation is essential for maintaining the balance of the DNA methylation level throughout the genome. One mechanism of active DNA demethylation involves the ten-eleven translocation (TET) family of enzymes which oxidize 5-methylcytosine (5mC) to 5-hydroxymethyl cytosine (5hmC). It is suggested that TET enzymes can mediate oxidation of 5mC to 5hmC and, then to 5-formylcytosine (fC) and 5-carboxylcytosine (caC), which might be converted to C by a decarboxylase. Another proposed mechanism of active DNA demethylation is by Thymine DNA glycosylase (TDG) which can initiate base excision repair (BER) of such intermediate modified cytosines, and of spontaneous deaminated 5mC [5]. There might be other mechanisms for active DNA demethylation by enzymes like activation-induced deaminase (AID) which deaminates 5mC or 5hmC, or Gadd45a, but this is still under debate [6, 7]. In addition to the DNMTs (writers) and DNA demethylases (erasers), there are proteins which can bind to the DNA methylation (readers) and recruit other factors and enzymes.

Histone modifications

The human genome contains around 3 billion base pairs (bp) of DNA which are organized in 23 chromosomes. The vast majority of our cells are diploid, therefore each cell with 46 chromosomes contain 6 billion bp of DNA. By considering the length of each base pair, we have approximately 2 meters of DNA in each diploid cell; interestingly, this long DNA is compacted and condensed in the nucleus of each cell. Histone proteins are responsible proteins to organize such long DNA into the nucleus.

Histones are small proteins with a positive charge, the main types of histones involved in compacting DNA are H1, H2A, H2B, H3, and H4; however, there are histone variants as well which have their own functions. DNA is negatively charged and wrapped around histone proteins. About 146 bp of DNA which is wrapped around dimers of H2A, H2B, H3, and H4 together with the linker DNA and H1 make up the nucleosome. Each chromosome consists of thousands of nucleosomes.

Histones have protruding N-terminal tails which can posttranslationaly undergo chemical modifications (so called histone modifications/marks). There are various types of modifications including acetylation (ac), methylation (me),

phosphorylation (P), ubiquitination (ubi), and SUMOylation (SUMO). According to the nomenclature for describing histone modifications, first the histone is named, then the modified amino acid residue along with its position in the protein is added, and finally the type of modification with a number showing the amount of the modification (only 'me' can be added more than 1 group per residue) [8]. Two well-investigated histone modifications are histone acetylation and histone methylation which are the consequences of addition of acetyl and methyl groups mainly to arginine (R) and/or lysine (K) residues of histone tails[9]. Histone modifications are dynamic and reversible; there are several epigenetic modifier enzymes (epi-enzymes) which are specifically responsible for adding (writers) or removing (erasers) histone modifications[10]. In addition, there are some proteins which can detect and bind to the epigenetic modifications (readers); they either have enzymatic activity or they only recruit the related enzymes to spread or change the epigenetic modification pattern of genes [11].

Histone modifications, depending on the residue they occur on, the type of modifications (chemical groups), and the number of modifications are associated with active or repressed state of genes [9].Indeed, certain histone modifications can be used to predict gene expression [12].However, since such findings are mainly derived from associative studies, the direct and instructive roles of histone modifications in gene expression is still under debate [13].

Epigenetics and cancer

Aberrant epigenetic modifications (epi-mutations) are implicated in initiation and/or progression of a broad range of diseases including cancer. Although research on cancer as a complex disease focused on genetic mutations for many years, it is now apparent that also epi-mutations contribute to cancer. The findings of aberrant global DNA hypomethylation and also observing DNA hypermethylation of promoters of silenced tumor suppressor genes in cancer[14] led to further investigation of the epigenetic features of cancer which still continues. Unraveling the link between aberrant DNA methylation and cancer stages seems to be promising in view of defining clinical applications for DNA methylation, for example, developing new diagnostic markers [14-16].

Epi-mutations in cancer are not limited only to the aberrant DNA methylation, various aberrant histone modifications including altered levels of histone

acetylation and/or histone methylation are associated with cancer [17, 18], and knowing the pattern of histone modifications might have clinical applications in cancer [19]. Epi-enzymes are also frequently found to be genetically or epigenetically mutated in several types of cancer which, in turn, cause epi-mutations [4, 20, 21].

Epigenetics and cancer therapy

Epigenetic modifications are potentially reversible and therefore the writers/erasers are considered as attractive targets in cancer therapy. Several epigenetic drugs (epi-drugs) have been developed and validated to inhibit epi-enzymes. Epi-drugs are mainly DNA methyltransferase inhibitors (DNMTis) and histone deacetylase inhibitors (HDACis). Recently, new epi-drugs for inhibiting histone acetyltransferases and histone methyltransferases have been developed and are being tested [22].

Several DNMTis and HDACis are currently Food and Drug Administration (FDA) approved and many more have entered clinical trials. Approved epi-drugs are used for treatment of hematological malignancies [23], but also treatment of solid tumors with epi-drugs is progressing [22, 24].

Epi-drug treatment potentially results in removal of the repressive epigenetic modifications which can lead to gene upregulation. In fact, upregulation of tumor suppressor genes is one of the mechanisms by which epi-drugs are thought to be beneficial in cancer therapy. The function of epi-drugs, however, is inevitably genome-wide which can have severe consequences such as upregulation of prometastatic genes[25,26]. In addition, epi-enzymes can also modify non-chromatin proteins. As a consequence, epi-drugs not only inhibit the aberrant writing or erasing of epigenetic modifications, but also affect unintended cellular pathways[27,28]. Besides epi-drugs which inhibit the epi-enzymes, a more specific approach is Epigenetic Editing which targets specific epigenetic modifications instead of inhibiting epi-enzymes.

Epigenetic Editing

Epigenetic Editing is the approach of writing or erasing epigenetic modifications at the gene of interest and modulate its expression. An Epigenetic Editing tool consists of a DNA binding domain coupled to an epigenetic effector domain. Epigenetic effector domains are the catalytic domains of epigenetic enzymes

which are used to write or erase epigenetic modifications. The DNA binding domain is designed to bind the target gene, so the epigenetic effector domains can change the given epigenetic modifications at the target gene, with the ultimate goals of down- or upregulation of expression of the gene of interest.

Targeting a gene directly at the DNA level to induce downregulation is more advantageous than targeting the protein or the RNA. Effective inhibition of proteins or RNAs requires continuous administration of their targeting tool/drug, whereas DNA targeting silences the source of expression. Moreover, only two copies of DNA need to be targeted compared with several (potential spliced isoforms of) RNAs or (potential diverse) protein molecules. With respect to upregulation, targeting DNA results in a more natural upregulation of the gene (all isoforms in natural ratios) than administration of ectopic cDNA, which results in (over)expression of only one isoform of the target gene.

Modulation of genes has been shown for several genes using artificial transcription factors (ATFs) which target genes at DNA level [29]. ATFs are composed of DNA binding domains fused to transcription effector domains. Transcription effector domains can be transient transcription repressor domains (e.g. super krab domain [SKD]) or transcription activator domains (e.g. the tetramer of herpes simplex virus protein VP16 [VP64]). Several DNA binding domains have been developed and investigated including triplex forming helix (TFO), zinc finger proteins (ZFPs), transcription activator-like domains (TALEs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) [29].

In this thesis, we used ZFPs as DNA binding domains. ZFPs were designed as 6-finger ZFPs where each single finger can recognize and bind to three base pairs of the target DNA, therefore 6-finger ZFPs potentially recognize 18 base pairs which is mathematically unique in the genome [30]. We used ZFPs as a part of our Epigenetic Editing tools or ATFs to target and modulate HER/neu, ESR1, and EPB43L1 genes. ATFs have been successful in modulating expression of several genes, but their effect on gene expression seems to be transient. The presence of the ATF at the target gene is necessary for gene modulation. When the ATF is bound to the gene, the transcription effector domain is able to recruit transcription activation or repressing factors to cause gene expression modulation. Upon

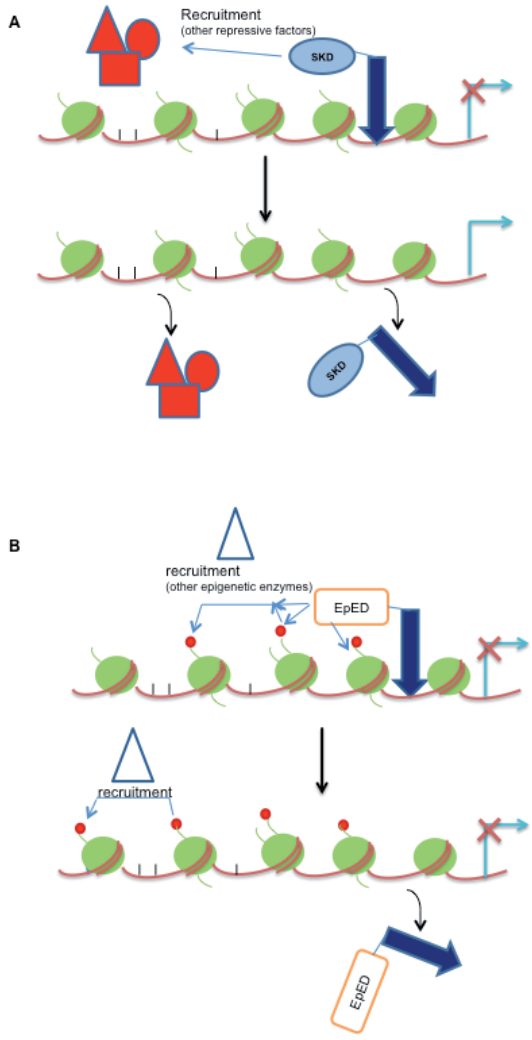

Figure 1. Epigenetic Editing effect on gene repression. A part of a gene is represented which is targeted by the ZFP (blue arrow). Histones (green circles) with their tails and CpGs (black short lines) are shown. (A) SKD domain (blue oval) fused to the ZFP binds to the target site. Upon its binding, it recruits other transcriptional repressive factors (geometric figures in red) and thus results in gene downregulation (Upper part), if the ZFP fused to SKD dissociates, there is no further recruitment and thus no downregulation (lower part). (B) An epigenetic effector domain like G9a or SUV39-H1 (shown as EpED in the rectangle) fused to the ZFP targets the gene. Upon binding to the gene, G9a or SUV39-H1 inducing methylation of H3K9 (methylation are shown as red small circles on the tails of histones) results in gene downregulation. G9a or SUV39-H1 and/or the induced H3K9 methylation marks are able to recruit other epigenetic enzymes (triangle) which might spread the methylation (upper part). When the ZFP fused to G9a or SUV39-H1 dissociates, the induced methylation marks might remain and continue recruiting the epigenetic enzymes which results in a sustained gene downregulation (lower part).

absence of the transcription effector domain, however, the former pattern of gene expression is likely to be restored [31] (Fig.1a). By contrast, the epigenetic effector domain of an Epigenetic Editing tool changes the epigenetic modifications at the target gene. Because of cellular epigenetic maintenance processes, edited epigenetic modifications are more likely to remain on the DNA or histone tails, even after removal of the Epigenetic Editing tool, therefore if written epigenetic modifications cause any gene expression modulation, this effect is expected to improve sustainability (Fig.1b). Moreover, written epigenetic modifications can spread along the target gene [32, 33] (Fig.1b) which can be due to the interaction of these modifications with epigenetic enzymes [34-36]. Written/erased epigenetic modifications are also potentially inherited to the next cell generations [37]. So, the effect of Epigenetic Editing seems to be more stable on gene expression than the effect of ATFs.

In this thesis, we aimed to use the Epigenetic Editing approach to write repressive epigenetic modifications on the target genes in order to modulate their expression. Epigenetic effector domains used in this research were catalytic domains of histone methyltransferases (G9a, Suvdel76) and a DNA methyltransferase (M.SssI). Using this approach we intended to write repressive epigenetic modifications (H3K9me2, H3K9me3, DNA methylation) and downregulate expression of target genes.

Aim of this thesis

The main goal of this thesis was to write epigenetic modifications on target genes and modulate their expression. The target genes of this research play crucial roles in cancer. We also exploited ATFs for modulating expression of our target genes. The effect of modulated genes were assessed in cancer cell growth.

In **chapter 2**, the importance of epigenetic mechanisms in breast cancer as well as current and potential treatment approaches are reviewed. In this review, by assessing advantages and disadvantages of the current epigenetic therapy, we introduce the innovative technology of Epigenetic Editing and we suggest that in the future, Epigenetic Editing technology might have the potential to be considered as an interesting alternative or an option for combination treatments.

In **chapter 3**, we produced Epigenetic Editing tools targeting the HER2/neu gene. Induction of histone methylation in a targeted manner was achieved, for the

first time, in 2002 [32] and there has not been any further follow up on it, therefore we set out to validate the approach for the HER2/neu gene [38]. In addition, we investigated whether the induced histone repressive modifications on the HER2/neu gene resulted in a downregulation of HER2/neu and whether the approach was efficient enough to result in less cell growth. Validation of binding of the ZFP to the target gene using genome-wide analysis is another interesting part of this research.

In **chapter 4**, we extrapolated the gene-targeting approach to up and downregulate the ESR1 gene in order to tune ESR1 expression levels in breast and ovarian cancer cells. We downregulated ESR1 in ER-positive cancer cells to investigate its role in cell growth and upregulated ESR1 in ER-negative cells for endocrine re-sensitivity purposes. Association of epigenetic modifications with gene expression led us to induce repressive epigenetic modifications on ESR1 using Epigenetic Editing technology.

In order to re-express silenced EPB41L3 gene in cancer cells, we used ATFs and epi-drugs in **chapter 5**. In addition, association of the re-expression of EPB41L3 with histone modifications was investigated. By setting up an inducible system, we intend to assess the synergic effect of epi-drugs and ATF on the EPB41L3 gene.

Chapter 7 is a general discussion of the achieved results in this thesis. In addition, some key factors influencing Epigenetic Editing technology and future perspectives are briefly discussed.

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Chapter 2

Current and upcoming approaches to exploit the reversibility of epigenetic mutations in breast cancer

Submitted to Breast Cancer Research journal

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Abstract

DNA methylation and histone modifications are important epigenetic modifications associated with gene (dys)regulation. The epigenetic modifications are balanced by epigenetic enzymes so-called writers and erasers, such as DNA (de) methylases and histone (de)acetylases. Aberrant epigenetic alterations have been associated with various diseases including breast cancer. Since aberrant epigenetic modifications are potentially reversible they might represent targets for breast cancer therapy. Indeed, several drugs have been designed to inhibit epigenetic enzymes (epi-drugs) thereby reversing epigenetic modifications. FDA approval has been obtained for some epi-drugs for hematological malignancies. However, these drugs have very modest anti-tumor efficacy in phase I and II clinical trials in breast cancer patients as mono-therapy. Therefore current clinical trials focus on the combination of epi-drugs with other therapies to enhance or re-store the sensitivity to such therapies. This approach has given some promising results in early phase II trials. The disadvantage of epi-drugs, however, is genome-wide effects which may cause unwanted upregulation of e.g. pro-metastatic genes. Development of gene-targeted epigenetic modifications (Epigenetic Editing) in breast cancer can provide a novel approach to prevent such unwanted events. In this context, identification of crucial epigenetic modifications regulating key genes in breast cancer is of critical importance. In this review, we first describe aberrant DNA methylation and histone modifications as two important classes of epigenetic mutations in breast cancer. Then, we focus on the preclinical and clinical epigenetic-based therapies which are currently explored for breast cancer. Finally we describe Epigenetic Editing as a promising new approach for possible applications towards more targeted breast cancer treatment.

Keywords

Epigenetic Editing, breast cancer, epi-drugs, DNA methylation, histone acetylation, histone deacetylase inhibitors, DNA methyltransferase inhibitors

Introduction

Cells in one organism generally contain the same genetic information, but present very different gene expression profiles. Epigenetic modifications underlie the cell identity by switching genes on or off during mammalian development, without altering the DNA sequence. The heritability of epigenetic modifications plays critical roles to maintain the cell-type specific gene expression during cell divisions [1]. DNA methylation and histone modification signatures, especially the ones on promoter regions of genes, are well known to be associated with gene expression [2].

DNA methylation, the first identified epigenetic modification, is written by a family of DNA methyltransferases (DNMTs). It occurs on carbon 5 of the cytosine mostly in the context of dinucleotide cytosine phosphate guanine (CpG); it is classically known that the DNA methylation status of promoter regions is inversely correlated with gene expression [2]. As such, DNA hypermethylation has been suggested to inhibit expression of retroposons/transposons, and to establish mono-allelic pattern of genes (e.g. imprinting and X-chromosome inactivation). In addition, DNA methylation is thought to be a key player in prevention of chromosomal instability, translocations and gene disruption [1]. DNA methylation was thought to be irreversible till the recent discovery of enzymes which oxidize the methylated cytosine and convert it to hydroxymethyl cytosine (hmC) [2] providing intermediates in the process of active DNA demethylation [3].

In addition to DNA methylation, various post-translational histone modifications have been described to be associated with gene expression [1]. In nucleosomes, histone proteins (generally two copies of H2A, H2B, H3, and H4 each) provide the scaffold around which 147 bp of nuclear DNA is wrapped. Histones tails (especially the N-terminal domains of histones) extensively undergo post-translational histone modifications (e.g. acetylation [ac], methylation [me], ubiquitination [ub], phosphorylation [P]) on some residues especially lysine (K) and arginine (R) [1](Figure 1).

Histone modifications as well as DNA methylation are reversible. A very dynamic form of post-translational histone modifications is histone acetylation which mainly occurs on lysine residues (K). It is modified by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Figure 1). There are four classes of HDACs with 18 members, HDACs 1-11 and Sirtuins 1-7. Acetylation of

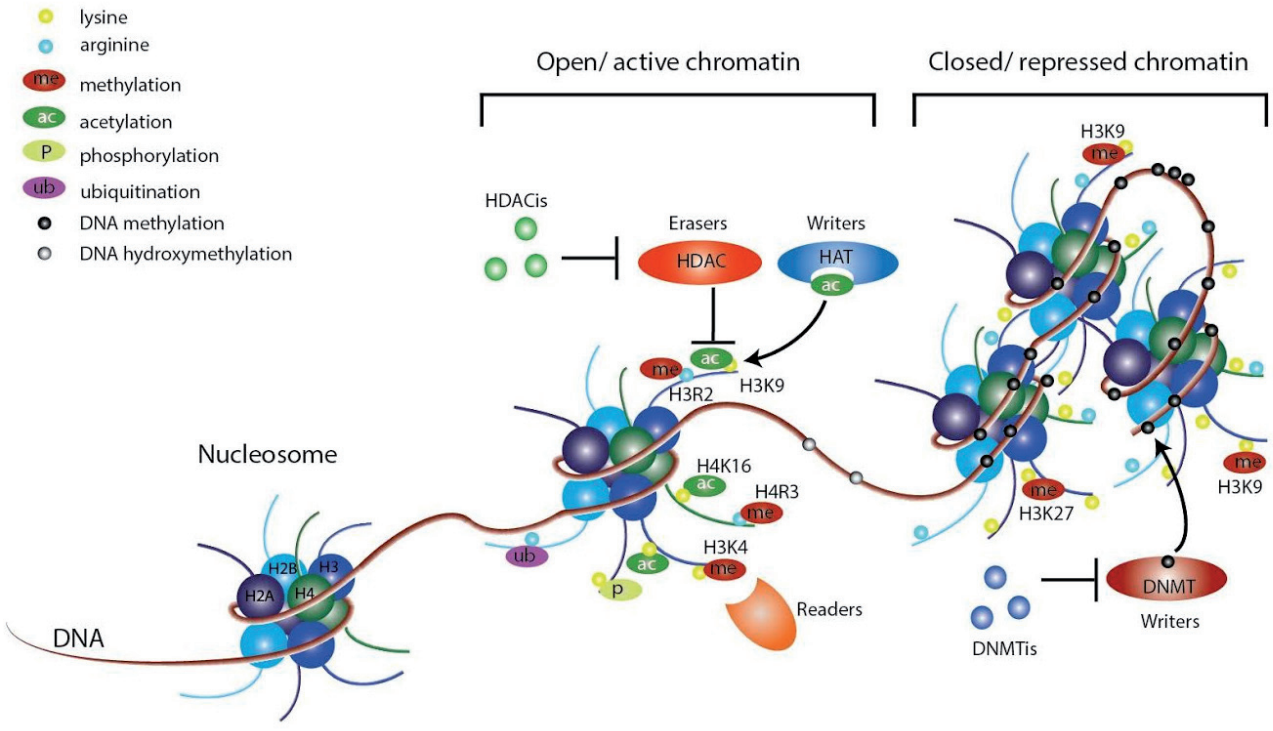

Figure 1. Epigenetic enzymes and their inhibitors. The schematic figure shows the interactions between epigenetic enzymes (writers, erasers, readers) and nucleosomes. The nucleosome core consists of a histone octamer (mainly two copies of each H2A, H2B, H3 and H4) which is wrapped by a nuclear DNA strand of 146 bp. DNA methylation and hydroxymethylation are depicted as black and grey circles, respectively. DNA methylation is induced by DNMTs. To inhibit DNA methylation, DNMTis are being used to target and suppress DNMTs. Histone tales can be posttranscriptionally modified using enzymes such as HATs. Histone acetylation can be inhibited by HDACs and HDACis can be used as HDACs suppressors.

histones reduces the negative charge of histones; thereby, according to early in vitro studies, the histone-DNA interaction is reduced and DNA is accessible to transcription factors. Although histone acetylation is still believed to be involved in regulation of gene transcription, addition of acetylation, alone, to histone tails would not be sufficient to regulate gene transcription in vivo and in the chromatin context. The effect of histone acetylation on gene regulation is dependent on various factors including, but not limited to, the position of acetylation [4].

Various epigenetic enzymes are continuously acting to retain the balance of epigenetic modifications by inducing ('writers') or removing ('erasers') epigenetic modifications. Other epigenetic players bind to epigenetic modifications ('readers') and recruit further re-enforcing complexes (Figure 1). Malfunctioning of the enzymes results in aberrant epigenetic modifications (epigenetic mutations). Since epigenetic enzymes interact with, recruit or suppress each other, while also epigenetic modifications recruit epigenetic enzymes [5, 6], malfunctioning of any epigenetic enzyme can be sufficient to severely affect the epigenome and disrupt the normal state of the cell. So, function of epigenetic enzymes is vital in retaining the normal state of cells.

In cancer, numerous epigenetic enzymes are frequently mutated and/or dysregulated, resulting in altered epigenetic modifications [1]. The dysregulated epigenetic enzymes in cancer are potential targets of several classes of inhibitors including DNMTs inhibitors (DNMTis), HDACs inhibitors (HDACis), and the recently developed inhibitors of histone methyltransferases (HMTis) and histone acetyltransferases (HATis). The inhibitors of epigenetic enzymes used in (pre)-clinical treatments are so-called epi-drugs.

Epigenetics and breast cancer

Extensive studies on epigenome changes in breast cancer have been undertaken to understand the role of epigenetics in breast cancer and to develop novel epigenetic therapies. Such studies demonstrated the association of aberrant DNA hypomethylation not only with cancer in general, but also with breast cancer[7]. In addition to global DNA hypomethylation which underlies chromosomal instability and disturbed gene expression patterns, hypermethylation of promoter regions of e.g. tumor suppressor genes is found in breast cancer [8]. Decreased levels of DNA hydroxymethylation is also observed in breast tumors versus

normal breast tissue [9].

Besides the hypermethylated tumor suppressor genes, genes involved in DNA repair, apoptosis, metabolism, cell cycle regulation, cell adherence, metastasis, cellular homeostasis, cell growth and genes encoding several epigenetic enzymes are frequently hypermethylated in breast cancer [2,8]. Aberrant DNA hypermethylation of some key genes in breast cancer might be useful as prognostic or diagnostic markers. For instance, aberrant hypermethylation of genes encoding estrogen receptor-alpha (ER-α) and progesteron receptor (PR) is correlated with silencing of these genes and with development of ER- and PR-negative breast cancer. Indeed, some hypermethylated genes such as RASSF1A are considered as potential diagnosis markers of breast cancer [2]. Also, aberrant DNA hypermethylation of paired like homeodomain transcription factor-2 gene in breast cancer was recently considered as a marker linked to tamoxifen resistance. Thus, the DNA methylation status of such genes might show value as predictive marker for therapy response [2].

Another common occurrence in cancer is the global reduction of monoacetylated lysine 16 of histone H4 (H4K16) [7]. The loss or low levels of H4K16 acetylation was suggested as an early event in breast cancer [10] and is associated with altered levels of HDACs [11]. Moreover, mutated HATs have been reported for breast cancer [1]. Altered histone methylation patterns [12] as well as mutated HMTs are also observed in breast cancer [1].

Altogether, maintained balance of epigenetic modifications by epigenetic enzymes is essential for the regulation of gene expression and the maintenance of the normal status of cells. Clearly, malfunctioning of epigenetic enzymes and their subsequent aberrant epigenetic modifications are involved in development and progression of different cancer types including breast cancer. Treatments to reverse the aberrant epigenetic modifications are currently under intensive preclinical and clinical investigations and will be discussed below.

Preclinical studies on epigenetic therapy for breast cancer

The reversible nature of epigenetic modifications makes epigenetic mutations attractive targets for epigenetic therapy of cancer. Currently, intensive research is focused on inhibiting epigenetic enzymes such as DNMTs and HDACs. Although there are aberrant histone methylation modifications in breast cancer,

to the best of our knowledge, there is no report describing the effects of any HMTis on breast cancer. DNMTis and HDACis have been tested as therapeutic interventions against several tumor types including breast cancer. Here, we discuss the different DNMTis and HDACis and their efficacy in preclinical breast cancer studies.

DNA methyltransferase inhibitors

Inhibitors of DNMTs are used to prevent DNA re-methylation after cell divisions and can be classified as nucleoside analogues and non-nucleoside analogues. Azacitidine (5azaC, Vidaza®) and decitabine (5azadC, Dacogen) are two well-known examples of nucleoside analogues [13]. Azacitidine and decitabine are incorporated into the DNA during replication. By forming covalent bonds with DNMTs, they trap DNMTs and block their functions [13].

Azacitidine is considered as a global DNMTi and can be incorporated into both DNA and RNA. For example, upon treatment of breast cancer cells with azacitidine, DNA re-methylation was inhibited for 23 out of 26 tested hypermethylated genes in breast cancer. Further analysis of five selected genes demonstrated their re-expression [14].

Animal studies further validated the potential therapeutic implications of such observations. Assessment of several therapeutic doses of azacitidine showed association of azacitidine with tumor size reduction of xenografts derived from breast cancer cells [15]. In such a study, administration of 0.5 mg/kg of azacitidine, for five days a week, was correlated with growth inhibition of patient-derived tumors which were engrafted orthotopically into immunodeficient mice [15].

Decitabine treatment also prevents DNA re-methylation and re-activates silenced genes [15]. For example, decitabine was able to induce tumor necrosis factor related apoptosis-inducing ligand (TRAIL) in triple-negative breast cancer cells[16], which can explain how this DNMTi makes breast cancer cells sensitive to chemotherapeutic agents [17]. Decitabine treatment of animals with orthotopically implanted breast cancer cells resulted in reduced tumor volume [18]. Similarly, decitabine pre-treated breast cancer cells showed diminished tumor growth upon xenografting [15].

Importantly, demethylation and re-expression of genes involved in endocrine therapy response such as ESR1 gene encoding ER-α can be exploited to overcome

endocrine therapy resistance in ER-negative breast cancer [2]. Such strategies open up new windows for otherwise difficult-to-treat breast cancers.
Non-nucleosides inhibitors include several classes of natural compounds like the polyphenols [13]. Epigallocatechin-3-gallate (EGCG), a major catechin found in green tea extract, was found to induce apoptosis in breast cancer via inhibiting expression of genes such as vascular endothelial growth factor (VEGF) [19] and it was shown to re-express ESR1 in breast cancer cells [20].
So regardless of the type of agent, inhibition of DNMTs results in re-expression of tumor suppressor genes associated with inhibition of growth of cancer cells.

Histone deacetylase inhibitors

HDACis chelate the zinc co-enzyme factor, thereby blocking the HDACs catalytic activity. HDACis are divided in four groups: short chain fatty acids (e.g. sodium butyrate, valproic acid), hydroxamic acids (e.g. trichostatin A, vorinostat, panobinostat), cyclic tetrapeptides (e.g. depsipetide, romidepsin [isostax]), and benzamides (e.g. entinostat, tacedinaline) [16].
HDACis as monotreatment in vitro and in vivo have several anticancer effects on breast cancer, among which growth arrest, the induction of apoptosis, and cellular differentiation [16, 21-25].
In addition to their efficacy as preclinical monotherapy in breast cancer cells, HDACis enhance the sensitivity to radiotherapy [16] and cytotoxic agents [24]. For example, the combination of vorinostat and TRAIL resulted in a significant growth inhibition, when compared to either treatment alone, in mice bearing TRAIL-resistant tumor xenografts [24]. Various HDACis, among which valproic acid, trichostatin A, and entinostat, have been shown to play a role in overcoming resistance to therapies. In this respect, HDACis can be exploited for overcoming resistance to HER2-targeted therapies [26]. Also, HDACis are well accepted for their anticancer activities by re-expressing silenced genes such as ER-α, in vitro and in vivo [27]. Moreover, the re-expression of ER-α re-sensitized breast cancer cells to the ER-targeted therapy tamoxifen, in vitro [20, 28]. Paradoxically, HDACis have non-selective effects on non-histone proteins which might cause opposite effects. For example, in ER-positive breast cancer cells ER-α expression was decreased upon treatment with vorinostat. This effect can be due to the increased acetylation level of heat shock proteins, which are known to stabilize the ER-α

protein and inhibit its degradation [29]. Despite these opposite effects, however, the combination of HDACis and endocrine therapy acted synergistically in ER-positive models [30].

FDA-approved epi-drugs in oncology

Azacitidine and decitabine are both approved by the United States Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome (MDS). Azacitidine is administered by subcutaneous or intravenous injections once daily during 7 days followed by 21 days without treatment. Decitabine is given intravenously thrice daily for three consecutive days followed by four days without treatment. In the setting of MDS, both treatments provide an objective response (complete + partial response) of 16-17% compared to no response in untreated controls. Both regimens show a comparable toxicity profile with myelo-suppression, gastro-intestinal complaints and constitutional symptoms as most common side effects [31].

Vorinostat and romidepsin are FDA-approved HDACis for the treatment of cutaneous T-cell lymphoma and in addition romidepsin is approved for the treatment of peripheral T-cell lymphoma. [32]. Vorinostat 400 mg orally once daily induced objective responses in approximately 30% of the patients. The most common adverse events include myelo-suppression, gastro-intestinal side effects and fatigue [33]. Administration of romidepsin as a 4-hour infusion on days 1, 8, and 15 of a 28-day cycle with a starting dose of 14 mg/m2, results in an objective response in 34% of patients with cutaneous T-cell lymphoma [34, 35] and in 38% of patients with peripheral T-cell lymphoma [36]. Side-effects are comparable to that of vorinostat.

Efficacy of epi-drugs in breast cancer patients

The efficacy of DNMTis and HDACis in breast cancer was evaluated in 21 phase I and II studies that enrolled 303 patients with breast cancer (Table 1). In eleven of these studies (n = 87 patients) epi-drugs were administered to the patient either as monotherapy or in combination with another epi-drug. Most of these studies were phase I studies (64%) in advanced solid tumors, and therefore not primarily aimed to evaluate anti-tumor efficacy with few patients enrolled who were in general heavily pre-treated. Nevertheless, the results with epi-drugs in breast

cancer are consistently showing very limited anti-tumor efficacy on its own.
In 87 patients receiving epi-drugs as monotherapy, in only 9 (10%) patients, objective responses were observed. The limited efficacy of epigenetic drugs at the maximum tolerated dose suggests that these drugs are not well suited as monotherapy in breast cancer. Biological efficacy at the epigenetic level was, however, observed; for instance, pre- and post-treatment tumor biopsies showed a significant reduction in tumor DNA methylation after decitabine monotherapy[37].
Given the fact that epi-drugs can alter the expression of therapeutic targets, this led to the hypothesis that they should especially be administered as a (re-) sensitizer for drugs to which intrinsic or acquired resistance exists. Also in clinical trials this novel approach has rendered promising results in other tumor types.

Table 1. Efficacy of epi-drug monotherapy and combination therapies in breast cancer patients

Epi-drug	Phase	Co-treatment	No. of patients	OR / CBR	Reference
Monotherapy					
Azacitidine	I	None	11	7 / na	[52]
Azacitidine plus valproic acid	I		4	0 / 0	[53]
Decitabine	I		4	0 / na	[37]
Fazarabine	I		3	1 / 1 *	[54]
-	II		14	0 / 0	[55]
Phenylbutyrate	I		5	0 / na	[56]
Vorinostat	II		14	0 / 3	[57]
-	II		3	0 / 0	[58]
-	II		26	1 / 1	[59]
-	biomarker study		-	Na	[60]
Vorinostat plus Decitabine	I		3	0 / 0	[61]
Total			**87**	**9 (10 %) / na**	
Combination therapies					
Azacitidine	I	Erlotinib	1	0 / 1	[62]

Decitabine	I	Carboplatin	5	0 / na	[63]
Entinostat	II	Exemestane	64†	4 / 18	[40]
Valproic acid	II	5-fluoruracil, epirubicin and cyclophosphamide	15	9 / na	[64]
-	I	Followed by epirubicine	10	3 / 7	[65]
Valproic acid plus hydralazine	II	Standard chemotherapy	3	0 / 0	[66]
-	I	Doxorubicin plus cyclophosphamide	16	13 / na	[42]
Vorinostat	I	Doxorubicin	5	1 / 1	[67]
-	I-II	Paclitaxel plus bevacizumab	54	26 / 42	[41]
-	II	Tamoxifen	43	8 / 17	[31]
Total			216	64 (30 %) / na	

OR = objective response (partial + complete remission); CBR = (OR + stable disease >6 months); na = not available; * = Clinical response † An addition 67 patients were randomized to exemestane plus placebo

Decitabine, was shown to allow the re-expression of the copper transporter CTR1, which plays a role in cellular platinum-uptake, in patients with solid tumors and lymphoma[37], and restore the sensitivity to platinum-based chemotherapy in ovarian cancer[38,39]. A combination of epi-drugs with cytotoxic or targeted therapies, such as ER-targeted therapy, was evaluated in 10 phase I/II studies in 216 breast cancer patients. The largest study so far is a phase II study in which 130 metastatic breast cancer patients were randomized to exemestane plus placebo (n = 67) or exemestane plus entinostat (n = 64). These patients had earlier progressed on a nonsteroidal aromatase inhibitor. The combination of exemestane plus entinostat significantly improved PFS (4.3 v 2.3 months) and OS (28.1 v 19.8 months) [40]. In another phase II study in 43 patients with metastatic breast cancer who progressed on at least one prior line of endocrine therapy, vorinostat 200 mg twice daily was combined with tamoxifen. In this study, objective response rate was 19% and clinical benefit rate (objective response or stable disease >6 months) 40% [31]. Baseline high HDAC2 levels correlated with response, which may prove valuable as a predictive biomarker to select patients for treatment with HDACis. Finally, in a phase I/II study in 54 patients with metastatic breast cancer, vorinostat

200-300 mg twice daily on day 1-3, 8-10, and 15-17 was added to paclitaxel plus bevacizumab. This combination resulted in a 49% objective response rate (partial + complete remission) and 78% clinical benefit rate (objective response + stable disease >6 months). Serial biopsies, available from seven patients, showed an increase in acetylation of heat shock protein 90, and α-tubulin [41].

Although there is preclinical evidence for enhanced efficacy of HER2-targeted therapies when combined with epi-drugs, results from clinical studies are awaited.

In conclusion, epi-drugs have limited anti-tumor efficacy in breast cancer patients at the maximum tolerated dose when administered as monotherapy, but can be administered safely. However, expected epigenetic changes, such as decreased tumor DNA methylation [37], increased histone acetylation [41], and upregulation of gene expression [42] are observed after their administration in clinical breast cancer studies. Current studies suggest a potential role for epi-drugs in combination with chemotherapeutics and targeted therapies, to enhance or restore the sensitivity to these drugs.

Current breast cancer trials evaluating epi-drugs

Ongoing trials increasingly apply epi-drugs to specific subgroups rather than to the general population of breast cancer patients. Much work is performed on (re-) sensitization of endocrine-resistant tumors to endocrine therapy. In patients with triple negative or hormone-refractory metastatic breast cancer, azacitidine is combined with entinostat; although the response rate is the primary endpoint in this study, the effects on ER- and PR expression will be evaluated as the secondary endpoints (NCT01349959). A novel non-invasive way to measure ER-expression is by molecular imaging using positron emission tomography (PET) and 18F-fluoroestradiol (FES) as a tracer [43]. This tool facilitates the assessment of ER-expression during treatment. In an NCI study, hormone-refractory patients are treated with daily vorinostat for 2 weeks, followed by a treatment with an aromatase inhibitor for 6 weeks (NCT01153672). Cycles are repeated every 8 weeks until progression. As a secondary endpoint, changes in ER-expression will be measured using serial FES-PET imaging. Panobinostat and decitabine are also evaluated to sensitize triple negative breast cancer patients to endocrine therapy in phase I/II studies (NCT01194908, NCT01105312).

The use of DNMTis and HDACis as chemo-sensitizers is also evaluated in various

Table 2. Overview of current clinical trials evaluating DNMT-inhibitors and HDAC-inhibitors in breast cancer

Drug	Condition	Co-treatment	Primary outcome measure	*N*	Phase	Status	NCT#
DNMT-inhibitor							
Azacitidine	Advanced BC	Entinostat*	Objective response rate	60	II	R	01349959
-	Advanced/ metastatic BC	Nab-Paclitaxel		45	I/II	R	00748553
Decitabine	Advanced/ metastatic TNBC	Panobinostat** (± tamoxifen)	The maximum tolerated dose of decitabine and panobinostat	60	I/II	R	01194908
FdCyd	Solid tumors, including BC	Tetrahydrouridine	To determine the safety of FdCyd	20	I	R	01479348
FdCyd	Solid tumors, including BC	Tetrahydrouridine	To determine PFS and/ or response rate of FdCyd plus Tetrahydrouridine	185	I	R	00978250
EGCG	Newly diagnosed BC	-	To determine whether EGCG can affect proliferation rate and induce apoptosis	20	II	R	00949923
-	Newly diagnosed BC	-	To evaluate the effects of EGCG on various biomarkers	32	II	A	00676793
-	Stage I-III BC	-	To determine the safety and maximum tolerated dose of EGCG	40	I	A	00516243
HDAC-inhibitor							
Vorinostat	BC	Lapatinib	Clinical benefit rate	47	I/II	R	01118975
-	Recurrent/metastatic BC	-	To evaluate the safety of vorinostat	49	I/II	A	00416130
-	Advanced BC	Capecitabine	The maximum tolerated dose, safety, and efficacy of vorinostat plus capecitabine	47	II	U	00719875
-	Local recurrent/ metastatic BC	Paclitaxel/ bevacizumab	The maximum tolerated dose, and objective response rate of vorinostat in combination with paclitaxel/bevacizumab	58	I/II	U	00368875
-	Metastatic BC	Ixabepilone	Dose limiting toxicity	56	I	A	01084057
-	Hormone-refractory BC	Aromatase inhibitor	Clinical benefit rate	14	II	R	01720602
-	Locally advanced BC	Paclitaxel/ trastuzumab	To determine the recommended phase II dose	54	I/II	U	00574587

-	Hormone-refractory BC	Aromatase inhibitor	Clinical benefit rate	20	II	R	01153672
-	Newly diagnosed BC	Nab-paclitaxel/ carboplatin	Pathologic complete response rate	74	II	A	00616967
-	HIV+ with solid tumor, including BC	Paclitaxel/ carboplatin	Maximum tolerated dose	66	I	R	01249443
-	Brain metastases, including from BC	(plus radiotherapy)	Maximum tolerated dose	24	I	A	00838929
Entinostat	Locally recurrent/metastatic ER+	± exemestane	Pharmacokinetics of entinostat in fasted and fed subjects	28	I	R	01594398
-	Newly diagnosed TNBC	Anastrozole	Safety, tolerability and recommended phase II dose (phase I cohort); Change in proliferation, ER/PR-expression (phase II cohort)	41	I/II	R	01234532
-	HER2-positive metastatic BC	Lapatinib	Recommended phase II dose (phase I cohort); Objective response rate (phase II cohort)	70	I/II	R	01434303
-	Advanced BC	Azacitidine*	Objective response rate	60	II	R	01349959
Panobinostat	TN metastatic BC	Letrozole	Maximum tolerated dose, adverse events (phase I cohort); Response rate (phase II cohort)	48	I/II	R	01105312
-	Advanced/ metastatic TNBC	Decitabine** (± tamoxifen)	The maximum tolerated dose of decitabine and panobinostat	60	I/II	R	01194908
-	HER2-negative locally recurrent/ metastatic BC	-	Objective response rate	118	II	A	00777049
VPA	Newly diagnosed locally advanced/ metastatic BC	FEC	Pathologic response rate	55	II	R	01010854
-	Newly diagnosed BC	-	To determine whether VPA levels correlate with leukocyte and tumor histone acetylation	33	NA	R	01007695
Depsipeptide	Solid or hematologic malignancy, including BC	-	Safety, tolerability, maximum tolerated dose and pharmacokinetics	132	I	R	01638533

*Study population: (TN)BC=(triple-negative) breast cancer; NSCLC=non small-cell lung cancer; N=estimated enrolment; Status: A= active, not recruiting; C=completed; R=recruiting; U=unknown. *,**=cross-referenced within table*

breast cancer trials (e.g. NCT00748553, NCT00368875). Among the evaluated combinations are azacitidine with Nab-paclitaxel (abraxane®), valproic acid with FEC, and vorinostat with paclitaxel plus bevacizumab. Finally, sensitization to HER2-targeted therapy will be evaluated in a limited number of studies. One phase I/II study evaluated 200 mg vorinostat twice daily on day 1-14, combined with trastuzumab 6 mg/kg once every three weeks. This study enrolled 16 patients and was terminated due to low response-rate (NCT00258349). Another study will evaluate the safety and efficacy of vorinostat combined with the tyrosine kinase inhibitor, lapatinib (NCT01118975). Also several studies using panobinostat to sensitize breast cancer to trastuzumab (NCT00788931, NCT00567879), and lapatinib (NCT00632489) have recently been completed and results are awaited. All trials were phase I or II. An overview of ongoing trials with DNMTis and/or HDACis in breast cancer is provided in Table 2.

Epigenetic Editing

Despite the above described promises of epi-drugs, they affect genes genome-wide, and in addition, epi-drugs inhibit writers and erasers which generally also modify non-chromatin targets, resulting in unwanted effects including upregulation of prometastatic genes [44] and genes encoding drug resistance-associated proteins[45]. To avoid the unwanted effects, epigenetic therapy can be improved using gene targeting approaches. Writing or erasing epigenetic modifications of selected target genes (Epigenetic Editing) is obtained by fusing a writer or eraser of a specific epigenetic modification to an engineered DNA binding domain [6]. As DNA binding domains, Zinc Finger Proteins (ZFPs), Triplex Forming Oligos (TFOs), Transcription activator-like effectors (TALEs), or Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) can be fused to the catalytic domains of epigenetic enzymes [6] or to epi-drugs [46].

The novel approach of Epigenetic Editing has been successful in modulating several genes [6]: we previously showed that targeted DNA methylation is instructive in gene downregulation (MASPIN [47] and VEGF-A [48]) and that targeted DNA demethylation could upregulate the target gene (ICAM-1) [49]. We also demonstrated that targeting the repressive histone methylation modification H3K9me2, to Her2/neu gene induced Her2/neu protein downregulation which inhibited cancer cell growth [50]. Moreover, targeted DNA methylation on the

SOX2 promoter prevented growth of breast cancer cells, also upon removal of the epigenetic writer [47]. Epigenetic Editing thus provides powerful tools for investigating the reversibility of epigenetic control in gene regulation [6, 51]. As targeting of genes has recently become widely feasible, Epigenetic Editing opens new avenues towards "the druggable genome for cancer therapy".

Conclusion

Epigenetic mutations including aberrant DNA methylation and histone modifications are associated with breast cancer development and therapy-resistance. Aberrant DNA methylation and histone acetylation can be reversed by DNMTis and HDACis. Several DNMTis and HDACis are FDA-approved, albeit not (yet) for the treatment of patients with breast cancer. These drugs can induce apoptosis, alter the gene expression, and reverse therapy-resistance in preclinical models. In clinical studies, DNMTis and HDACis have shown very modest anti-tumor activity as monotherapy, although effects on gene expression can be observed. Current clinical trials, therefore, mainly focus on the combination of these drugs with chemotherapeutics and targeted-therapies. Despite their promise, a disadvantage of DNMTis and HDACis is their genome-wide function and non-chromatin effects. Epigenetic Editing of a single gene results in gene modulation, and thereby fully exploits the reversibility of epigenetic modifications as therapeutic targets. Epigenetic Editing and other targeted approaches thus provide alternatives for current epigenetic therapies of breast cancer.

Abbreviations

DNMTs, DNA methyltransferases; DNMTis, DNA methyltransferase inhibitors; HATs, histone acetyltransferases; HDACis, histone deacetylase inhibitors; epi-drugs, epigenetic enzymes; ER, estrogen receptor; PR, progesteron receptor; ac, acetylation; me, methylation; P, phosphorylation; ub, ubiquitination; K, lysine; R, arginine; hmC, hydroxymethyl cytosine

Competing interests

The authors declare no conflicts of interest.

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Chapter 3

Towards sustained silencing of HER2/neu in cancer by epigenetic editing

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Abstract

The human epidermal growth factor receptor-2 (HER2/neu/ERBB2) is overexpressed in several cancer types. Although therapies targeting the HER2/neu protein result in inhibition of cell proliferation, the anticancer effect might be further optimized by limiting HER2/neu expression at the DNA level. Towards this aim, epigenetic editing was performed to suppress HER2/neu expression by inducing epigenetic silencing marks on the HER2/neu promoter. HER2/neu expression and HER2/neu promoter epigenetic modification status were determined in a panel of ovarian and breast cancer cell lines. HER2/neu-overexpressing cancer cells were transduced to express a zinc finger protein (ZFP), targeting the HER2/neu gene, fused to histone methyltransferases (G9a, SUV39-H1)/super KRAB domain (SKD). Epigenetic assessment of the HER2/neu promoter showed that HER2/neu-ZFP fused to G9a efficiently induced the intended silencing histone methylation mark (H3K9me2). Importantly, H3K9me2 induction was associated with a dramatic downregulation of HER2/neu expression in HER2/neu- overexpressing cells. Downregulation by SKD, traditionally considered transient in nature, was associated with removal of the histone acetylation mark (H3ac). The downregulation of HER2/neu by induced H3K9 methylation and/or reduced H3 acetylation was sufficient to effectively inhibit cellular metabolic activity and clonogenicity. Furthermore, genome-wide analysis indicated preferential binding of the ZFP to its target sequence. These results not only show that H3K9 methylation can be induced but also that this epigenetic mark was instructive in promoting downregulation of HER2/neu expression.

Implications: Epigenetic editing provides a novel (synergistic) approach to modulate expression of oncogenes. Mol Cancer Res; 11(9); 1029–39. _2013 AACR.

Introduction

Her2/neu is a transmembrane tyrosine kinase receptor and an important member of the EGF receptor family. Upon ligand binding to the extracellular domain, the Her2/neu receptor heterodimerizes to other members of this family [1]. Heterodimerized, activated receptors autophosphorylate specific tyrosine residues of their cytoplasmic tails, thereby triggering signaling pathways, which regulate cell proliferation [2].

Besides its crucial roles in normal cells, Her2/neu has been found to be amplified and/or overexpressed in several types of cancer inducing tumor growth [3]. Her2/neu overexpression is associated with a poor prognosis for breast, gastric, and ovarian cancer [4–6]. On the basis of these characteristics, Her2/neu is an attractive therapeutic target. The first U.S. Food and Drug Administration (FDA)-approved therapeutic to target Her2/neu in HER2-positive breast cancer is trastuzumab[7,8]. Trastuzumab, a recombinant humanized monoclonal antibody, targets the extracellular domain of the Her2/neu receptor thereby inhibiting its dimerization and therefore its activation. Apart from breast cancer, this antibody is also successfully used in patients with HER2-positive gastric cancer [9]. Another tyrosine kinase inhibitor of Her2/neu receptor (lapatinib) also showed an improved survival in patients with metastatic breast cancer [10].

Despite the successes of Her2/neu-targeting therapies, resistance to Her2/neu-targeting agents remains an obstacle and several trastuzumab resistance mechanisms have been proposed [11]. An important mechanism of resistance is thought to be the reduced receptor–antibody binding which can be caused by nonaccessibility of the Her2/neu protein to trastuzumab, due to, for instance, alternative translation start sites of Her2/neu protein [12]. Alternatively, tight attachment of epithelial cancer cells via upregulation of epithelial proteins in the intercellular junctions can result in the escape of the Her2/neu receptor from trastuzumab and other receptor-targeted therapies. Indeed, loosening the tight lateral junctions increased the surface presence of the Her2/neu receptor in vitro and in vivo, subsequently improving Trastuzumab efficiency [13].

Because of the potential of Her2/neu to serve as a potent anticancer therapeutic target, many studies are ongoing to find the most efficient way to exploit Her2/neu. Approaches to improve blocking of the Her2/neu function through combination of Her2/neu targeting therapies indeed showed improved response

rates compared to monotherapy in vitro, in vivo [14, 15], and in clinical studies[16]. Another application is through exploiting Her2/neu as a targeting device for antitumor agents. In this regard, TDM-1 (Trastuzumab conjugated to a derivative of Maytansine 1, a potent antitumor agent) showed improved antitumor effect compared to Trastuzumab alone in HER2-positive locally advanced or metastatic breast cancer patients [17].

Achieving an optimal inhibition of Her2/neu function, however, remains a problem and this is partially due to the high turnover rate of the Her2/neu receptor [18]. We reasoned that the high turnover rate of Her2/neu limits its therapeutic potential when targeted for inhibition at the protein level and we set out to silence its expression directly at the DNA level. Gene expression can be regulated at the DNA level by so called Artificial Transcription Factors (ATFs), composed of a DNA binding domain fused to an effector domain [19]. As DNA targeting tools, engineered Zinc Finger Proteins (ZFPs) have shown great flexibility in gene targeting [20]. Modulation of expression of various endogenous genes has been achieved with upregulation of C13ORF18 [21], Maspin [22], down/upregulation of EpCAM [23], and downregulation of SOX2 [24] as some examples. Also, regulation of the Her2/neu gene expression has been achieved by ATFs [25, 26], resulting in a satisfactory downregulation associated with cell growth inhibition[25]. However, as the effector domains generally used in ATF approach e.g. Super KRAB Domein (SKD) and four copies of the viral protein VP16 (VP64) have no catalytic activity, the effect on transcription relies on the recruitment of other proteins and therefore the ATF needs to be continuously present.

To exert a more permanent downregulation of gene expression, epigenetics provides a promising avenue and changing epigenetic signatures might result in mitotically stable changes in gene expression. To induce epigenetic modifications on a target gene (epigenetic editing), catalytic domains of epigenetic enzymes can be targeted to the DNA sequences of interest. Using integrated reporter sequences, this strategy indeed resulted in gene expression modulation for several epigenetic domains [27]. Modulation of expression of endogenous genes by epigenetic editing would open up exciting venues. Targeted DNA methylation to repress gene expression was recently reported for three endogenous genes [28, 29]. Inducing repressive histone modifications has been reported for one gene; upon targeting of catalytic domains of histone methyltransferase enzymes (G9a, SUV39-H1) to

the Vascular Endothelial Growth Factor (VEGF-A) promoter, repressive histone marks were induced and result in downregulation of gene expression [30].
These considerations tempted us to explore downregulation of Her2/neu via epigenetic editing and we thus set out to induce repressive histone marks onto the Her2/neu promoter. We show here that dimethylation of lysine 9 of histone H3 (H3K9me2) on the Her2/neu gene resulted in downregulation of this gene. As DNA targeting domains can be engineered for virtually any gene, epigenetic editing provides a generally applicable approach to silence (overexpressed) genes.

Materials and Methods

Cell culture

A panel of four cancer cell lines consisting of breast cancer cell lines (SKBR3, MDA-MB231, and MCF7), and an ovarian cancer cell line (SKOV3), as well as HEK293T cells were obtained from from American Type Culture Collection. All cell lines have been authenticated and match their expected DNA fingerprints (STR profiling, BaseClear). All cells were cultured in Dulbecco's modified Eagle medium (BioWhittaker) supplemented with 2mM L-glutamine, 50 µg/ml gentamycin, and 10% FBS (BioWhittaker) and incubated at 37 °C in a humidified 5% CO2-containing atmosphere.

Constructs and retroviral transduction

ATFs consisting of the ZFP targeting Her2/neu (designated E2C) fused to transcription effector domains (SKD/VP64) were reported before [25, 31, 31] and generously provided by Dr. Barbas (La Jolla, USA) in the pMX-IRES-GFP retroviral backbone containing a HA-tag, a nuclear localization signal, and the GFP sequence. In this study, catalytic domains of two histone methyltransferases were cloned as reported previously [30]. For G9a, the N-terminal domain (aminoacid 1-829) was not included (excluding most of the ankyrin repeats); for SUV39-H1 the C-terminal region encoding amino acids 76-412 was amplified, lacking the N-terminal HP1 Interaction domain. Primers were derived from Snowden and colleagues, 2002, with introduced AscI and PacI restriction sites allowing swapping with VP64 [30]. Catalytic mutant G9a (ref. 32; pMX-E2C-G9a-W1050A) was amplified using sense: 5'-GCCAAGATGGGCGCGGGGGTCCGCGC-3' and

antisense: 5'-CGCGGACCCCCGCGCCCATCTTGGC-3' primers. pMX vectors were co-transfected with the viral packaging plasmids encoding gag-pol and the vesicular stomatitis virus G protein into HEK293T cells using the calcium phosphate transfection method [21]. pMX empty vector and the backbone with the ZFP (E2C) only (pMX-E2C) served as controls. Supernatant of HEK293T cells containing virus was harvested 48 and 72 hours after transfection. Host cells were seeded in 6-well plates and transduced on two consecutive days with the supernatant of transduced HEK293 cells supplemented with 6 µg/ml polybrene (Sigma, St Louis, MO, USA) and 10% FCS. Indications of transduction efficiency (GFP) and the effect of the constructs on transcription of Her2/neu were read out 4 days after the first transduction.

Fluorescence Activated Cells Sorting (FACS)

Transduced host cells were washed and stained with the antibody against Her2/neu (APC anti-human-CD340, BioLegend, Uithoorn, The Netherlands). Calibur flow cytometry (Beckton Dickinson Biosciences, San Jose, CA, USA) was used to detect GFP expression and to determine Mean Fluorescence Intensity (MFI). For the sorting experiment, SKBR3 cells were stained with Her2/neu antibody 15 days after transduction with pMX empty and pMX-E2C-Suvdel76 constructs and sorted based on GFP and Her2/neu expression using a Beckman Coulter MoFlo XDP cell sorter (Becton Dickinson Biosciences).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the transduced and non-transduced host cells using the Qiagen RNeasy plus mini kit (Qiagen, Venlo, The Netherlands) and 1 µg RNA of each sample was used for the reverse transcription reaction using the Fermentas Revertaid cDNA synthesis kit with random hexamer primers (Fermentas, Leon-Rot, Germany). Per reaction set, one RNA sample was prepared without Reverse Transcriptase as a control for absence of DNA contamination in the subsequent PCR.

A subsequent qRT-PCR reaction was performed (ABIPrism 7900HT, Applied Biosystems, Nieuwekerk, the Netherlands) with 10 ng cDNA using ROX enzyme mixture (Abgene, Surrey, UK), and a Taqman gene expression assay for the quantification of Her2/neu expression (Hs01001599_m1, Applied Biosystems)

or primer and probes for GAPDH expression (Fw 5'-CCACATCGCTCA-GACACCAT-3', Rv 5'-GCGCCCAATACGACCAAAT-3', and probe 5'-6FAMCGTTGACTCCGACCTTCACCTTCCCMGBNFQ-3' (Eurogentec, Maastricht, the Netherlands)). Data were analyzed with SDS 2.1 RQ software (Applied Biosystems) and relative expression was calculated by the comparative delta Ct method.

Bisulfite sequencing

The methylation status of 29 CpGs in Her2/neu gene flanking the E2C binding site was examined using bisulfite sequencing. First, genomic DNA of cells was isolated using a standard protocol and was bisulfite-converted (EZ DNA Methylation-GoldTM Kit, Zymo research, Irvine, CA, USA). PCR was performed for the 400 bp-region amplified by primers displayed in Fig. 3A (Fw 5'-AAAGTGAAGTTGGGAGTTGTTAT-3' and Rv 5'- ACCAAACCCACCT-TAAATACTC -3'), the PCR product was ligated into pCR2.1 vector (Invitrogen, Leusden, the Netherlands) and sequenced with M13 reverse primers 5'– CAGGAAACAGCTATGAC -3'. The DNA methylation analysis was performed by Bisulfite sequencing DNA Methylation Analysis (http://biochem.jacobs-university.de/BDPC/BISMA/).

Chromatin ImmunoPrecipitation (ChIP) and qPCR

Cells were harvested and treated for protein-DNA crosslinking using formaldehyde as described previously [34]. Cells were lyzed and chromatin was sonicated for 15 minutes by Bioruptor (Diagenode, Liège, Belgium) (High, 30" on, 30"off), sheared chromatin was pelleted at 4°C (18000g, 10 min). 5 µg of specific antibodies (acH3 (06-599), H3K4me3 (07-473), and H3K9me2 (07-441) (Milipore, Massachusetts, USA), normal rabbit IgG (ab46540), H3core (ab1791), H3K9me3 (ab8898), (Abcam, Cambridge, UK), and HA-tag (101P-200), (Covance, the Netherlands)) in 0.02% PBS-Tween-20 were bound to magnetic Dynabeads (Invitrogen) during 15 minutes incubation, then unbound antibodies were washed off and diluted sheared chromatin was added to the complex of magnetic Dynabeads-antibody (rotating overnight at 4°C). After separation of magnetic Dynabeads and supernatant using DynaMag™-2 magnet rack (Invitrogen) and washing off unbound chromatin with PBS, chromatin was

eluted with 2%SDS and 50mM NaHCO3 and treated with RNAse (Roche, Mannheim, Germany) and high salt concentrations at 65ºC overnight. After reversing crosslinks and digestion of protein, DNA was purified as described previously [34].

To assess the induction of histone marks and their spreading, several primer pairs were used for the Her2/neu gene around the E2C-binding site and the transcription start site (TSS; Fig.3A): region A (Fw 5'-TCAAGACCAGCCT-CACCAAC-3', Rv 5'- ACCTCCTCCTTCTCCTGTG-3'), region B (Fw 5'-GTTGCCACTCCCAGACTTG -3', Rv 5' CTCTGCTCACCACAAC-CTCTG -3'), and region C (Fw 5'- CGCCGCGCGCCCGGCCCC -3', Rv 5'-GCACAAGGCCGCCAGCTC -3'). qPCR was performed using AbsoluteTM QPCR SYBR green ROX Mix (Abgene) on an ABI7900HT and analyzed. To calculate the fold induction/reduction of histone marks we used the formula: percentage input=2^(Ctinput -CtChIP)*dilution factor*100.

To calculate the fold induction/reduction of histone marks we normalized % of input for each mark to % of input of pMX empty.

To determine the specificity of the ZFP, we performed ChIP sequencing with the antibody against the HA-tag. For ChIP-Seq, the DNA fraction obtained by ChIP was purified and subjected to massive parallel sequencing. Sample preparation including barcoding was performed using a Mondrian SP (NuGEN Technologies Inc, CA, USA) and the Ovation SP Ultralow Library system (NuGEN). Fragments were selected ranging from 250 to 370 bp (including primer sequences) and subjected to paired-end sequencing on an HiSeq2000 (Illumina). Resulting reads were aligned to the human reference genome (NCBI37.2) using NextGENe V2.3.3 (SoftGenetics, LLC, PA, USA). Peak regions were identified using the peak identification algorithm in this software package.

Cell proliferation

To analyze the effects on cellular metabolism after inducing histone marks, seeded cells in 96-well plates were transduced and incubated at 37°C. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reagent (Sigma), was added to the wells every 24 hours (for 24-120 hours or for five days as indicated). After 3 hours and 45 minutes incubation at 37°C, the medium was aspirated and MTT crystals were dissolved in Dimethyl sulfoxide (DMSO)

(Merk, Darmstadt, Germany). The optic density was detected at a wavelength of 520 nm using Varioskan microplate spectrophotometer (Thermo scientific).

Clonogenic assay

The clonogenic assay was conducted to determine the capability of a single cell to grow into a colony. Transduced cells (3000 cells/well) were plated in 6-well plates and incubated at 37°C for 4 weeks. The colony-forming capacity was detected by staining colonies with Coomassie blue (Sigma). The number of colonies (including at least 50 cells) was counted using phase-contrast microscopy.

Co-treatment with lapatinib

To analyze the effect of pMX-constructs on sensitivity of cells to lapatinib, SKOV3 cells were seeded in 96-well plates; treated with 1 μM/L of lapatinib and transduced with pMX-constructs. Co-treated cells were incubated 120 hours at 37°C. MTS reagent (Promega) was added to the wells after 120 hours. After an incubation of 3 hours and 45 minutes at 37°C, the optic density was detected at a wavelength of 490 nm using Varioskan Flash (Thermo scientific, Fermentas).

Statistics

Results were analyzed for significance using the Student's t test or paired t test for sorted and co-treated cells. Significance was determined as $P < 0.05$.

Results

Her2/neu expression, epigenetic modifications and modulation of Her2/neu gene expression by ATFs in the panel of cell lines

We selected SKBR3 and SKOV3 as Her2/neu-over expressing cell lines, MDA-MB-231 and MCF-7 as Her2/neu low-expressing cell lines. Her2/neu expression was measured at protein and RNA level (Fig. 1A & B). The 29 CpGs located in a 400 bp region (-143 to +251) surrounding the E2C-binding site were unmethylated in all cell lines (data not shown). Assessment of histone H3 acetylation mark at the Her2/neu promoter (-146bp to -60bp relative to the transcription start site) showed that this mark enriched in Her2/neu positive cell lines (SKBR3, SKOV3) and not in Her2/neu negative cell lines (MDA-MB231, MCF7; data not shown).

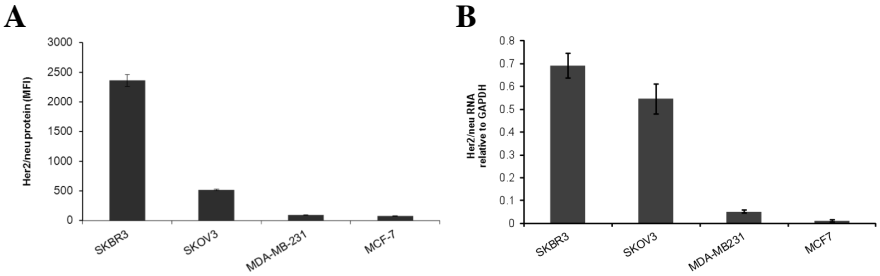

Figure 1. Her2/neu expression in the cancer cell line panel. (A) Measurement of Her2/neu expression at protein level was performed by flow cytometry. The mean fluorescence intensity (MFI) of Her2/neu protein level in cell line panel is the average (±SEM) of three independent experiments. (B) Measurement of Her2/neu expression at RNA level was performed by q-RT-PCR. The mean is the average (±SEM) of three independent experiments.

The effects of E2C-based ATFs on Her2/neu expression were determined in the Her2/neu positive- and Her2/neu negative cell lines (Fig. 2). The expression of Her2/neu in ATF-transduced cells was normalized to cells transduced with the pMX empty vector. Downregulation of Her2/neu at protein level was very efficiently obtained by pMX-E2C-SKD in the high Her2/neu-expressing cell lines (SKBR3: 73 ± 4%, P<0.001; SKOV3: 64.5 ± 6%, P< 0.001) and, although to a lower degree, repression was also detectable in the low Her2/neu expressing cell lines [23 ± 8% for MDA-MB231 (P<0.05) and 19 ± 6% for MCF-7 (P<0.05)]. Upon treatment with pMX-E2C-VP64, Her2/neu protein upregulation was obtained in all cell lines ranging from 1.4 fold in MCF7 to 2.2 fold in SKBR3 cells (P<0.001) although significance was not reached in the low expressing cell line MCF7 (P=0.12). Expression of pMX-E2C only did not affect the Her2/neu expression. No effect of expression of pMX-E2C-VP64 was observed on an irrelevant gene (supplementary Fig. S1).

To validate the binding of the E2C ZFP to the Her2/neu gene, we conducted ChIP using a HA-tag antibody. Strong enrichment was observed for region B (131% of input), and some for region A (47% of input), while hardly any enrichment was detected for irrelevant genes (Supplementary Fig. S2, also Fig.3A for the regions). In addition, ChIP-Seq data showed that the Her2/neu ZFP preferably binds to the Her2/neu gene. Focusing on CDS (coding sequences) genes, the highest peak (558 reads) was identified for the Her2/neu gene segment (chr17:37856415-37856847) that indeed contained the target sequence, whereas second CDS gene in ranking showed only about half of the reads (294 reads).

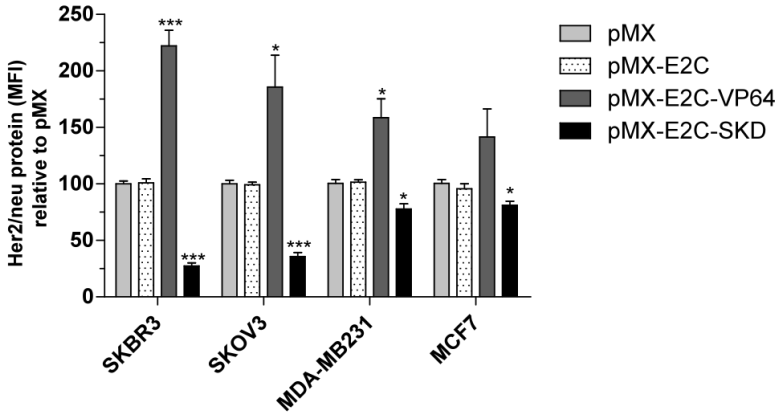

Figure 2. Modulation of Her2/neu expression by Artificial Transcription Factors (ATFs). Modulation of Her2/neu expression at protein level by ATFs was measured by flow cytometry. MFI is the average (±SEM) of three experiments. MFI of all samples in every cell line was normalized to pMX empty (* P<0.05, ** P<0.01, *** P<0.001).

Targeted histone methyltransferase induced H3K9 methylation

Upon transduction of SKOV3 cells with pMX-E2C-G9a, H3K9me2 was efficiently induced on the Her2/neu gene ; 3.2 ± 0.47% of input was recovered for pMX-E2C-G9a transduced cells versus 0.016 ± 0.003% of input for pMX empty transduced cells (P<0.01) at the zinc finger binding site (region C). Also for region B, 5.3 ± 0.38% of input was associated with H3K9me2 versus 0.019 ± 0.0019% for pMX empty transduced cells (P<0.001). For region A, about 1 kb upstream of the ZF binding site, only 0.46 ± 0.083% was recovered for pMX-E2X-G9a transduced cells, which was still 30-fold higher than the recovery of 0.014 ± 0.011% for pMX-transduced cells (P<0.05). There was no induction of H3K9me2 in the tested regions of Her2/neu gene in SKOV3 cells transduced with pMX-E2C, nor with pMX-E2C-SKD (Fig.3B).

H3K9me3 induction was assessed for regions A, B, and C of the Her2/neu gene in SKOV3 cells transduced with pMX-E2C-Suvdel76, pMX-E2C-SKD, pMX-E2C, and pMX empty (Fig. 3C). There was a slight and significant induction of H3K9me3 mark to 0.58 ± 0.15% of input DNA at region C for cells transduced with pMX-E2C-SKD versus 0.11 ±0.12 % of input for pMX empty transduced cells (P<0.05). For pMX-E2C-Suvdel76 or pMX-E2C transduced cells, no significant induction of H3K9me3 mark was detected in any of the regions (Fig. 3C).

Also, levels of H3 acetylation (H3ac) and H3K4me3 marks were assessed in cells transduced with the different pMX constructs. In cells transduced with pMX-E2C-G9a, presence of H3ac was reduced with 87 ± 9.9% in region B ($P<0.01$) and 83 ± 9.5% in region C ($P<0.05$) compared to pMX empty. Also in 1 kb upstream (region A) there was 70 ± 25% reduction of H3ac mark ($P=0.07$). Interestingly, H3ac was almost absent in the targeted region of cells transduced with pMX-E2C-SKD (region A: 98 ± 1.4% ($P<0.05$); region B: 94 ± 9.04% ($P<0.001$); region C: 95 ± 4.3%, ($P<0.05$)). In cells transduced with pMX-E2C-Suvdel76 or pMX-E2C, there was no significant reduction/induction of H3ac detected for regions A, B, nor C (Fig. 3D).

In line with the H3ac data, also the H3K4me3 mark was reduced with 63 ± 12% (region B: $P<0.01$) and 67 ± 23% (region C: $P<0.05$) of the Her2/neu gene in pMX-E2C-SKD transduced SKOVE3 cells (Fig.3D). The observed reduction of H3K4me3 in SKOV3 cells transduced with pMX-E2C-G9a, however, did not reach significance. H3K4me3 mark was not reduced in any of the tested Her2/neu regions in cells transduced with pMX-E2C-suvdel76, nor with pMX-E2C (Fig.3D).

Also for SKBR3 cells transduced with pMX-E2C-Suvdel76, the H3K9me3 mark was only slightly induced in region B (Fig.S.3A). To increase the time of exposure to the E2C fusion constructs and to diminish the diluting effects of analyzing also the suboptimally transduced cells, pMX empty- and pMX-E2C-Suvdel76-transduced SKBR3 cells were sorted 15 days after transduction based on GFP expression (an indicator of transduction) and Her2/neu expression. Cells were classified into 3 subpopulations: GFP+, GFP- , and cells with very low expression of Her2/neu (Supplementary Fig. S4A). For sorted pMX-E2C-Suvdel76-transduced SKBR3 cells, GFP- and GFP+ cells showed 12% and 40% downregulation of Her2/neu protein, respectively, compared to the sorted pMX empty (Supplementary Fig. S4B). Cells with very low expression of Her2/neu (7.3% of total viable cell population) showed 50- to 500-fold less expression of Her2/neu compared to GFP+ and GFP- cell populations (Supplementary Fig. S4A). All three cell populations were sub-cultured but Her2/neu-low expressing cell population grew slowly and died. During sub-culturing, sorted cell populations were assessed for Her2/neu- and GFP expression in different time points for 45 days; during this period of time, the GFP+ cells maintained their lower expression profile

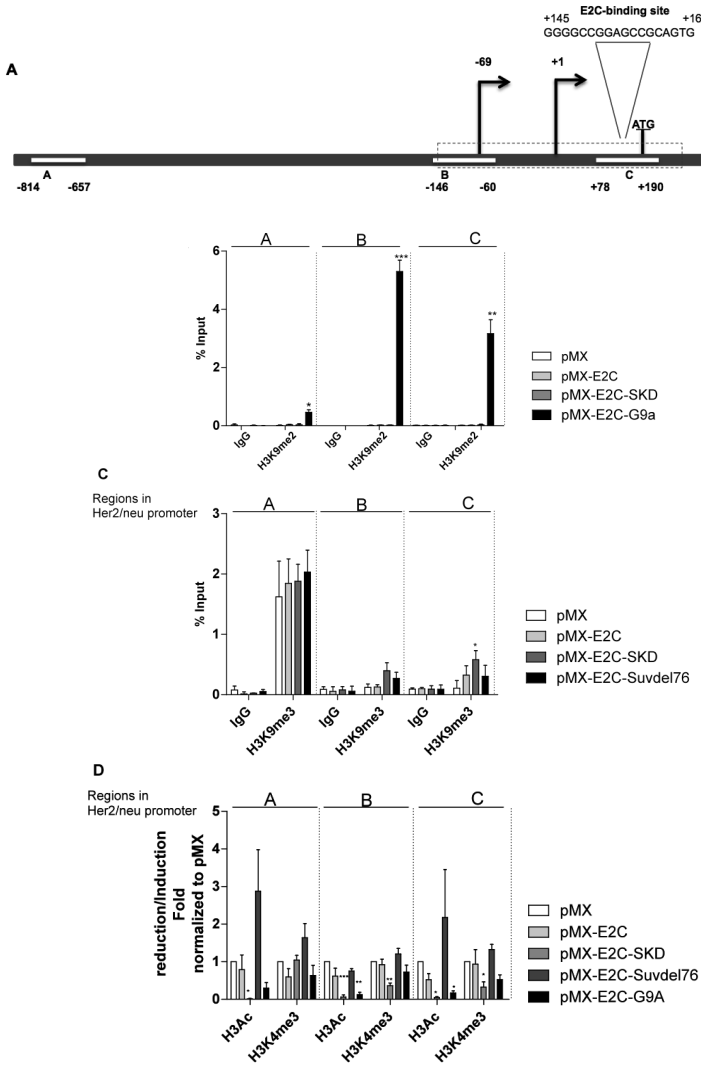

Figure 3. Histone modification changes after targeting histone methyltransferases to the Her2/neu gene. Induction of histone marks by E2C-fusions was assessed by quantitative ChIP for regions A, B, and C of the Her2/neu promoter: data are presented as % of input. The bars represent the average number of (±SEM) of 3 independent experiments. IgG was used as negative control for immunoprecipitation. (A) Scheme of Her2/neu promoter upstream of the E2C-binding site and Exon1 of Her2/neu gene. Transcription start sites (TSS) are located at +1 and -69, the translation start site shown as ATG is at position +178 [49]. The triangle represents Her2/neu-ZFP (so called E2C) binding site (+145 to +163) and the target sequence of E2C is shown. The dashed rectangle box represents the amplified region by bisulfite primers containing 29 CpGs (black lines). The regions amplified in ChIP assays (A, B, and C) are depicted in white. Effect of the different pMX-constructs on (B) H3K9me2; (C) H3K9me3 and (D) Histone acetylation and H3K4me3. To compare reduction of H3-acetylation and H3K4me in different constructs, % of input of these marks was normalized to % of input of pMX empty (* P<0.05, ** P<0.01, *** P<0.001).

for Her2/neu (P<0.05) although a slight increase was observed (Supplementary Fig. S4C). Interestingly, GFP expression of the GFP+ cells compared to the GFP- cells maintained at a constant ratio during sub-culturing (Supplementary Fig. S4D). 30 days after sorting, ChIP on sorted cell populations showed that H3K9me3 mark in the region B of Her2/neu promoter was more pronounced in the GFP+ cell population compared to the GFP- cell population; also there was more enrichment of H3K9me2 in GFP+ cells compared to GFP- cells (Fig. S3B).

Methylation of histone H3 of lysine 9 is associated with Her2/neu downregulation

To assess the effect of inducing repressive marks on Her2/neu gene expression, Her2/neu protein expression was assessed in cell lines transduced with pMX-E2C fused to epigenetic effector domains (Fig. 4A). Her2/neu was significantly downregulated in transduced cancer cells with pMX-E2C-G9a. Downregulation ranged from 25 ± 6% in SKBR3 (P<0.01) and 31 ± 9% in MDA-MB231 (P<0.01) to 54 ± 2.5% in SKOV3 (P<0.0001) which was comparable to the effect of SKD in SKOV3. There was no downregulation of Her2/neu in SKOV3 cells transduced with inactive catalytic G9a, pMX-G9a W1050A (Fig 4B). Transduction with pMX-E2C-Suvdel76 also resulted in downregulation of Her2/neu expression [20 ± 3.5% in SKBR3 (P<0.01), 27 ± 10% in SKOV3 (P<0.05), and 21 ± 5% in MDA-MB231 (P<0.01)], but downregulation was less efficient than observed for pMX-E2C-G9a. For the very low Her2/neu expressing cell line MCF7, no further downregulation of Her2/neu protein could be detected after transduction with pMX-E2C-G9a, but there was a 11 ± 1.11 % downregulation of Her2/neu induced by pMX-E2C-Suvdel76 (P<0.05; Supplementary Fig. S5). Downregulation of Her2/neu protein was reflected by the decrease in RNA expression: for pMX-E2C-G9a, RNA levels were decreased by 54 ± 6.7% (P<0.001) in SKOV3 which was comparable to the effect of E2C-SKD and 21 ± 6.1% in SKBR3 (P<0.01). pMX-E2C-Suvdel76 resulted in 33 ± 11.1% downregulation of Her2/neu in SKOV3 (P<0.01) and 30 ± 23% in SKBR3 (Fig. 4C).

To determine whether repression could be further increased, the GFP+ SKBR3 cells (sorted pMX-E2C-suvdel76 transduced SKBR3, showing 42% downregulation of Her2/neu protein compared with pMX empty) were supertransduced

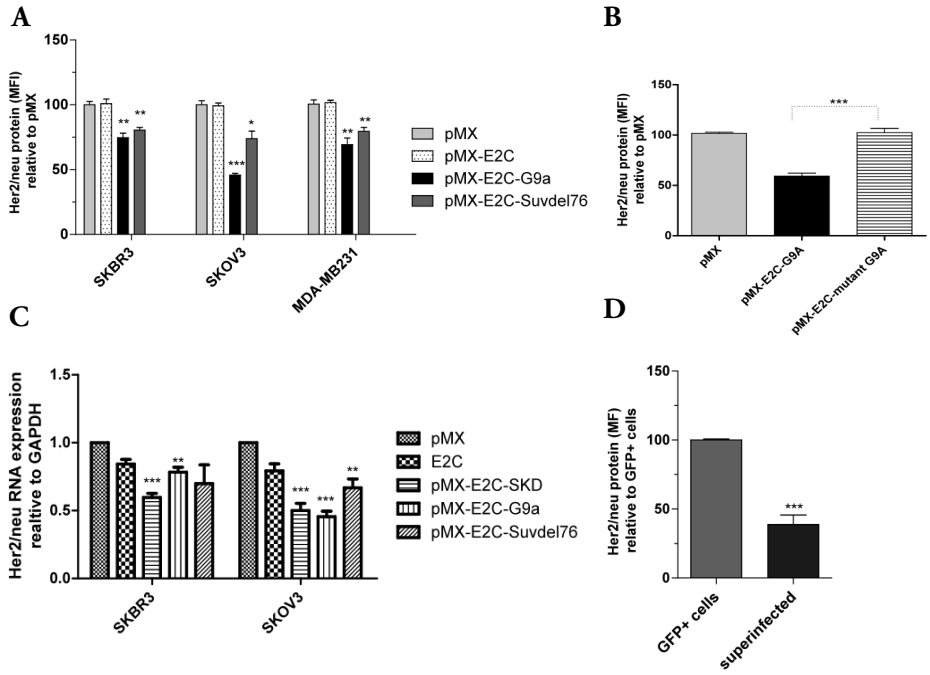

Figure 4. Downregulation of Her2/neu by epigenetic editing. **(A)** MFI representing Her2/neu expression was measured by flow cytometry. MFI is the average (±SEM) of at least three independent experiments. MFI of all samples was normalized to pMX epmty. (B) Effect on Her2/neu expression after targeting mutant G9a to Her2/neu in SKOV3 cells (C) Her2/neu expression at RNA level measured by q-RT-PCR is shown for SKBR3 and SKOV3. The relative measured RNA was normalized to pMX empty. The mean is the average (±SEM) of three independent experiments. (D) MFI representing Her2/neu expression is the average (±SEM) of three independent experiments. MFI of supertransduced cells with pMX-E2C-G9a was normalized to MFI of high transduced sorted SKBR3 cells (GFP+). (* P<0.05, ** P<0.01, *** P<0.001).

(retransduced) with pMX-E2C-G9a. Supertransduced cells showed a further 61 ± 12% (p< 0.001) downregulation of Her2/neu compared with GFP+ cells (Fig. 4D). Thus the observed downregulation is expected to reach above 80% downregulation when it is compared with pMX empty as a control.

Induced downregulation of Her2/neu inhibited cell growth

The effect of induced Her2/neu downregulation on metabolic activity and colony-forming capacity was investigated, SKOV3 cells were seeded and transduced in 96-well plates with pMX empty, ZFP only (pMX-E2C), pMX-E2C-SKD, pMX-E2C-VP64, pMX-E2C-G9a, and pMX-E2C-Suvdel76,

and measured every 24 hours. Her2/neu downregulation was associated with a significant decreased in metabolic activity at day five (42 ± 3% for -G9a, 40 ± 2% for -Suvdel76, and 35 ± 1% for SKD fused to pMX-E2C compared to pMX empty; P< 0.05) (Fig. 5A). Such growth inhibitory effects were confirmed in the clonogenic assay, where equal numbers of SKOV3 cells transduced with pMX empty, pMX-E2C fused to SKD, VP64, G9a, and Suvdel76 were replated in 6-well plates. pMX-E2C-G9a transduced cells showed the lowest numbers of colonies (on average 37 ± 6, in comparison to 76 ± 8 for pMX empty transduced cells (P<0.001) and 55 ± 13 for pMX-E2C-SKD (P<0.05))(Fig.5B).

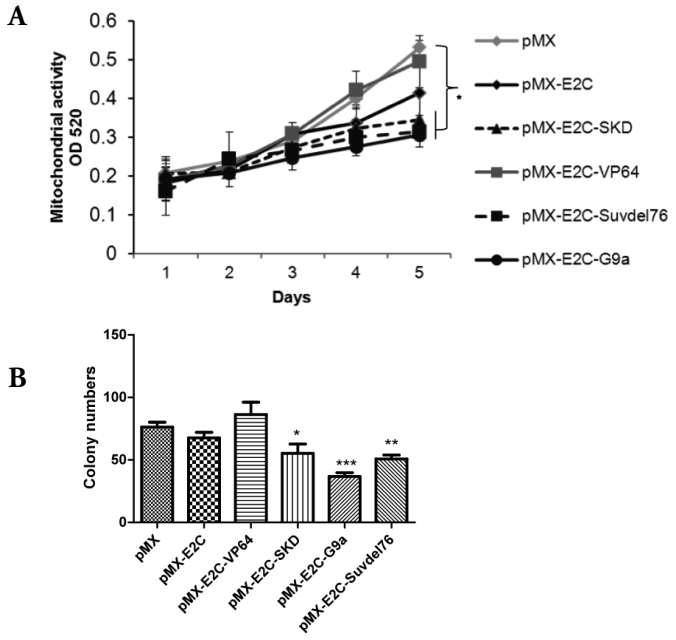

Figure 5. Decrease of cell proliferation upon induction of H3K9 methylation. (A) A five day-cell proliferation assay was performed on transduced SKOV3 cells with pMX empty, pMX-E2C, pMX-E2C fused to transcription effector domains (SKD, VP64) and histone methyltransferases (G9a, Suvdel76). Absorption is the average (±SD) of three independent experiments (* P<0.05). **(B)** Transduced SKOV3 cells were replated and allowed to form colonies for 4 weeks. Number of colonies is the average (±SEM) of at least three independent experiments and representing colonies consisted of at least 50 cells.

Induced downregulation of Her2/neu improved the cell growth inhibitory effect of lapatinib

To assess whether combination of targeted downregulation of Her2/neu at DNA level and protein level results in an improved cell growth inhibition, we cotreated SKOV3 cells with the different pMX constructs and lapatinib. 1µmol/L lapatinib did not reduce growth of treated cells but there was an improvement in cell growth inhibition induced by 1µmol/L lapatinib when combined with pMX-repressive constructs. When compared to cell growth inhibition induced with repressive pMX-constructs, the cotreatment results in 27 ± 16% ($P<0.05$) for pMX-E2C-SKD, 25 ± 3.8%, ($P<0.05$) for pMX-E2C-G9a, and 26 ± 4.1%, ($P<0.05$) for pMX-E2C-Suvdel76.

Overall, the cotreatment resulted in an efficient growth inhibition when compared to pMX empty and lapatinib cotreatment [(pMX-E2C-SKD: 43 ± 8.0% ($P<0.05$), pMX-E2C-G9a: 49 ± 10%, ($P<0.05$), pMX-E2C-Suvdel76: 45 ± 11%, ($P<0.05$); Fig.6)].

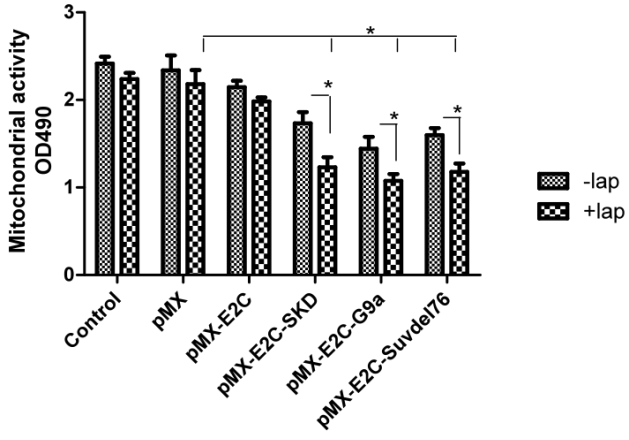

Figure 6. Cell proliferation inhibition upon co-treatment of SKOV3 cells with ZFP constructs and lapatinib. Cell proliferation assay was performed on SKOV3 cells co-treated with pMX-constructs and Lapatinib. Untreated SKOV3, SKOV3 single treated with lapatinib, and transduced SKOV3 cells with pMX-constructs were used as controls. Absorption is the average (±SEM) of three independent experiments.

Discussion

Using a Her2/neu-targeting ZFP fused to a histone methyltransferase, we showed that the intended repressive histone mark was induced. This epigenetic editing was associated with a decrease in H3ac and resulted in Her2/neu downregulation. The degree of repression was comparable with repression obtained for SKD (a transient noncatalytic transcriptional repressor). For SKD, the gene repression was associated with H3 deacetylation and a lowering in H3K4me3.

The repression of Her2/neu by induced H3K9me2 is in line with descriptive studies of Her2/neu epigenetics in breast cancer. For example, Lim and colleagues showed that Her2/neu is a direct target of the histone demethylase KDM1, which removes methyl groups of dimethylated H3K9 [34]. In their study, siRNA-mediated knockdown of KDM1 lowered the accumulation of KDM1 on the Her2/neu promoter resulting in an increase in H3K9 methylation, a decrease in Her2/neu expression, and an inhibition in proliferation of the treated breast cancer cell lines [34]. Another study by Mishra and colleagues described phosphorylation on serine 10 of histone H3 and acetylation of histone H3 and H4 in the promoter region of Her2/neu gene to be positively associated with Her2/neu expression[35]. The role of histone acetylation was confirmed by treating the breast cancer cells with a histone deacetylase inhibitor, trichostatin A, which increased Her2/neu expression and was associated with H4 acetylation, but not H3ac of Her2/neu promoter [35]. Our data on reduced H3ac association with Her2/neu seem in contrast with this study which showed that H3ac was not decreased upon Her2/neu downregulation, but this might be explained by the different causes of repression [35].

In contrast to the study conducted by Mishra and colleagues, Fuino and colleagues showed that histone deacetylase inhibitor LAQ824 downregulates Her2/neu expression without an increase of H3 and H4 acetylation on Her2/neu promoter [36]. Further research clarified that LAQ824 treatment resulted in activation of a transcriptional repressor of Her2/neu, attenuation of pAKT, c-Raf-1, phosphorylated mitogen-activated protein kinase levels, as well as in acetylation of HSP90. Because hyperacetylation of HSP90 results in an unstable chaperon complex, LAQ824 indirectly marked Her2/neu protein for proteosomal degradation [36].

On the basis of these and other similar studies, agents to inhibit epigenetic

enzymes are currently extensively explored. Some of these inhibitors, so called epi-drugs, have been FDA approved for treatment of hematologic malignancies[37,38]. Functioning genome-wide, however, these enzyme inhibitors are hardly predictable in terms of their effect in up- or downregulation of genes. So, unwanted upregulation of tumor related genes by epidrugs is one of their disadvantages [39]. Despite such disadvantages, epidrugs have recently also been shown to be beneficial for patients with solid tumors [40]. Also in patients with breast cancer, many clinical trials are ongoing to test epi-drugs [41]. In view of their genome-wide effects, together with reported effects on nonchromatin proteins, the gene-specific modification of epigenetic marks as presented here might be advantageous.

In the present study, catalytic domains of epigenetic enzymes were fused to a previously validated Her2/neu targeting ZFP (E2C; [25]) to rewrite the epigenetic context of the Her2/neu gene. The Her2/neu ZFP has previously been indicated to be quite selective for Her2/neu (ErbB2) as upon fusion to KRAB no effect was observed on the expression of ErbB1 or ErbB3 [25], even though their promoters contain similar sequences. Here, we added that also on irrelevant genes no enrichment of the fusion protein could be detected and interestingly genome-wide data showed that the Her2/neu ZFP is preferentially bound to Her2/neu gene. The domains of G9a and SUV39-H1 used in our ZFP fusion complexes have been previously reported to result in H3K9 methylation of the promoter of VEGF-A gene and in downregulation of this gene [30]. We showed here that the approach is also suitable for downregulation of an overexpressed oncogene.

Cells transduced with pMX-E2C-SKD showed efficient downregulation of Her2/neu, which was associated with an efficient removal of H3ac and H3K4me3, but not with relevant induction of H3K9me. Transduction with pMXE2C-G9a induced H3K9me2 and repressed gene expression almost as efficiently as SKD. This G9a-induced repression was again associated with a reduction in H3ac, which was not found for the Suv39-H1 domain. A role of SKD in epigenetic remodeling has been suggested but it is controversial, and even upregulation of expression by targeting SKD has been reported [42]. With respect to repression, it has been shown in embryonic stem cells that KRAB (as part of SKD) can indirectly induce de novo DNA methylation via recruiting its cofactor, KAP1[43].

In addition, KRAB/KAP1 can spread H3K9me3-containing heterochromatin in adult and embryonic stem cells [44]. Despite these reported functions of KRAB in epigenetic regulation, KRAB-induced repression is generally considered to be transient. Indeed, SKD fused to ZFP targeting the SOX2 promoter has been shown to induce DNA methylation, but thisDNA methylation was not lasting when ZFP-SKD was no longer expressed in cells [24]. Interestingly, targeting of a DNA methyltransferase to this gene results in more sustained downregulation and DNA methylation compared with targeting the repressive transcriptional modulator SKD [28].

The induction of H3K9me2 in cells transduced with pMX-E2C-G9a in our study was associated with a reduction in H3ac. Interestingly, the G9a-induced H3K9me2 mark was more specifically localized to the TSS proximal region than the E2C-SKD or E2C-G9a induced hypoacetylation, which is spreading across the 1 kb region analyzed upstream. It is tempting to speculate that the acetylation effects are the indirect consequence of the Her2/neu gene being repressed, whereas the methylation mark is directly written by the E2C-G9A domain and therefore restricted to the target site. In addition to the induction of repressive histone mark on Her2/neu promoter and reduction of histone active marks, we showed that the degree of decreased Her2/neu expression is efficient enough to give rise to significant metabolic activity inhibition and also less colony-forming capacity. These results are consistent with the study conducted by Lim and colleagues [34] confirming that H3K9me2 plays a critical role in Her2/neu modulation in breast cancer.

Transduction with pMX-E2C-Suvdel76 was not efficient in inducing its intended mark, which might be explained by the fact that SUV39-H1 preferentially uses monomethylated H3K9 as the substrate to induce H3K9 trimethylation [45]. SKD is also known to indirectly induce H3K9me3 marks, but no efficient enrichment of H3K9me3 by SKD was observed. As Suvdel76 has been used to efficiently repress VEGF-A expression [30], the effect of epigenetic editors might be gene- and/or chromatin context-dependent warranting systematic studies to provide more insights into the general applicability of the approach. Indeed, there are various factors affecting the downregulation/upregulation of a gene by a ZFP fused to a transcriptional effector domain, and even positiondependent effects have been described for one gene (ErbB2; [46, 47]).

To address the added value of gene repression for conventional protein targeting therapies, we combined Her2/neu gene repression with treatment with lapatinib, which is a drug targeting the Her2/neu receptor. We could show a sensitization of cells by lapatinib for the repressive effects of ZF-constructs (pMX-E2C-SKD, pMXE2C-G9a, pMX-E2C-Suvdel76). Although, 1 µmol/L lapatinib was not effective in inhibiting the growth of SKOV3 cells by itself, the combination of Her2-repressive pMX constructs and lapatinib resulted in an increased inhibition of metabolic activity. Novel approaches to target gene expression as described here might thus improve the effect of conventional protein-targeting drug treatment regiments.

As many (onco)genes are overexpressed in all different stages of cancer, epigenetic editing can be extrapolated to other genes playing important roles in breast and ovarian cancer, including undruggable genes. In this respect, the undruggable SOX2 successfully repressed by a ZFP-SKD [24] was more permanently repressed by targeting a DNA methyltransferase [28]. Also, other epigenetic effector domains have been fused to ZFPs or to other types of DNA-targeting tools and were effective in gene expression modulation via their capacity for induction or reduction of their specific epigenetic marks. These effector domains include DNA methylases, histone methyltransferases, and histone acetyltransferases [27]. In conclusion, the induction of epigenetic marks on a target gene using efficient rewriters of epigenetic context can result in gene expression modulation. As epigenetic marks have the potential of being mitotically stable, this study describes a powerful approach to efficiently silence (onco)genes.

Author's contributions

Fahimeh Falahi has contributed by designing, setting up, performing experiments, data analysis, and writing the manuscript. Christian Huisman has participated in setting up the experiments and editing the manuscript. Hinke G K Kazemier has participated in data collection. Pieter van der Vlies has participated in ChIP sequencing experiment. Klaas KoK has analyzed ChIP seq data and edited the manuscript. Geke A. P. Hospers has participated in editing the manuscript. Marianne G. Rots has contributed by designing and supervising the project and editing the manuscript.

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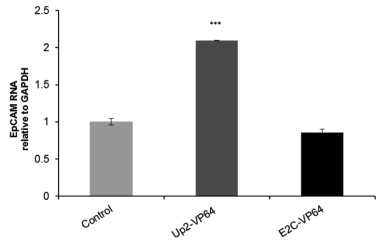
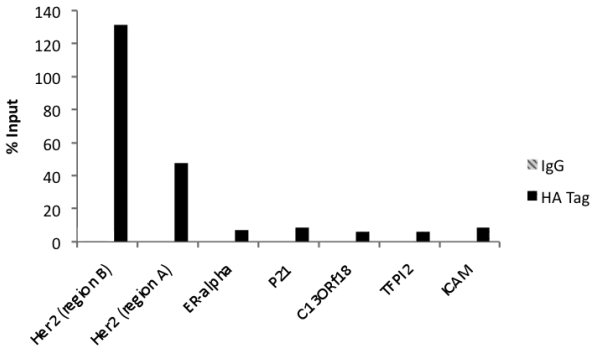
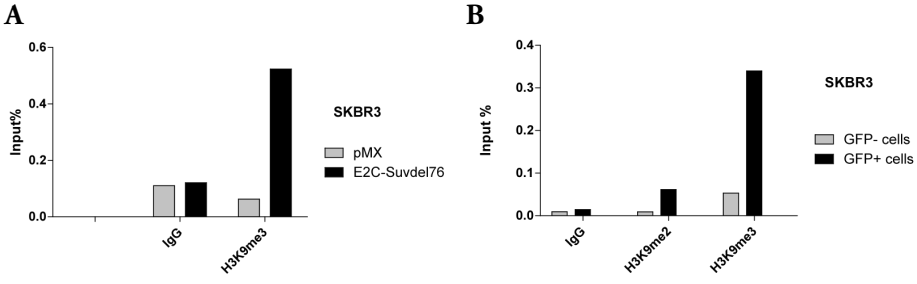


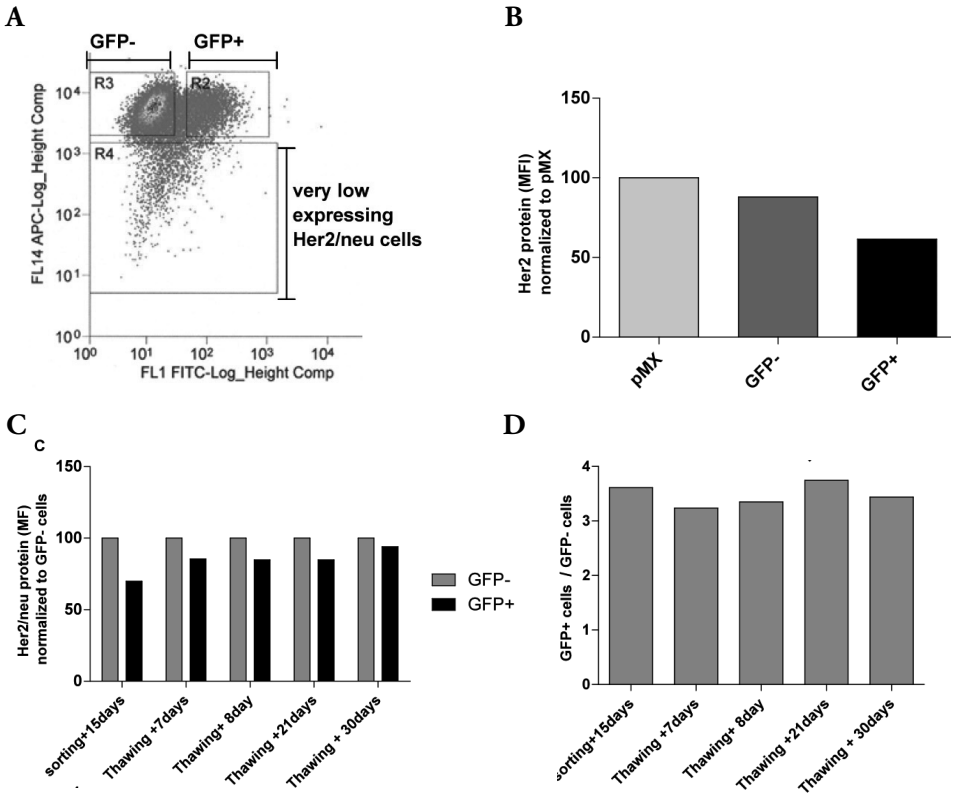
Supplementary Figure 1: Her2/neu Zinc Finger Protein fused to VP64 does not have any effect on an irrelevant gene.



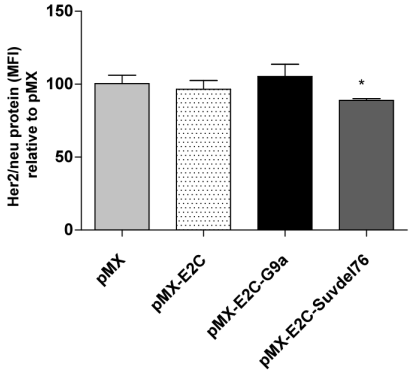
Supplementary Figure 2 : Specific binding of HER2/neu ZFP (E2C).



Supplementary Figure 3: Induction of H3K9me3 in region B of Her2/neu gene in SKBR3 cells.



Supplementary Figure 4: Sorted cells maintain their profile of Her2/neu and GFP expression.



Supplementary Figure 5: Her2/neu downregulation status in MCF7 by pMX-E2C fused to histone methyltransferase domains.

Chapter 4

Epigenetic Editing of estrogen receptor-alpha gene in breast cancer as an innovative tool to modulate its expression level

To be submitted

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Abstract

Estrogen receptor alpha (ER-α) gene (ESR1) is overexpressed in ~75% of breast cancers. Breast tumors expressing ER-α can benefit from endocrine therapy. Loss of ER-α expression is one of the mechanisms by which resistance to endocrine therapy can develop. Moreover, 25% of breast cancer patients have tumors that lack ER-α expression at diagnosis, and these patients can thus not benefit from endocrine therapy.

As epigenetic modifications such as DNA hypermethylation are known to silence ESR1, we aimed to downregulate ESR1 using induction of repressive epigenetic modifications (DNA methylation and H3K9me2). We also aimed to upregulate ESR1 expression using artificial transcription factors which might be interesting, in the future, as a gene-targeting-strategy for re-sensitizing cancer cells to endocrine therapy. In order to target ESR1, we designed three 6-finger zinc finger proteins (ZFPs). Retroviruses containing genes encoding ZFPs fused to transcription effector domains (super KRAB domain [SKD]/VP16 tetramer [VP64]) or epigenetic effector domains (a DNA methyltransferase/a histone methyltransferase and its mutant) were used for transducing cancer cells. The expression and epigenetic modifications status of ESR1 were assessed in transduced cells as well as in untreated cells. DNA methylation was induced at the ESR1 locus using the ZFP fused to a DNA methyltransferase and it was correlated with 24.4 ± 3.81 % downregulation of ESR1, whereas, the ZFP fused to a histone methyltransferase did not induce ESR1 downregulation. ZFPs fused to SKD also resulted in up to 39.4 % ESR1 downregulation. ESR1 downregulation was associated with the reduced colony formation capacity of cells. ESR1 was successfully upregulated using the ZFPs coupled to VP64. This study suggests that, targeted DNA methylation of ESR1 can be used for downregulating this gene. Moreover, our results showed feasibility of both down- and upregulation of ESR1 in a targeted manner.

Introduction

The ESR1 gene encodes estrogen receptor-alpha (ER-α) from multiple promoters. Because of the several promoters regulating ESR1, there are various ESR1 transcripts, and together with a multitude of spliced isoforms, this makes ESR1 to be known as a complex gene [1].

ER-α functions as a transcription factor which is activated upon binding to 17ß-estradiol (E2). The active form of ER-α can bind to estrogen response elements (EREs) in promoter regions of target genes. In such a way, ER-α mediates the proliferative effect of E2 to several tissues, especially breast [1]. However, not all of the genes regulated by ER-α contain the EREs; for these genes, ER-α does not bind directly to DNA but interacts with transcription factors [2]. Alternatively, ER-α can interact with epigenetic modifier enzymes which result in epigenetic changes on the ER-α target genes [3]. Moreover, there are interactions between ER-α and critical cell growth signaling pathways. For instance, crosstalk exists between ER-signaling pathway and HER tyrosine kinase family [4]. Altogether, ER-α seems to be a key regulator of cell growth and has a vital role in the maintenance of normal state of cells.

Dysregulation of ESR1 has been found in several types of cancers including breast cancer. ER-α is overexpressed in about 75% of breast cancers (ER-positive) [5]. Alternatively, ~25% of breast cancers lack ER-α at initial diagnosis (ER-negative[5], and 16-40% of ER-α positive primary tumors will lose ER-α expression in metastatic disease [6, 7].

Interestingly, ER-α is a strong predictor for response to endocrine therapy. Endocrine therapy is a very commonly used therapy for patients with ER-positive breast cancer by blocking the function of ER-α [8] and, thereby inhibiting ER-α-dependent growth of cancer cells. Adjuvant endocrine therapy nearly halves the recurrence risk (relative risk 0.53) for patients with ER-positive primary breast cancer [9]. As a first line-treatment in metastatic disease, objective response (partial and complete response) is observed in about 33% of patients[10], and clinical benefit rate (including stable disease >6 months) can be as high as 67-73% [11]. However, eventually all patients will become resistant to endocrine therapy.

Endocrine therapy resistance has been extensively investigated to reveal the resistance mechanisms [12]. There are several described resistance mechanisms

including overexpression of HER2/neu, overactivation of growth signaling pathways, and overexpression of ER-α coactivators [13]. These mechanisms are involved in phosphorylation of ER-α and its coactivators, hence ER-α activation[4]. Since endocrine therapy targets and blocks ER-α, but leaves ESR1 gene active which can continuously produce ER-α, we reasoned that the resistance of ER-positive breast cancer can be partly due to the hyperactive gene and, therefore, we aimed to target and repress ESR1 gene.

ER-negative breast cancer patients do not benefit from endocrine therapy. In this type of breast cancer, ESR1 is not expressed mainly because of DNA hypermethylation of the promoter and exon 1 of ESR1 [14-18]. This suggests that epigenetic modifications are involved in the regulation of ESR1 expression. As epigenetic modifications are reversible and mitotically inheritable, they are attractive therapeutic targets. To overcome endocrine therapy resistance in ER-negative breast cancer, epigenetic drugs (epi-drugs) which target epigenetic enzymes have been used to re-express ESR1 [19-22]. In this regard, application of DNA methyltransferase inhibitors and histone deacetylase inhibitors are associated with re-expression of ESR1 in breast cancer and re-sensitivity to endocrine therapy [19, 23, 24]. Despite the advantages of epi-drugs, their function is genome-wide and they can upregulate prometastatic genes [25]. Therefore, the development of approaches with more gene-specificity is required. An alternative to re-express the gene of interest in a targeted manner can be provided by artificial transcription factors (ATFs). An ATF is a DNA binding domain coupled to a transcription effector domain. ATFs are able to target and transiently modulate target gene expression.

Moreover, the modulation of gene expression can be achieved via changing epigenetic modifications of the target gene (Epigenetic Editing). To change (write or erase) epigenetic modifications, catalytic domains of epigenetic enzymes can be fused to DNA binding domains such as triplex forming oligos (TFO), zinc finger proteins (ZFPs), transcription activator-like domains (TAL domains), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) [26]. The epigenetic effector domains can be either catalytic domains of epigenetic enzymes[26] or epi-drugs [27]. So far, feasibility of Epigenetic Editing is shown by us in modulation of several genes for instance by inducing DNA methylation[28,29]. We also previously induced an inactive histone methylation mark on HER2/

neu[30] and active localized DNA demethylation on ICAM-1 [31] to modulate gene expression. Other studies, recently validated this approach [32-35].

In this study, we aimed to target ESR1 and modulate its expression in ER-positive and ER-negative cancer cells using ATFs. In addition, because of the correlation of epigenetic modifications with ESR1 expression, we set out to use Epigenetic Editing to downregulate ESR1 expression. After modulating ESR1 using ATFs, we demonstrated induction of DNA methylation on ESR1 which was correlated with downregulation of ESR1. The induced downregulation of ESR1 using Epigenetic Editing and the feasibility of both up- and downregulation of ESR1 suggest that Epigenetic Editing can be used to re-express ESR1 in ER-negative cancer cells, in future.

Methods and materials

Cell culture

Breast cancer cell lines with different levels of ESR1 expression (SKBR3, MDA-MB231, and MCF7), ovarian cancer cell line (SKOV3), as well as HEK293T cells were obtained from ATCC (Manassas, VA, USA). All cells were cultured in DMEM (BioWhittaker, Walkersville, MD, USA) supplemented with 2mM L-glutamine, 50 µg/ml gentamycin, and 10% FBS (BioWhittaker) and incubated at 37 °C in a humidified 5% CO2-containing atmosphere.

Constructs and retroviral transduction

Three ZFPs were designed to target ESR1 at exon 1 and its proximal promoter[36]; these three ZFPs are called ER1, ER2, and ER3 throughout this manuscript (Figure1). Binding sites of ER1 and ER2 are close to transcription start site 1 (TSS1). TSS1 stands for the TSS of ESR1 transcript variant 1 [37]. Target sequences on ESR1 gene are ER1: GCAACAGTCCCTGGCCGT, ER2: GGAGCTGGCGGAGGGCGT and ER3: GAACGAGCTGGAGCCCCT. ATFs or Epigenetic Editing tools were ZFPs fused to repressor/activator transcriptional effector domains (super KRAB domain [SKD]/ tetramer VP16 [VP64]) or epigenetic effector domains. The ZFP coupled to the effector domain was cloned into the pMX-IRES-GFP retroviral vector containing a HA-tag, a nuclear localization signal, and the Green Fluorescent Protein (GFP) sequence.

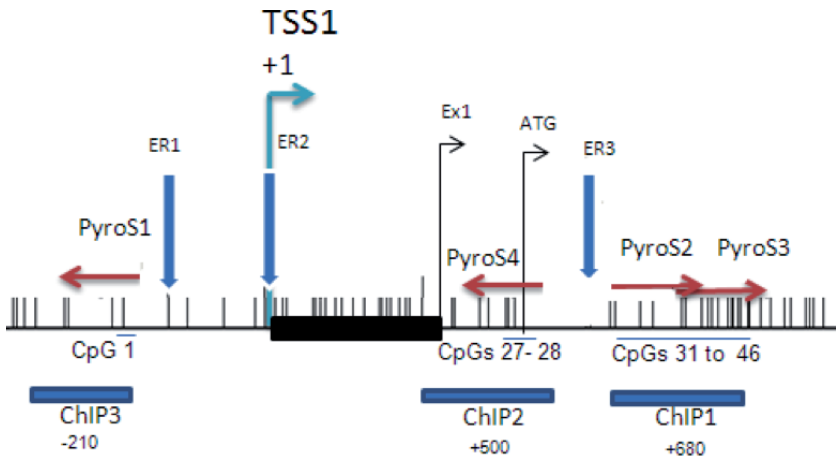

Figure 1. Scheme of ESR1 gene. TSS1 represents the transcription start site of transcript variant 1, EX1 stands for exon 1, and the black box represents the proximal promoter to exon 1. Blue vertical arrows represent binding sites of ER-ZFPs (ER1, ER2, and ER3). Black vertical lines are symbols for CpGs. Regions sequenced by pryosequencing are shown by red arrows; number of assessed CpGs are mentioned. The regions amplified in ChIP assays are depicted as blue rectangles.

Epigenetic effector domains were the catalytic domains of DNA methyltransferase M.SssI [38, 39] and histone methyltransferase G9a [30]. The mutant G9a as described previously [30] was used as a control for the function of G9a. pMX empty vector and pMX-ZFP with no effector domain (pMX-ER-NoED) served as controls of experiments. Hek293 cells were transfected with pMX-constructs. Supernatant of HEK293T cells containing virus was harvested 48 and 72 hours after transfection. Host cells seeded in 6-well plates were transduced on 2 consecutive days with the supernatant of transduced HEK293 cells supplemented with 6 mg/mL polybrene (Sigma) and 10% fetal calf serum. Indications of transduction efficiency (GFP) and the effect of the pMX-constructs on ESR1 RNA were read out 4 days after the first transduction.

Fluorescence Activated Cells Sorting (FACS)

Transduced host cells were harvested 4 days after the first transduction. Calibur flow cytometry (Beckton Dickinson Biosciences, San Jose, CA, USA) was used to assess GFP expression.

Western Blotting

Transduced MCF7 cells were lysed using RIPA buffer and proteinase inhibitor complex. Using standard western blotting, the membrane was blocked with 5% dried milk in TBS supplemented with 0.1% Tween-20 (TBS-T) for one hour. Then, the blot was incubated with mouse monoclonal anti-HA tag antibody (Covance, Rotterdam, the Netherlands) at 4°C overnight, followed by the detection with horseradish the peroxidase-conjugated (HRP) secondary rabbit anti-mouse and swine anti-rabbit antibodies (Dako, Glostrup, Denmark). Visualization was done using the Pierce ECL2 chemoluminoscence detection kit (Thermo Scientific, Rockford, USA).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted as described previously [30]. A subsequent qRT-PCR reaction was performed (ABIPrism 7900HT, Applied Biosystems, Nieuwekerk, the Netherlands) with 10 ng cDNA using ROX enzyme mixture (Abgene, Surrey, UK), and a commercial primer-probe for the quantification of ESR1 expression (Hs00174860_m1;Applied Biosystems) or primer and probes for GAPDH expression [30]. Data were analysed with SDS 2.1 RQ software (Applied Biosystems) and the gene expression relative to GAPDH expression for each replicate in the same experiment was calculated by the comparative delta Ct method. The relative expression for each sample was compared to that of the control sample and the fold of expression was calculated using the formula ($2^{\wedge(-\Delta\Delta Ct)} = 1/2^{\Delta\Delta Ct}$).

Bisulfite treatment and pyrosequencing

First, genomic DNA of cells was isolated using a standard protocol and DNA was bisulfite-converted (EZ DNA Methylation-GoldTM Kit, Zymo research, Irvine, CA, USA). PCR was performed (Qiagen Pyromark PCR kit) using three Pyro-primer pairs (Pyro1: Fw 5'- biotinylated-AGGGAAGTTGTTTTTT-GGGA-3', Rv 5'- CCCCAACCTCCAACTTTAAATACTAATC -3; Pyro2: Fw AGGGAAAGAGTTGGAGTTTTTGAA-3', Rv5'- biotinylated-TCTCCAAATAATAAAACACCTACT-3'; Pyro3: Fw 5'- biotinylated-GGTAGGGTAGGGGTTAGAGT-3', Rv5'-CTAATACAATAAAACCATC-CCAAATACTT-3'). PCR products were sequenced using Pyromark Q24 machine (Qiagen) and pyro sequencing primers (PyroS1:5'- CTAATCTCCCCAACT-

CATATAC-3'; PyroS2: 5'-TTTGAATAGTTAGTAGTTTAAGAT-3'; PyroS3: 5'-GTAAGTTAGTAGTGTATAATTA-3' (Figure 1); LINE-1 (Long Interspersed Element–1): 5'-AGTTAGGTGTGGGATATAGT-3'; and ICAM-1: 5'-ATTTCCCAACTAACAAAATACCC-3'). ICAM-1 primer was designed to sequence 5 CpGs, however, in our experiments, sequencing result of the first 2 CpGs was reliable.

Chromatin Immunoprecipitation (ChIP) and PCR

ChIP assay was performed using 5 μg of specific ChIP antibodies (histone H3 lysine9 dimethylation, H3K9me2 [07-441], Millipore, Massachusetts, USA, and normal rabbit IgG [ab46540], Abcam, USA) and magnetic beads (Invitrogen) as described previously [30]. Standard and SYBRgreen PCR was performed using designed ChIP primer pairs for the ESR1 (ER1 ChIP Fw: 5'-GAACCGTCCGCAGCT-CAAGATC-3', ER2 ChIP Rv: 5'-GTCTGACCGTAGACCT-GCGCGTTG-3'; ER2 ChIP Fw: 5'-CCTCTAACCTCGGGCTGTG-3', ER3 ChIP Rv: 5'-ATCCCAGATGCTTTGGTGTG-3'; ER3 ChIP Fw: 5'-GCCGTGAAACTCAGCCTCT-3') (Figure1).

Colony forming assay

In order to evaluate the effect of downregulation of the ESR1 in cancer cell growth, transduced cells (1500 cells/well) were plated in 6-well plates and incubated at 37°C for 11 days (MCF7) or 4 weeks (SKOV3). The colony forming capacity was detected by staining colonies with Coomassie blue (Sigma). Counting of colonies (including at least 50 cells) was counted using phase-contrast microscopy.

4-OH-Tamoxifen treatment and cell proliferation assay

To assess the response of cancer cells to 4-OH Tamoxifen (4-OH-TAM) (Sigma), 500 cells were seeded in 96 well plates. 24 hours after seeding, cells were treated with 10 μM of 4-OH-TAM, or with ethanol (EtOH) (controls). 48 hours after treatment, mitochondrial activity as an indicator of cell proliferation was measured using MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) reagent (Sigma), which was added to the wells and after an incubation of 3 hours and 45 minutes at 37°C, the optic

density was detected at a wave length of 490 nm using Varioskan Flash (Thermo scientific).

Fulvestrant treatment and cell proliferation assay

To assess the response of cancer cells to fulvestrant (Sigma), 2000 cells per well were seeded in 96 well plates. Then cells were treated with 10 μM fulvestrant; DMSO was added to the control cells. 72 and 96 hours later, mitochondrial activity as an indicator of cell proliferation was measured using MTS reagent, which was added to the wells and after an incubation of 3 hours and 45 minutes at 37°C, the optic density was detected at a wave length of 490 nm using Varioskan Flash (Thermo scientific).

Statistics

Results were analyzed for significance using the unpaired Student t test (two-tailed). Significance was determined as $P < 0.05$.

Results

Down- and upregulation of ESR1 expression using ATFs

We selected a panel of cell lines with different levels of ESR1 expression: MCF7 (highly ER-positive breast cancer cell line), SKOV3 (an intermediate ER-expressing ovarian cancer cell line), and MDA-MB-231, SKBR3 (ER-negative breast cancer cells) (Supplementary Figure 1). These cells were transduced with ATFs to express the ZF-constructs. ATFs composed of ER2 and ER3, but not ER1, could induce down- and upregulation of ESR1 in ER-positive and ER-negative breast cancer cells, respectively. ESR1 was 30.4 ± 3.55 % and 39.4 ± 5.44 % downregulated ($P<0.01$) in MCF7 cells transduced with pMX-ER2-SKD and pMX-ER3-SKD, respectively, compared to ESR1 expression in cells transduced with pMX empty vector. ESR1 was 21.2 ± 5.97 % downregulated ($P<0.05$) in MCF cells transduced with pMX-ER2-NoED compared to MCF7 cells transduced with pMX-empty vector. ESR1 downregulation by pMX-ER3-NoED did not reach significant (Figure 2A). In contrast, ESR1 was not downregulated in SKOV3 cells transduced with any of ATFs (Figure 2A).

The expression of ESR1 was upregulated up to 1.85 ($P<0.01$) and 3.32 fold

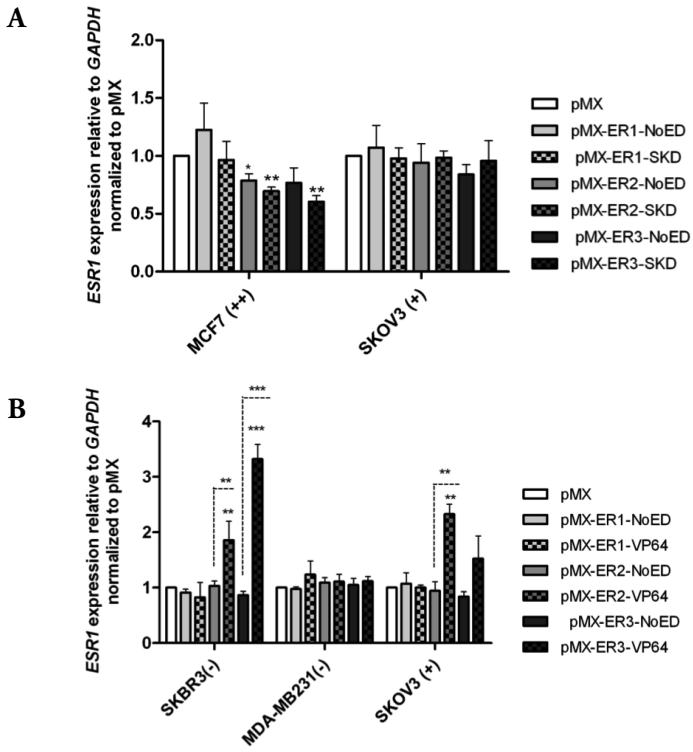

Figure 2. Down- and upregulation of ESR1 using ATFs. (A) Downregulation of ESR1 expression in ER-highly and intermediate positive cell lines. (B) Upregulation of ESR1 in ER-negative and ER-intermediate positive cell lines. ESR1 expression was measured using q-RT-PCR. ESR1 expression is the average (±SEM) of three independent experiments. Expression of ESR1 in different transduced cells was normalized to that of the cancer cells transduced with pMX-empty vector. pMX-empty vector and pMX-ER3-NoED were served as control (* P<0.05, ** P<0.01, ***P<0.001, the dashed lines were used to show the significant difference with pMX-NoED).

(P<0.001) in SKBR3 cells transduced with pMX-ER2-VP64 and pMX-ER3-VP64, respectively, compared to ESR1 expression in SKBR3 cells transduced with pMX-empty vector; the observed upregulation of ESR1 was significant compared to ESR1 expression in SKBR3 cells transduced with pMX-ER2-NoED (P<0.01) and pMX-ER3-NoED (P<0.001) (Figure 2B).

In SKOV3 cells, pMX-ER2-VP64 was more efficient than pMX-ER3-VP64 in upregulation of ESR1; ESR1 was upregulated up to 2.32 fold (P<0.01) in SKOV3 cells transduced with pMX-ER2-VP64 compared to SKOV3 cells transduced with pMX-empty vector. The observed upregulation was also significant compared to SKOV3 cells transduced with pMX-ER2-NoED. ESR1 upregulation resulted

by pMX-ER3-Vp64 was not significant compared to controls (Figure 2B). In contrast, none of ATFs resulted in upregulation of ESR1 in MDA-MB231 cells (Figure 2B).

ESR1 downregulation in cells transduced with DNA methyltransferase

In MCF7 cells transduced with pMX-ER3-M.SssI, ESR1 expression was 24.4 ± 3.81 % ($P<0.01$) downregulated compared to ESR1 expression in MCF7 cells transduced with pMX-empty vector (Figure 3). Downregulation of ESR1 by pMX-ER3-M.SssI was less efficient than downregulation of ESR1 by pMX-ER3-SKD (38.6 ± 5.76). The observed downregulation was consistent with the assessed GFP expression which showed more than double GFP expression in cells transduced with pMX-ER3-SKD (about 40%) compared to the GFP% in cells transduced with pMX-ER3-M.SssI (<20%) (data not shown). ESR1 expression in MCF7 cells transduced with pMX-ER3-M.SssI was not significantly different than ESR1 expression in MCF7 cells transduced with pMX-ER3-NoED. There was no downregulation of ESR1 upon transduction of MCF7 cells with pMX-ER1-M.SssI or pMX-ER2-M.SssI (Figure 3).

Assessment of ESR1 in SKOV3 cells transduced with pMX-ER3-M.SssI, or with pMX-ER3-SKD again did not show downregulation of this gene (Supplementary Figure 2).

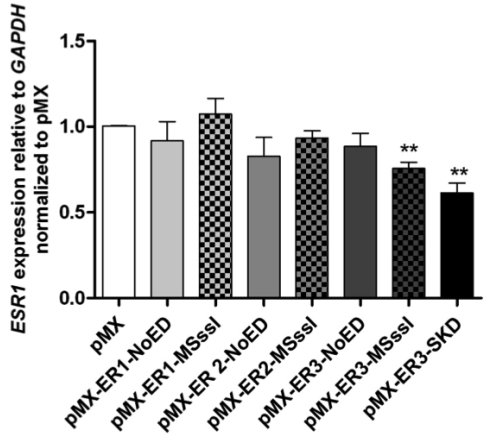

Figure 3. ESR1 was downregulated in cells transduced with DNA methyltransferase. Downregulation of ESR1 expression was measured using q-RT-PCR and ESR1 expression in transduced cells was normalized to ESR1 expression in MCF7 cells transduced with pMX-empty vector. The mean is the average (±SEM) of three independent experiments. (**P<0.01).

Targeted DNA methyltransferase induced DNA methylation on ESR1 gene

An overview of DNA methylation status of ESR1 in the cell line panel showed that assessed CpGs in four regions of ESR1 are more methylated in untreated MDA-MB231 than in other untreated cell lines (Supplementary table 1). DNA methylation of ESR1 downstream of ER3 binding site in transduced cells was assessed using PyroS3. Upon transduction of MCF7 cells with pMX-ER3-M.SssI, percentage of methylation of CpG-40, CpG-41, CpG-42, CpG-43, CpG-44 and CpG-46 was significantly increased compared to their counterparts in MCF7 cells transduced with pMX-empty vector. ER3-NoED did not induce DNA methylation for either of CpGs (Figure 4A).

Assessment of the same CpGs of ESR1 in SKOV3 cells transduced with pMX-ER3-M.SssI showed highly increased DNA methylation (up to 12 fold) compared to their counterparts in SKOV3 cells transduced with pMX-empty vector (n=1) (supplementary Figure 3A). The methylation of these CpGs of ESR1 in HEK293T cells transfected with pMX-ER3-M.SssI was slightly increased compared to their counterparts in HEK293 cells transfected with pMX-empty vector (n=1) (Supplementary Figure 3B).

To evaluate the off-target effect of M.SssI on the genome, DNA methylation of CpGs in the repetitive sequence of LINE1 (Figure 4B) and ICAM-1 was assessed (Figure 4C). The result showed that one of the assessed CpGs of each gene has significantly increased methylation in MCF7 cells transduced with pMX-ER3-M.SssI compared to cells transduced with pMX-empty vector.

Reduction of cell colony forming capacity of cancer cells was associated with downregulation of ESR1

In correlation with downregulation of ESR1, there was a reduction of the cell colony formation of MCF7 cells transduced with pMX-ER3-SKD (57.5 ± 5.12 %; $P < 0.01$) and MCF7 cells transduced with pMX-ER3-M.SssI (30.3 ± 12.3 %; $P<0.05$). As also observed for gene expression downregulation, the effect of ER3-NoED on reduced colony formation did not reach significance (Figure 5). SKOV3 cells transduced with pMX-ER3-SKD and pMX-ER3-M.SssI had a remarkable reduction in cell colony formation (88.6 % and 92.6 %, respectively) (Supplementary Figure 4).

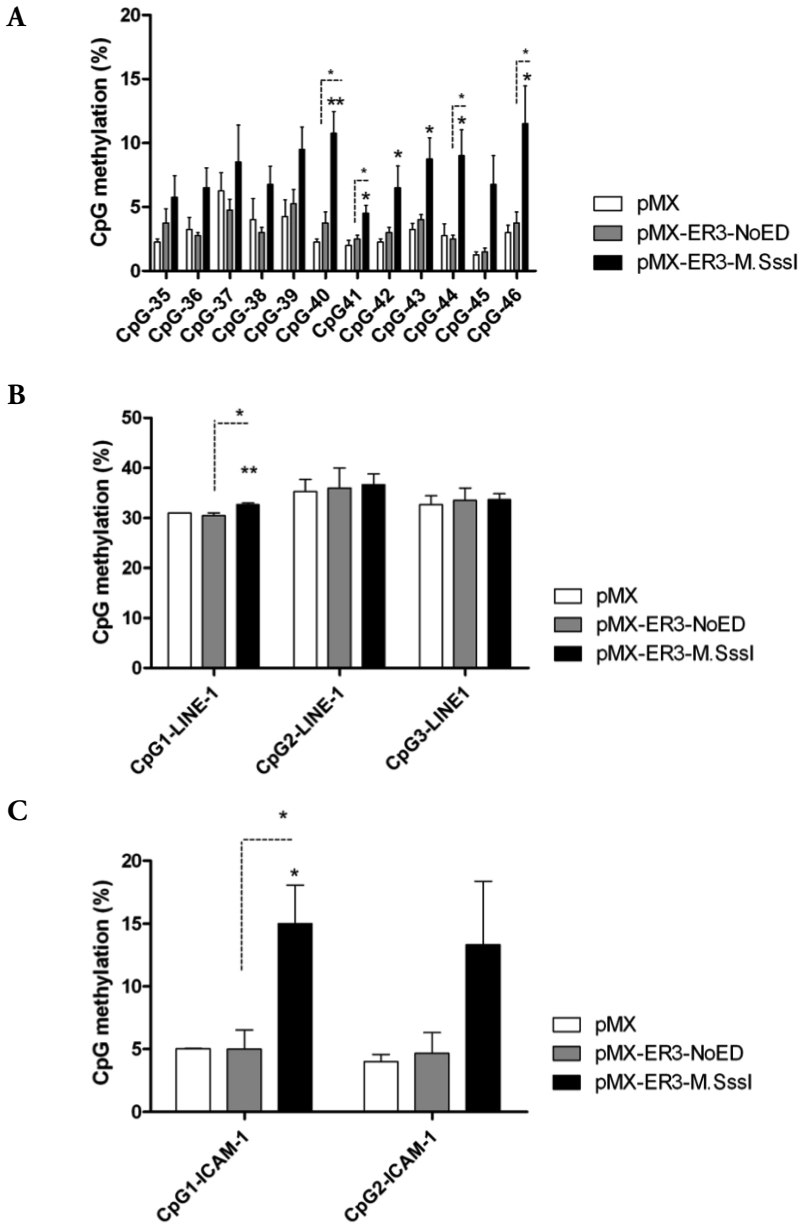

Figure 4. DNA methylation was induced on CpGs located downstream of the binding site of ER3-ZFP upon transduction of MCF7 with pMX-ER3-M.SssI. Induced DNA methylation by pMX-ER3-M.SssI on CpGs of (A) ESR1, (B) the repetitive sequence LINE1, and (C) ICAM-1. The assessed region was amplified by Pyro primer 2 and the % of methylation was the result of pyrosequencing using primer Pyro 3. pMX-empty vector and pMX-ER3-NoED were served as control. The mean is the average (±SEM) of three independent experiments. (**P<0.01, *P<0.05, the dashed lines were used to show the significant difference with pMX-NoED).

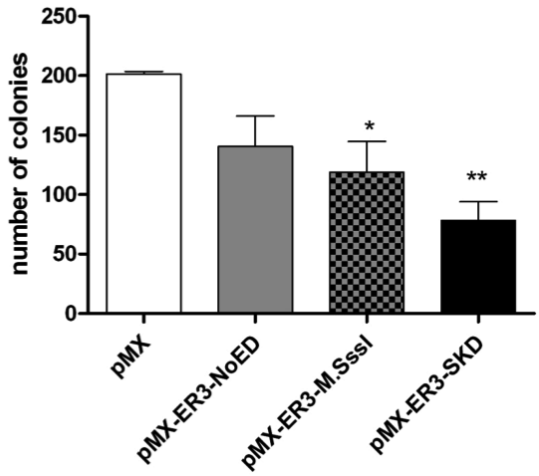

Figure 5. Reduction of cell colony formation upon downregulation of ESR1. Transduced MCF7 cells were replated and allowed to form colonies for 11 days. Number of colonies is the average (±SEM) of three independent experiments and each colony consists of at least 50 cells (* P<0.05; **P<0.01).

Induction of histone H3K9me2 using histone targeted histone methyltransferase G9a

In order to induce the inactive histone H3K9me2 mark on ESR1, MCF7 cells were transduced with pMX-ER3-G9a. Protein expression of ER3-G9a in transduced MCF7 cells was detected (Supplementary Figure 5) and H3K9me2 mark was enriched immediately downstream of the ER3-binding site (P=0.052) on ESR1 in MCF7 cells transduced with pMX-ER3-G9a compared to cells transduced with pMX-ER3-NoED. The observed enrichment was not significant compared to pMX-ER3-mutantG9a (Figure 6A). There was no enrichment detected on the adjacent region 500bp downstream of TSS1.

The observed enrichment on about 200 bp upstream of TSS1 was not significantly different compared to the enrichment in the same region in MCF7 cells transduced with pMX-ER3-NoED, or with pMX-ER3-mutantG9a (Figure 6A). Despite the induction of H3K9me2, ESR1 was not significantly downregulated in MCF7 cells transduced with pMX-ER3-G9a compared to ESR1 expression in MCF7 cells transduced with pMX-empty vector, pMX-ER3-NoED, or with pMX-ER3-mutantG9a. in this set of experiments, ESR1 was significantly downregulated in MCF7 cells transduced with pMX-ER3-NoED compared to pMX-empty vector (Figure 6B).

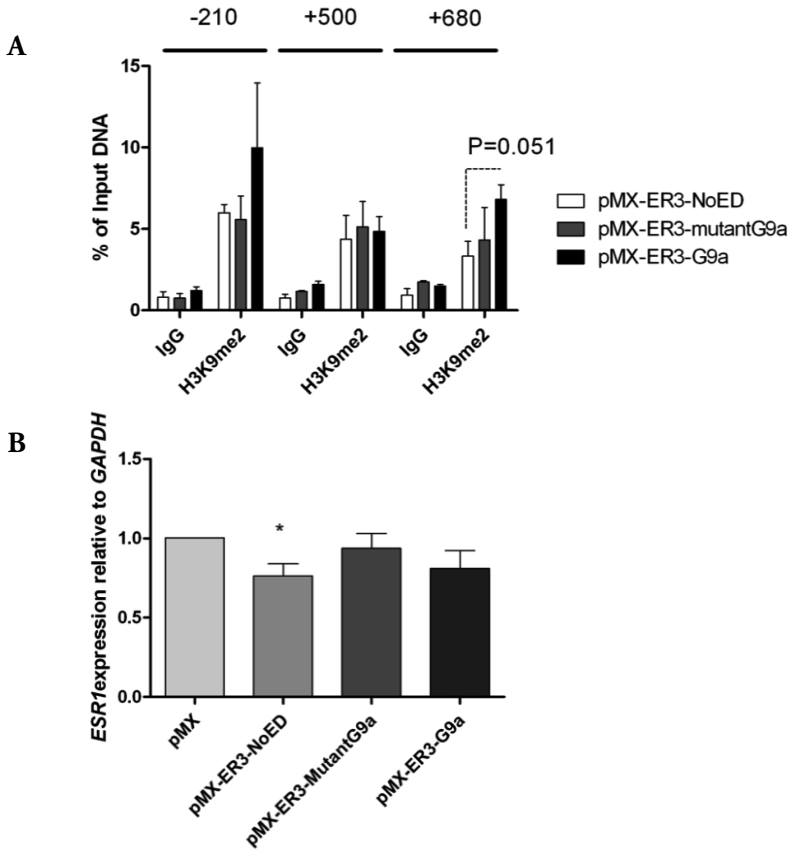

Figure 6. Induction of H3K9me2 on ESR1 gene using pMX-ER3-G9a. (A) Enrichment of H3K9me2 mark by pMX-constructs was assessed by quantitative ChIP for regions ChIP1, ChIP2 and ChIP3 of the ESR1 gene: % of input DNA represents the amount of immunoprecipitated DNA compared to the input. The bars represent the average number of (±SEM) of 3 independent experiments. IgG was used as a negative control for immunoprecipitation. Cells infected with pMX-ER3-NoED and pMX-ER3-mutantG9a were considered as controls. (B) ESR1 expression at RNA level was measured by q-RT-PCR. The relative measured RNA was normalized to pMX. The mean is the average (±SEM) of three independent experiments (*P<0.05).

Towards re-sensitization of ER-negative cells to endocrine therapy

Some studies previously demonstrated that re-expression of ER-α can lead to re-sensitization of ER-negative breast cancer to endocrine therapy [19-21]. We aimed to exploit our targeted upregulation approach to re-sensitize ER-negative cancer cells, but first we set out to find the proper dose of endocrine therapy agents for which their effect on cells is because of targeting and suppressing ER-α and not because of high dose toxicity. In an attempt to choose a low and effective dose of

fulvestrant or 4-OH-TAM, we tested several different concentrations (data not shown). Treatment with different concentrations showed that 10 μM fulvestrant was the only concentration that ER-positive breast cancer cells (MCF7) responded to. In the panel of cell lines, the cellular metabolic activity of MCF7 cells was reduced about 26.0 ± 5.43 % ($P<0.05$) upon treatment with 10 μM fulvestrant, compared to the control cells treated with DMSO (Supplementary Figure 6A) and there was no response for SKBR3, nor SKOV3 (data not shown). To achieve a higher response we evaluated the effect of 4-OH-TAM.

Upon treatment with 10 μM 4-OH-TAM, cellular metabolic activity of MCF7 was reduced by 57.1 ± 4.08 % ($P<0.05$) compared to MCF7 cells treated with EtOH. However, there was also a reduction of metabolic activity in response to 10 μM 4-OH-TAM for SKOV3 (34.5 ± 6.55%), and ER-negative breast cancer cells (MDA-MB231 (32.3 ± 6.80%), and SKBR3 (27.5 ± 3.82%)) compared to that of cells treated with EtOH. (Supplementary Figure 6B). The observed response of ER-negative cancer cells (SKBR3/MDA-MB231) forced us to assess lower concentrations (7, 5, 1 μM). Treatment of ER-positive breast cancer cells (MCF7) with these concentrations, however, resulted in no response (data not shown).

Discussion

We could up- and downregulate ESR1 in ER-negative and ER-positive breast cancer cells, respectively, using ATFs composed of VP64/SKD fused to ER2 and ER3. We also succeeded to induce DNA methylation on CpGs on ESR1 gene using pMX-ER3-M.SssI. The induced DNA methylation was associated with downregulation of ESR1 and reduction of cell colony formation.

In our study, we designed three ZFPs targeting ESR1; ER2 and ER3, but not ER1 could up- and down regulate ESR1 in breast cancer cells. However, ER3 seemed to be more efficient than ER2 in view of its effect, especially, on upregulation of ESR1 in breast cancer cells. Regardless of complexity of ESR1 regulation by several promoters, efficiency of function of ZFPs can be explained by the positions that they are designated to bind to. ER2 binding site is located adjacent to TSS1 (+2 to +19) and ER1 binding site is 69 bp further upstream (-68 to -86). Although regions around the TSSs are potentially very important regions for regulation of genes, these parts of genes can be highly competitive

for transcription factors which may compete with the binding of our ATFs and be a potential reason for the lack of effect of ER1, and lower efficiency of ER2 compared to ER3. ER3 binds to exon 1 which is 293 to 311 bp downstream of TSS1 and might be of less competition. However, TSSs were shown to be proper positions to target and regulate other genes [28, 40, 41].

It is known that DNA methylation status of the proximal promoter to TSS1 and exon 1 of ESR1 are important regions in view of the DNA methylation association with ESR1 expression [42]. It is also appreciated from our data showing that ESR1, especially exon 1 and downstream of ER3 binding site, was more methylated in MDA-MB-231 as ER-negative breast cancer cells compared to MCF7. However, this distinct pattern was not observed in SKBR3, another ER-negative breast cancer. This observation can be well explained by the fact that the epigenetic pattern of one gene may vary between different cell types. In previous studies, the DNA methylation pattern of ESR1 in SKBR3 is indeed different from MDA-MB231 as it contains less methylated regions [16]. In our study the methylation pattern of ESR1 in SKBR3 is comparable to MCF7, ER-positive breast cancer, and not to MDA-MB231. Such difference might be because of the fact that epigenetics of cancer cell lines might have some changes in response to different growth conditions. The different epigenetic patterns are mainly linked to the cell type-specific expression of genes [43], whereas our observation of the lack of DNA methylation on exon 1 of ESR1 in SKBR3 is not linked to the expression pattern. No ESR1 expression in SKBR3 might be linked to histone modifications and it remains to be addressed.

Association of the induced DNA methylation of ESR1 with its downregulation in our study is in line with studies which describe the role of DNA methylation in regulation of ESR1. Ottaviano et al. was the first to show that the aberrant DNA methylation of ESR1 is correlated with no transcription of ESR1 [14]. Further investigation by Yoshida et al. unraveled that the aberrant DNA methylation of CpGs in exon 1 and promoters of ESR1 has an inverse correlation with ESR1 expression in breast cancer [42]. Nowadays, several studies confirm this correlation. ESR1 downregulation using pMX-ER3-M.SssI, was slightly less efficient than ESR1 downregulation by pMX-ER3-SKD. This observation is in line with the study of targeted DNA methylation of SOX2. SOX2 was more efficiently downregulated using SKD than using catalytic domain of a DNA

methyltransferase, DNMT3a[28]. In our study, the lower efficiency of ER3-M.SssI compared to ER3-SKD might be due to the less efficient delivery of pMX-ER3-M.SssI, as assessment of GFP expression, an indicator of pMX-constructs delivery, showed higher GFP expression in cells transduced with pMX-ER3-SKD than in cells transduced with pMX-ER3-M.SssI. Moreover, a higher efficiency of SKD might be explained by the fact that SKD is able to recruit different transcription repressors and causes a dramatic reduction of H3 acetylation as also shown by our previous study [30]. However, the effect of a catalytic domain of an epigenetic enzyme is expected to be more stable as indeed demonstrated earlier [28].

In contrast to the descriptive studies assessing effects of DNA demthylating epi-drugs on ESR1 regulation, we used a direct targeted approach. Consistent with the previous study showing the function of prokaryotic M.SssI in eukaryotes (yeast) [38], we confirmed that prokaryotic M.SssI can be functional in chromatin context of human cells. Although DNA methylation was induced significantly on ESR1, it was also detected on one of assessed CpGs in either LINE1 or ICAM-1 which were used as controls. These off-targets confirmed previous findings [26] indicating that although the ZFP causes M.SssI to function more preferential for the target site [38], M.SssI fused to the ZFP still retained its potential for methylation with no sequence restrictions [44]. To have more concentrated DNA methylation on the targeted site, we previously used a M.SssI mutant with a lower activity (so-called C141s) [41] and also some other studies used eukaryotic DNA methyltransferases with low catalytic activity [44]. Another factor for the observed off-target effect can be the ZFP, the absolute specificity of the ZFP to its target site is under debate and we previously showed that although the ZFP preferentially binds to its target site, it also binds to unwanted sites in the genome[30].

The level of ESR1 downregulation induced by pMX-ER3-M.SssI or pMX-ER3-SKD was not significantly different from the effect of pMX-ER3-NoED or pMX-ER2-NoED; however downregulation of ESR1 by either of NoEDs was not significant compared to the pMX-empty vector. In the other set of experiments for inducing H3K9me2, ESR1 downregulation by pMX-ER3-NoED reached significance. This observation can be explained by the fact that the binding of the ZFP, itself, may impede the progress of RNA polymerase [45]. The varied effects of PMX-ER-NoEDs in different sets of experiments might be due to the variable

transduction efficiency between different experiments.

The resulted downregulation of ESR1 in MCF7 cells transduced with pMX-ER3-SKD or pMX-ER3-M.SssI was enough for a reduced cell colony formation in transduced MCF7 cells. However, in SKOV3 cells transduced with either pMX-ER3-SKD or pMX-ER3-M.SssI, we observed an extreme inhibition of cell colony formation (n=1), while a comparable ESR1 downregulation did not occur in these cells. Such an effect might be because of the off-target effects of M.SssI and requires further investigation.

Now that we showed the feasibility of induction of DNA methylation on ESR1 in ER-positive breast cancer cells, targeted DNA methylation can be further exploited using DNA methyltransferase enzymes with more specificity[46]. In addition, a similar approach for targeting ESR1 can be used to induce targeted demethylation of this gene in ER-negative breast cancer cells. Targeted demethylation is recently shown to be feasible [31, 34]. For instance, DNA demethylase TET2 fused to the ZFP was shown by our group to upregulate ICAM-1 [31]. So targeted demethylation can be exploited, in future, to specifically re-express hypermethylated ESR1 in ER-negative breast cancer cells. Currently, epi-drugs are used to demethylate and re-express ESR1 in ER-negative breast cancer which indeed leads to endocrine therapy re-sensitization [19-21]. The epi-drugs have genome-wide function and inevitably they potentially upregulate prometastatic genes [25, 47]; so, developing more specific approaches for targeting ESR1 is of importance. In this respect, ATFs are good options for developing gene-targeting approaches; however, it should be taken into account that the effect of a single ATF may vary in different cell types. For instance, we showed that ESR1 was upregulated using ATFs in SKBR3 cells, but not in MDA-MB231 cells which suggests that the target gene behaviour/response can differ from one cell line to another. Unravelling the epigenetic and genetic factors affecting the target gene in different cellular contexts would shed light on the way of targeting genes.

ATFs can be potentially used, in the future, to re-sensitize ER-negative breast cancer cells to the treatments including fulvestrant and 4-OH-TAM. The response of MCF7 to fulvestrant was weak in our study. To evaluate the cell growth inhibitory effect of fulvestrant, we assessed the mitochondrial activity as it was used by other studies [48]; however, measuring mitochondrial activity may be not a good indicator of cell growth inhibition [49], so assessing the apoptosis or the

cell cycle arrest may be better options. Assessing different doses of 4-OH-TAM in the ER-positive cell line did not result in an efficient low dose response in ER-positive cells with no cell toxicity effect on ER-negative cells, therefore we could not further progress to evaluate the re-sensitivity of ER-negative cells using our targeted approach. This observation is in contrast with other studies showing that MCF7 cells respond efficiently to low doses of 4-OH-TAM [50]. The lack of an efficient response of MCF7 cells in our study suggests that ESR1 expression is not the only factor for the response and there are other factors involved. For instance, it was shown that the expression of the oncogene ZNF703 in ER-positive breast cancer cells causes the resistance of ER-positive breast cancer cells to tamoxifen[51]. In addition, ER-α interacts with several proteins and growth factors such as HER2/neu. Targeting and downregulating HER2/neu was found to improve the response of ER-positive ovarian cancer cells to endocrine therapy, in vivo [52]. This emphasizes that developing of multi-targeting approaches is necessary.

In this study, we also aimed to induce H3K9me2 mark on ESR1 and evaluate its effect on ESR1 expression. There is not much known about histone modifications which underlie or are associated with ESR1 expression, except for the information from a few descriptive studies [23, 24]. The information about histone modifications of ESR1 is mainly from studies which investigate effect of histone deacetylase inhibitors on ESR1 expression. In our study, G9a could enrich H3K9me2 mark on exon 1 but the enrichment of H3K9me2 mark was not significantly different than H3K9me2 mark on exon 1 in cells transduced with either pMX-ER3-NoED or pMX-ER3-mutantG9a. H3K9me2 at the promoter is generally associated with transcription repression [53]. We detected this mark in the gene body (Exon 1) of ESR1 and the function of epigenetic modifications in the gene body is not completely clear [54]. In our previous study, we could successfully induce the H3K9me2 mark also in exon 1 of HER2/neu [30], in contrast to ESR1, the HER2/neu gene did not show the presence of this mark in the control cells. It might be possible that the expressing ESR1 gene carries both active and repressive histone marks including H3K9me3. However, to the best of our knowledge, the presence of inactive histone marks including H3K9me2/3 on ESR1 has not been reported. So, assessing histone marks of ESR1 is very important to find the key histone marks associated with ESR1 expression. In addition, targeting more regions

of ESR1 e.g. promoter using Epigenetic Editing approach will provide useful information for unraveling the epigenetic mechanisms involved in ESR1 gene expression.

In conclusion, targeted modulation of gene expression either by transcription effector domain or targeted Epigenetic Editing are efficient in modulation of different genes. In addition, Epigenetic Editing can be very informative for investigating the role of epigenetic modifications on gene expression, particularly, for genes which have a complex genetic regulation system like ESR1. Since the control of ESR1 expression is essential for maintaining the normal state of cells, this study provides an approach to exploit reversibility of epigenetic modifications for modulating ESR1 which is extremely dysregulated in cancer.

List of abbreviations

ER-a, estrogen receptor-alpha; ER-a gene, ESR1; epi-drugs, Epigenetic drugs; ATFs, Artificial Transcription Factors; PyroS, Pyrosequencing primer; ChIP, Chromatin Immuno Percipitation; DMSO, Dimethyl sulfoxide; FCS, fetal calf serum; GFP, Green Florescent Protein; MTs, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; qRT-PCR, Quantitative Reverse Transcription Polymerase Chain Reaction;. ZFP, Zinc Finger Protein.

Author's contributions

Fahimeh Falahi has contributed by designing, setting up, performing experiments, data analysis, and writing the manuscript. Geke A. P. Hospers has contributed by designing, supervising the project, and revising the manuscript. Marianne G. Rots has contributed by designing, supervising the project, and revising the manuscript.

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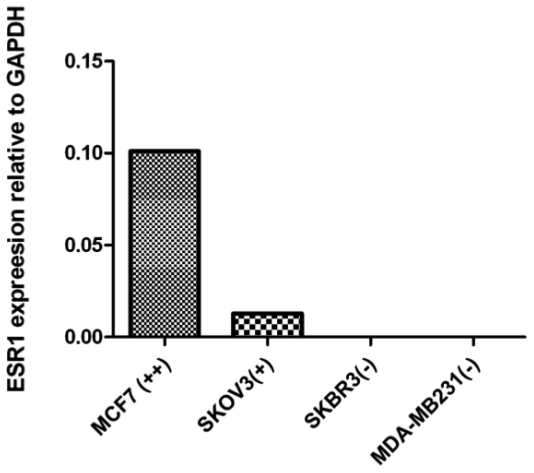
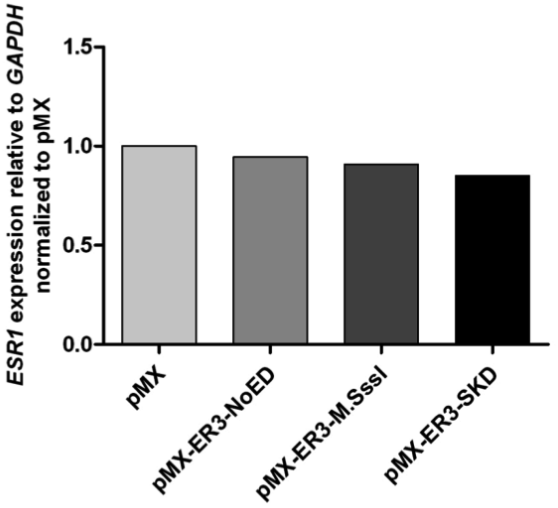
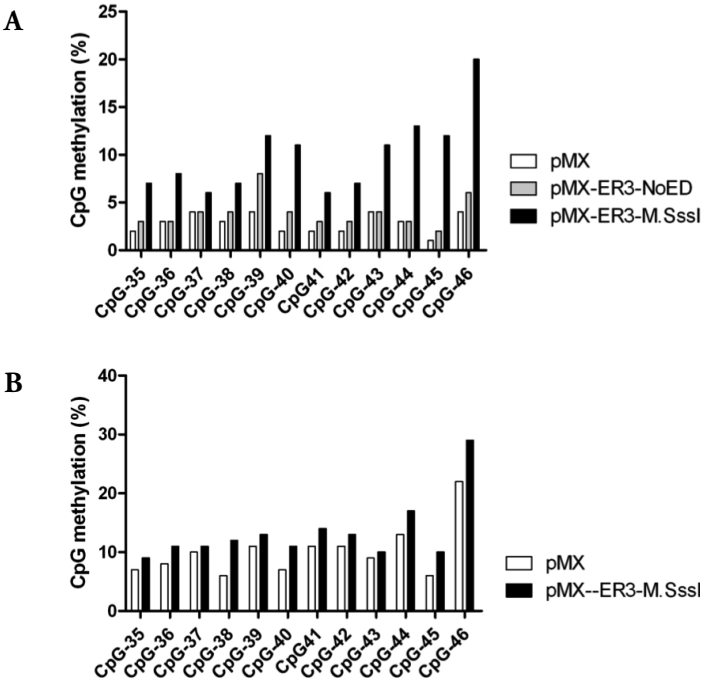


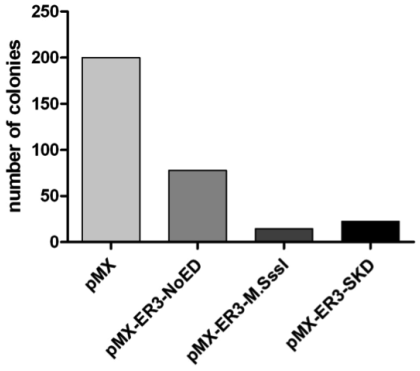
Supplementary Figure 1. ESR1 expression in a panel of cell lines. ESR1 expression was measured by q-RT-PCR. ESR1 expression was assessed relative to GAPDH.



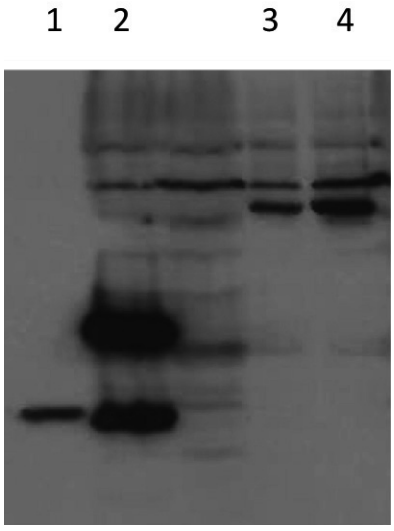
Supplementary Figure 2. Expression of ESR1 in SKOV3 cells transduced with pMX-ER3-M.SssI. ESR1 expression was measured using q-RT-PCR. ESR1 expression in transduced cells was normalized to ESR1 expression in the cancer cells transduced with pMX-empty.



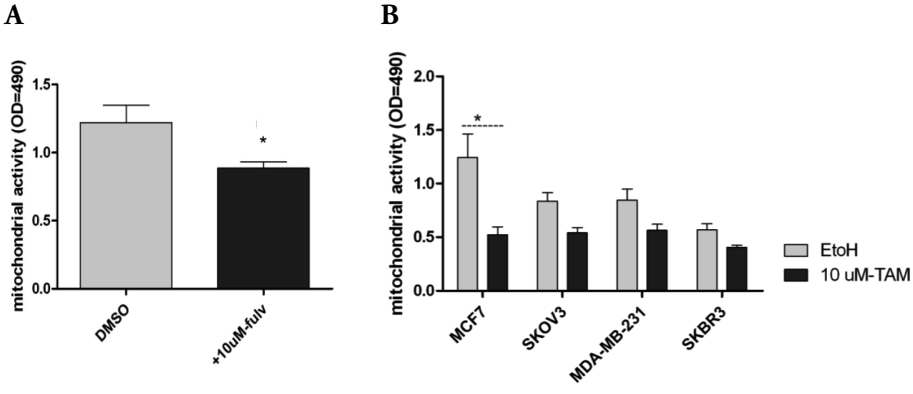
Supplementary Figure 3. DNA methylation was induced on CpGs located downstream of binding site of ER3 ZFP. Induced DNA methylation by pMX-ER3-M.SssI on CpGs of ESR1 in (A) transduced SKOV3 with pMX-ER3-MS.ssI and (B) HEK293T cells transfected with pMX-ER3-MS.ssI .pMX-empty vector and pMX-ER3-NoED were served as control.



Supplementary Figure 4. Reduction of cell colony forming capacity upon downregulation of ESR1. Transduced SKOV3 cells were replated and allowed to form colonies for 4 weeks. Each colony consists of at least 50 cells.



Supplementary Figure 5. Detection of protein of pMX-ER3 constructs in transduced MCF7 cells. Protein was detected using standard western blotting and antibody against HA-tag. 1)pMX-ER3-NoED, 2) pMX-ER3-SKD, 3) pMX-ER3-G9a, 4) pMX-ER3-mutantG9a.



Supplementary Figure 6. Response of cancer cells to endocrine therapy. Mitochondrial activity of treated cells with (A) fulvestrant and (B) 4-OH-Tam was measured using MTS. Absorption is the average (±SEM) of three independent experiments (* P<0.05).

	Sequenced by Pyro1 Upstream TSS1 ←	Sequenced by Pyro4 Downstream TSS1 ←	Sequenced by Pyro2 Downstream TSS1 →							Sequenced by Pyro3 Downstream TSS1 →											
	CpGs (% methylation)	CpGs (% methylation)	CpGs (% methylation)							CpGs (% methylation)											
CpG number	1	27	28	31	32	33	34	35	36	37	35	36	37	38	39	40	41	42	43	44	45	46
MCF7 (++)	13	4	10	3	5	10	3	7	3	6	3	2	3	3	3	3	2	4	4	3	2	4
SKOV3(+)	15	NA	NA	2	3	6	2	3	1	2	3	4	4	6	5	2	3	3	3	4	2	5
SKBR3(-)	17	NA	NA	3	5	9	7	4	2	3	3	5	5	8	8	2	5	4	3	5	1	8
MDA-MB231(-)	19	29	15	3	3	12	3	21	4	4	12	8	3	15	7	13	8	14	29	13	6	25

Supplementary Table1. DNA methylation status of ESR1 gene in the panel of cell lines. DNA methylation was assessed using pyrosequencing; arrows show the direction of sequencing. The first assessed CpG was numbered as 1.

Chapter 5

Epigenetic sustained re-expression of EPB41L3 in cancer using ATFs and epigenetic drugs

Manuscript in preparation

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Abstract

Epigenetic silencing of tumor suppressor genes (TSGs) is considered a significant event in the progression of cancer. EPB41L3, often silenced by hypermethylation, functions as a TSG in various types of cancer. In cervical cancer, EPB41L3 was reported as methylation marker, although its function as TSG has not been explored. Artificial Transcription Factors (ATFs) are uniquely suited to re-express silenced TSGs thereby reprogramming cancer cells to a less malignant phenotype. In this study, we aim to specifically re-express EPB41L3 using ATFs to inhibit tumor growth. Two ATFs, targeting the EPB41L3 promoter, were designed and delivered into breast, ovarian and cervical cancer cells displaying various degrees of EPB41L3 promoter hypermethylation. Doxycycline-inducible ATF-transfectants were created to measure EPB41L3 re-expression in course of time, also after co-treatment with epigenetic drugs. In all seven cell lines, EPB41L3 could be significantly upregulated, resulting in growth reduction (apoptosis), altered expression of cell cycle regulators and a change in histone marks. Upon doxycycline removal, EPB41L3 re-expression levels decreased overtime; interestingly, this decline could be prevented by co-stimulation with epigenetic drugs. These data demonstrate the potency of ATFs to re-express the hypermethylated EPB41L3. Furthermore, this is the first time that EPB41L3 is reported as functional TSGs in cervical cancer.

Introduction

Erythrocyte membrane protein band 4.1-like 3 (EPB41L3, DAL-1) is part of the 4.1 family of proteins, which actions are implicated in cell adhesion, cell motility and cell growth [1, 2, 3]. The 4.1 family shares a highly conserved FERM (4.1/Ezrin/Radixin/Moesin) domain that localise to the cytoplasmic side of the plasma membrane and link membrane proteins with the spectrin/actin cytoskeleton [4]. NF2 and EPB41L3 are two members of the 4.1 family that have been reported as tumor suppressor genes (TSGs) in various types of cancer [1]. Upon down-regulation of EPB41L3, a functional disruption in the organization in the cytoskeleton organization is believed to underlie the increased metastasis and invasion of cancer cells. Overexpression of EPB41L3 dramatically decreases cell growth in vitro and in vivo [2, 5, 6, 7], for example in breast cancer and ovarian cancer cells, EPB41L3 re-expression causes strong growth suppression, in part through the induction of apoptosis. These properties make EPB41L3 an attractive target for upregulation in cancer therapy.

Intensive efforts to map the cancer methylome have revealed many genes which are aberrantly methylated in cancer [8, 9]. In that respect, EPB41L3 was found to be frequently methylated in many cancer types, including breast- [10], ovarian- [5] and cervical cancer [11, 12]. An important application of differentially hypermethylated genes, such as EPB41L3, is their promise as a biomarker for early detection of cancer [11]. Another application lies in the reversible nature of epigenetic silencing, which in contrast to genetic mutations, allow for re-expression of the silenced TSG. Upon re-expression, the TSGs can enforce a less malignant phenotype in the cancer cells. In cervical cancer, 83% of frozen cervical scrapings of cancer patients are methylated for EPB41L3 versus 14% of normal cervices [11]. It may very well be that these methylation patterns indicate a strong suppression of EPB41L3, and a possible role of EPB41L3 in the pathology of cervical cancer. However, the potent tumor suppressive role of EPB41L3 has not been studied yet in cervical cancer.

Epigenetic drugs are successfully exploited to reverse TSG silencing [13], as also reported for EPB41L3 [14]. However, disadvantages of these drugs include their lack of gene-specificity and non-chromatin effects [15]. An interesting development in the last years is the specific targeting of methylated TSGs using engineered polydactyl zinc-finger proteins (ZFP). These naturally occurring DNA binding

proteins can be engineered to target virtually any gene in the human genome[16]. The DNA binding domains can be attached to gene activators, repressors or epigenetic enzymes for modulation of gene-expression[17], directed mutagenesis[18], functional analysis [19, 20, 21] or epigenetic editing [22]. For modulation of gene expression by ZFP-ATFs, high target specificity can be achieved close the single gene regulation as recently demonstrated for elastin [23] and phospholamban [24]. TSGs which have been successfully re-activated using ZFPs linked to a strong gene-activator (VP64), include maspin [25, 26, 27], CDKN2A [28] and C13ORF18[19]. Re-expression of these genes was associated with a strong decrease in tumor growth. Interestingly, re-activation of these three TSG genes was associated with site-specific DNA demethylation, while re-expression of the latter two was also accompanied with a decreased repressive histone methylation status. Such indirect effects of ATFs on local epigenetic structures of genes would have therapeutic benefits, if inherited to daughter cells. Promising strategies are being developed at the moment to make ATFs clinically attractive tools [29, 30].

In this study, we aim to re-express EPB41L3 using specifically designed ATFs in order to decrease tumor growth in various types of cancer. We validated the role of EPB41L3 in breast and ovarian cancer and demonstrated the effects of EPB41L3 on apoptosis, cell growth and cell cycle regulators in cervical cancer cell lines. As ATF induced expression may change the epigenetic state of gene transcription, we also addressed the kinetics of EPB41L3 re-expression.

Results

EPB41L3 mRNA expression and DNA methylation status

Expression levels of EPB41L3 mRNA were evaluated in breast (MDA-MB-231, SKBR3), ovarian (SKOV3, A2780) and cervical (HeLa, CaSki, C33A) cancer cell lines (Fig. 1a). EPB41L3 mRNA expression was detected in SKBR3 and C33A (mRNA relative to GAPDH: 3.8x10-5 (SKBR3) and 1.5x10-4 (C33A)), but not in the other cell lines. Examination of the DNA methylation status showed that EPB41L3 silencing was associated with extensive promoter hypermethylation in SKOV3 (75±9%), HeLa (88±1%) and CaSki (90±1%), but less in MDA-MB-231 (28±1%) and A2780 (58±14%) (Fig. 1b). The expressing cell lines showed lower degrees of promoter methylation (SKBR3 (6±3%) and C33A (19±5%)).

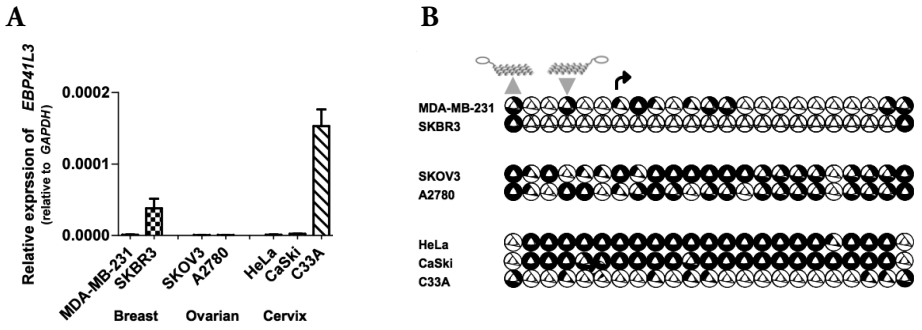

Figure 1. Association between EPB41L3 expression and DNA methylation status. EPB41L3 mRNA expression (a) and DNA methylation (b) in a panel of breast (MDA-MB-231, SKBR3), ovarian (SKOV3, A2780) and cervical (HeLa, CaSki and C33A) cancer cell lines. 3 clones per cell line were analyzed by bisulfite sequencing and each third of a circle represents a clone. Also shown are the predicted binding sites of EPB41L3 targeting ATFs (21ab-VP64 (▲), 22ab-VP64(▼) and the TSS (↱).

Targeted re-expression of EPB41L3

Next, we investigated whether EPB41L3 induction from the endogenous locus can be achieved using zinc finger-based ATFs. Expression of EPB41L3 targeting ATFs was monitored using qRT-PCR (Fig. S2). ATF 21ab-VP64 reached higher expression levels than 22ab-VP64 in all tested cell lines after retroviral transduction. Then we examined the ability of the two ATFs to re-express EPB41L3 in the seven cancer cell lines (Fig. 2). EPB41L3 was significantly upregulated ($p<0.05$) in all seven cell lines by 21ab-VP64 compared to the effects of an empty vector (pMX) (MDA-MB-231 11±2.7 fold, SKBR3 3.6±0.6 fold, A2780 8.5±2.5 fold, SKOV3 11±3.3 fold, HeLa 14±4.3 fold, CaSki 13±3.1 ($p<0.01$) and C33A 26±8.0 fold). Also 22ab-VP64 significantly induced EPB41L3 expression in SKBR3 (11±1.4 fold ($p<0.01$)), CaSki (5.1±1.7 fold ($p<0.05$)) and C33A (6.5±1.1 fold ($p<0.01$)). Controls, consisting of zinc fingers lacking the VP64 effector domain (NoEf) did not induce EPB41L3 expression different from the pMX. C33A cells were transduced to express two irrelevant ATFs, which did not influence gene expression of EPB41L3 (data not shown).

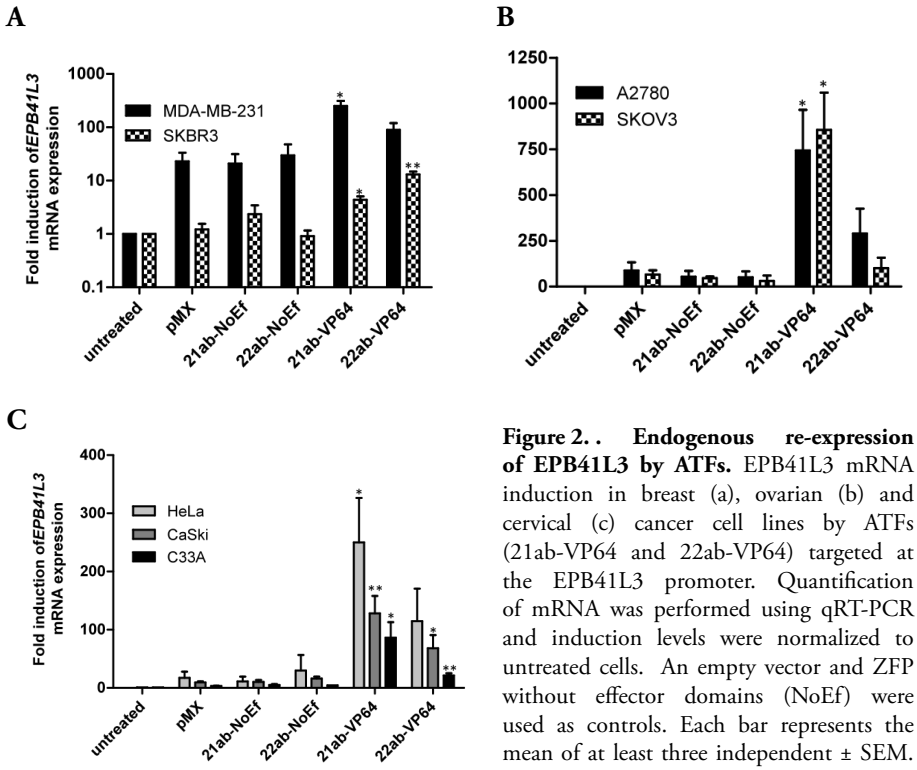

Figure 2. . Endogenous re-expression of EPB41L3 by ATFs. EPB41L3 mRNA induction in breast (a), ovarian (b) and cervical (c) cancer cell lines by ATFs (21ab-VP64 and 22ab-VP64) targeted at the EPB41L3 promoter. Quantification of mRNA was performed using qRT-PCR and induction levels were normalized to untreated cells. An empty vector and ZFP without effector domains (NoEf) were used as controls. Each bar represents the mean of at least three independent ± SEM.

EPB41L3 as tumor suppressor gene

To study if re-activation of EPB41L3 decreases growth in the EPB41L3 methylated cervical cancer cells, a 5 day MTT assay was performed for HeLa (Fig. 3a) and CaSki. The highest re-expressor of EPB41L3, 21ab-VP64, significantly decreased cell growth compared to controls at day 5 (pMX 100±15%, 21ab-NoEf +25±16%, 21ab-VP64 -59±10% ($p<0.05$), 22ab-VP64 -50±16% (ns)). For CaSki, growth was even further decreased at day 5 (-76%) compared to pMX (data not shown). Next, we studied if the ATF-mediated decrease in growth upon re-expression of EPB41L3 in methylated cell lines could be partially explained by the induction of apoptosis (Fig. 3b), as demonstrated by others for breast and ovarian cancer cells using EPB41L3 cDNA [5, 7]. Indeed, apoptosis was induced by 21ab-VP64, the highest re-expressor of EPB41L3, in the methylated breast- and ovarian cancer cells (MDA-MB-231 19±5%, SKOV3 11±3%, A2780 66±18%) compared to pMX ($p<0.05$). Interestingly, also for the methylated cervical cancer cells,

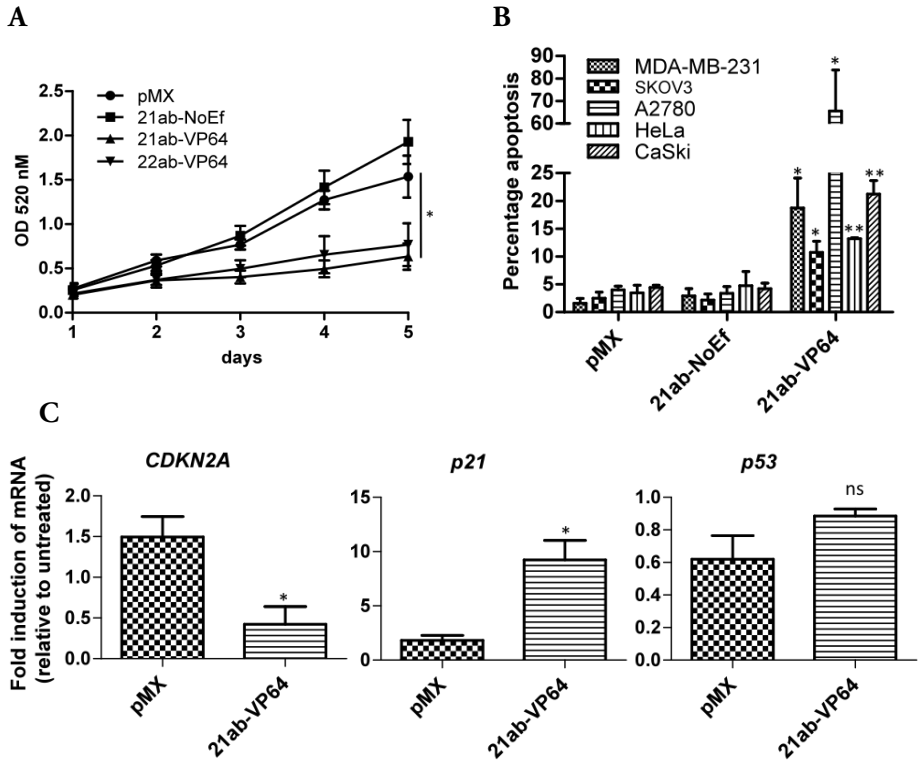

Figure 3. ATF 21ab-VP64 decreases cell growth and induces apoptosis. (A) Relative cell proliferation was measured with a MTT assay in HeLa cells after transduction with the EPB41L3 targeting constructs. Each data point represents the mean of five independent experiments ± SEM. (B) Percentage of apoptotic cells in methylated MDA-MB-231, A2780, SKOV3, HeLa and CaSki after transduction with the 21ab-VP64 and controls (pMX and 21ab-NoEf) measured by a DilC staining. All bars represent the mean of three independent experiments ± SEM. (C) mRNA expression of the cell cycle regulating genes CDKN2A, p21 and p53 also after transduction of pMX and 21ab-VP64 in CaSki cells. Quanficication and representation is similar as in Figure 2.

significant apoptosis was induced by 21ab-VP64 (HeLa 13±0.1% ($p<0.01$); CaSki 21±2% ($p<0.05$)). ZFPs with no effector domain had similar levels of apoptosis compared to pMX.

To gain better understanding of how EPB41L3 targeting ATFs induce the effects on cell growth in cervical cancer, we analyzed the expression of the cell cycle regulators cyclin-dependent kinase inhibitor 1 (p21) and cyclin-dependent kinase inhibitor 2A (CDKN2A) and tumor protein 53 (p53) in CaSki cells (in which 21ab-VP64 induced the most pronounced apoptotic and growth effects)

(Fig. 3c). All three proteins are deregulated in most cancers and play critical roles in cell cycle progression [31, 32]. However, in cervical cancer, p53 is degraded by the human papillomavirus (HPV) E6 protein, and considered functionally inactive[32]. We observed that p21 expression levels are further upregulated by 21ab-VP64 compared to pMX (5.2±0.30 fold ($p<0.05$)), while CDKN2A levels are down-regulated (3.5±1.8 fold ($p<0.05$)). P53 expression levels were not changed, as could be expected based on its functional inactive states in cervical cancer. These results suggest that EPB41L3 influences cell cycle regulators (CDKN2A, p21), which could be associated with the less malignant phenotype of the cells.

EPB41L3 re-expression affects histone marks

First, we confirmed successful association of the engineered ATF 21ab with its target site in the EPB41L3 promoter. An enrichment of 10% of input DNA was obtained for 21ab-NoEf expressing CaSki cells (0.06% for pMX) (Fig. 4a). To determine if EPB41L3 re-expression by ATFs influenced the histone marks at the ATF target site in methylated or unmethylated cells, H3K9me3 and H3Ac levels were assessed in CaSki (Fig. 4a) and C33A cells (Fig. 4b) after treatment with a control (21ab-NoEf) and 21ab-VP64. In control cells, CaSki cells showed more association of the repressive H3K9me3 mark with the EPB41L3 promoter than C33A (CaSki 21ab-NoEf 2.4±0.2% of input, C33A 21ab-NoEf 0.5±0.1% of

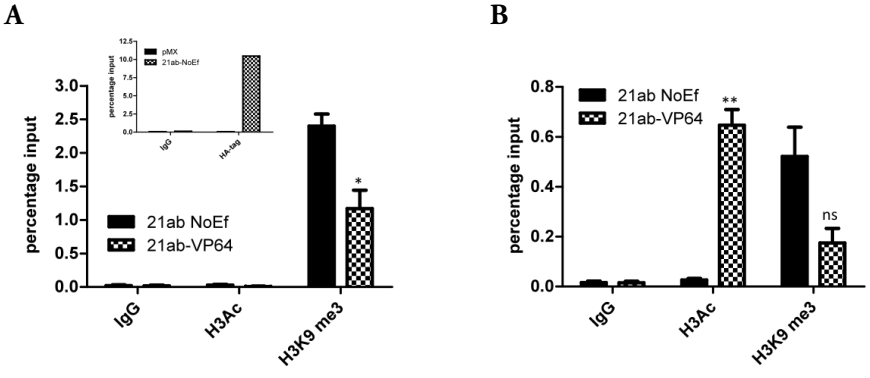

Figure 4. Change in histone marks after re-expression of EPB41L3 in methylated CaSki and unmethylated C33A cells. Quantitative ChIP for H3Ac, H3K9me3 (a,b) and HA tag (insert a) after treatment with 21ab-VP64 and/or 21ab-NoEf in CaSki (a) and C33A cells (b). Values represent the mean percentage of input of three independent experiments ± SEM, (HA tag one experiment).

input ($p<0.01$)), which is consistent with the more silenced/methylated state of EPB41L3 in CaSki.

EPB41L3 re-expression by 21ab-VP64 significantly decreased the repressive mark H3K9me3 by 2.3±0.5 fold ($p<0.05$) in CaSki cells and 3.4±0.6 fold ($p=0.056$) in C33A cells. Interestingly, increased EPB41L3 expression in unmethylated C33A cells was associated with a strong increase in the H3Ac mark (23±2.5 fold ($p<0.01$)), while enrichment of H3Ac in the methylated CaSki cells could not be detected upon EPB41L3 re-expression.

Sustained re-expression of EPB41L3

Next, we studied the kinetics of EPB41L3 re-expression during a period of five days. We constructed 21ab-VP64 DOX-inducible cell lines (HeLa, SKOV3) and studied expression of EPB41L3 and ATF 21ab-VP64 over time, also after removal of DOX. DOX-treatment resulted in re-expression of EPB41L3, with highest level one day after removal of DOX (HeLa 86±19 fold (Fig. 5a), SKOV3 6.5±1.5 fold (Fig. 5b). Then, EPB41L3 levels decayed overtime, similar to DOX-induced

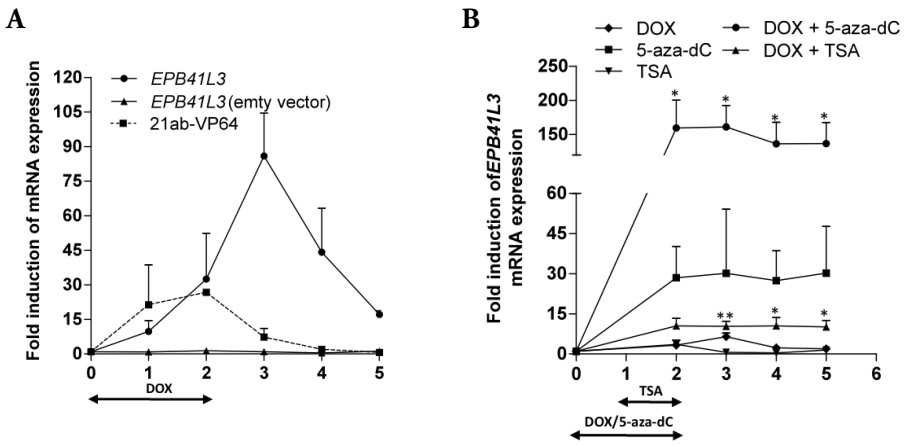

Figure 5. Kinetics of re-expression of EPB41L3 using ATFs and epigenetic drugs. Re-expression of EPB41L3 (and 21ab-VP64) mRNA in HeLa (a) and SKOV3 (b) cells stably transduced with 21ab-VP64 (a,b) or an empty vector (a) after treatment with DOX (HeLa), and DOX together with 5-aza-dC (5 μM) or TSA (400 nM)(SKOV3) (see treatment schedule at the bottom). Expression of EPB41L3 mRNA was measured during a period of 5 days. Quantification of mRNA was performed using qRT-PCR and induction levels were normalized to untreated cells. Each data point represents the mean of at least three independent experiments (empty vector two experiments) measured in triplicate ± SEM. Statistical differences (b) were determined between single treatment (5-aza-dC, TSA) and co-treatment with DOX (5-aza-dC + DOX, TSA+ DOX).

21ab-VP64 expression levels, only with a delay of 24 hours. Stable transfectants with an empty vector (HeLa) did not induce EPB41L3 expression (Fig. 5a). To induce higher and/or longer-term re-expression of EPB41L3, the SKOV3 21ab-VP64 stable transfectants were co-treated with DOX and/or epigenetic drugs (5-aza-dC or TSA) (Fig. 5b). Co-treatment strongly increased EPB41L3 re-expression compared to single treatment with 5-aza-dC (day 2: 5.6±1.4 fold ($p<0.05$) and TSA (day 2: 2.9±0.8 fold ($p=0.064$). Moreover, co-treatment with 5-aza-dC or TSA resulted in stable elevated expression levels of EPB41L3 for at least three days after removal of DOX (day 5: 5-aza-dC 4.5±1.0 fold ($p<0.05$), TSA 7.0±1.7 fold ($p<0.05$). This effect could also be observed for the 5-aza-dC only treated cells (although to a lower extend), but was less clear for this dose of TSA only.

Discussion

Here we show that EPB41L3 can be re-activated using ATFs, in breast, ovarian and cervical cancer cell lines with different degrees of promoter methylation. For breast- and ovarian cancer cells, we validated the role of EPB41L3 as TSG[1,2,3,5,6,7] by the ATFs. For cervical cancer, this is the first study demonstrating that EPB41L3 can induce tumor suppressive effects like apoptosis and reduction of cell growth. Based on its differential methylation profile in cervical cancer versus normal tissues [11] and the functional effects shown here, EPB41L3 might represent an interesting therapeutic target also for cervical cancer.

Several mechanisms have been described how EPB41L3 may mediate the inhibition of cell growth, including activation of the of Rac1-Dependent c-Jun-NH2-kinase[33], increasing activity of caspase-8 [7] and a broader mechanism by mediating post-translational protein modifications through the interaction with arginine N-methyltransferase 3, which functions in multiple pathways important in cell growth [34]. Additionally, we found that ATF-mediated re-expression of EPB41L3 influences expression of the cell cycle regulators CDKN2A (down) and p21 (up). CDKN2A is down-regulated in most cancers, however, in HPV-induced cervical cancer CDKN2A is overexpressed as a result of functional inactivation of pRB by the HPV E7 protein and may contribute to malignancy [32]. Therefore, down-regulation of CDKN2A may further contribute to reverse the malignant potency of cells. P21 is a cell cycle arrest gene in response to many stimuli, but can

also act as an oncogene depending on the cellular context [31]. In cervical cancer, p21 expression can be upregulated upon the activation of apoptotic pathways, e.g. expression of p21 is correlated with apoptosis after chemotherapy using DOX in CaSki cells [35]. It may very well be that the observed induction of p21, as well as the down-regulation of CDKN2A, leads to a less malignant phenotype and it could be an important explanation for the observed EPB41L3-induced decreased cell growth. The observed apoptotic effects are probably mediated by p53-independent pathways, as p53 is functionally inactive in cervical cancer. These and other studies demonstrate the potency of ATF technology as a tool for elucidating gene-function and pathways in cancer cells [19, 20, 21, 36].

Recently, transcription activator–like (TAL) effectors have been presented as an alternative for gene-targeting, promising higher success with regard to specificity and predictability [37, 38]. However, their potency to induce expression of methylated genes does not seem robust [39]. A recent study demonstrates activation of promoter activity for the pluripotency genes (Sox2, Klf4, c-Myc and Oct4) by TAL effectors from a reporter plasmid. However, endogenous upregulation in cells could only be achieved for Sox2 and Klf4, but not for Oct4 and c-Myc [40]. The authors argued that epigenetic repression of these genes may have prevented the TAL effectors ability to activate transcription, indicating possible limitations for TAL effectors to induce gene transcription. Contrary, ZF-ATFs are able to induce Oct4 levels in the highly methylated and silenced MDA-MD-231 cell line, which even resulted in a small activation of Oct4's downstream target nanog[41]. As ZFPs are relative small proteins (6F-ZFP < 200 aa, (TAL effectors > 800 aa)), they may have more efficient access to epigenetically silenced regions, and therefore better suited for gene re-expression. Here, we also show that only two engineered ATFs targeting a chosen TSS in close proximity can both have high success rate when it comes to gene induction. Additional advantages of their small size include low engineering prices and superior suitability for delivery in vivo. An improvement in the field of TAL effectors was the targeting of combinations of various TAL effectors to a single gene, which resulted in a great improvement in gene activation compared to a single TAL effector [39]. Also combination of an ATF with a targeted epigenetic enzyme has shown these improved effects on gene-regulation [42], but single ZF-ATF treatment often already leads to satisfactory gene-inductions and the desired functional effects, as also demonstrated here for EBP41L3.

Previously, it was shown that ATF based re-expression can have an impact on local epigenetic structures of genes, resulting in decreased DNA and H3K9me3 methylation levels [19, 26, 28]. Additionally, here we showed that ATFs can also induce H3Ac in an unmethylated cell line. Despite these epigenetic modifications, we observed a decline in EPB41L3 expression after removal of the ATF. Previously, a similar decline was observed for maspin using maspin specific ATFs [27]. However, we showed that prolonged re-expression could be achieved, when cells were co-treated with the DNA methylation inhibitor 5-aza-dC or the histone deacetylase inhibitor TSA. Epigenetic drugs are known to synergize with ATFs[19,43], and together may induce a strong change in histone/DNA methylation level enabling stable epigenetic programming and leading to sustained re-expression of a gene. 5-aza-dC itself has long been considered a transient drug, but recently this view has been revised as transient exposure of 5-aza-dC could produce an anti-tumor 'memory' response in leukemic and epithelial tumor cells [44]. In the current study, we also observed sustained re-expression of EPB41L3 after 5-aza-dC treatment, although higher expression levels could be induced by co-treatment with ATFs. An improvement of the ATFs may be the replacement of VP64 with epigenetic enzymes (DNA demethylases [45] or histone modifiers), enabling epigenetic editing at a chosen target site in a more efficient way [22]. Drugs influencing epigenetic features offer attractive prospects, as stable reprogramming of cells can be achieved by epigenetic changes, even after clearance of the drug (hit and run approach)[22]. However, often a combination of different classes of epigenetic drugs is required to achieve a favorable outcome on the desired epigenetic effect. While four epigenetic drugs are already clinically approved for malignancies [13], efforts are ongoing to make ZFP-based ATFs more suitable for clinical application [46], including protein- [30, 47] and RNA delivery [25]. A promising development in the field is the recent approval for the treatment of lipoprotein lipase deficiency by an adeno-associated viral vector engineered to express lipoprotein lipase [48]. Advances in such gene or protein delivery systems are very beneficial for developing save therapeutic strategies utilizing ATFs for future clinical applications.

In conclusion, this study demonstrated that the ATF-technology is uniquely suited to re-express endogenous EPB41L3 in various types of cancer cell lines and that EPB41L3 exerts tumor suppressive effects, also in cervical cancer. Moreover,

we showed that ATF mediated re-expression is associated with the induction of H3Ac in unmethylated cells and a reduction of H3K9me3 in methylated cells. Furthermore, re-expression of EPB41L3 could be sustained for several days when co-treated with epigenetic drugs. Therefore, the growth inhibitory effects associated with re-expression of EPB41L3 could be prolonged, which may be beneficial for future therapeutic applications.

Materials and Methods

Cell lines

All human breast cancer cell lines (MDA-MB-231, SKBR3), human ovarian cancer cell lines (SKOV3, A2780) and human cervical cancer cell lines (HeLa, CaSki and C33A) were obtained from ATCC (Manassas, VA) and cultured in DMEM (BioWhittaker, Walkersville, MD) supplemented with 10% FBS (BioWhittaker), 2mM L-glutamine and 50 µg/ml gentamycin. All cell lines were confirmed by STR profiling (BaseClear, Leiden, the Netherlands).

ATF retroviral transduction/development of stable cell lines

Two target regions of ATFs, designated 21ab and 22ab, were selected based on close proximity to the transcription start site (TSS) and high affinity predictions (www.zincfingertools.org). Double stranded DNA oligos (BIO BASIC, Markham, Canada) coding for the two 6-finger ZFPs predicted to bind the target sequences (21ab: GCAACAGGGGGCGGGGGG, 22ab: GGGGAGGAAGCCGCAGCC) were subcloned into the pMX-IRES-GFP containing either the gene activator VP64 or no effector domain (NoEf) [16, 19]. ATF-21ab-VP64 (21ab-VP64) was subcloned in the Retro-X Tet-On advanced inducible expression system (Clontech, Mountain View, CA) according to the manufacturer's instructions. Transduction of host cells with pMX-IRES-GFP or pRetroX-Tight-Pur was performed as previously described [19]. To obtain stable RetroX-Tet-On double transfectants, cells transduced with pRetroX-Tet-On/ pRetroX-Tight-Pur (ratio 1:3) were placed under selection with G418 sulfate (InvivoGen, San Diego, CA) (600 µg/ml) and puromycin (InvivoGen) (1 µg/ml) for two weeks. To express 21ab-VP64 in double transfectants, cells were treated with doxycycline (DOX) (Clontech) (500 ng/ml) for 2 days. DOX-treated cells

were also co-treated with 5-Aza-2'-Deoxycytidine (5-aza-dC) (Sigma, St Louis, MO) (5 μM) or trichostatin A (TSA) (Sigma) (400 nM).

Quantitative real-time PCR

RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Venlo, The Netherlands) and converted into cDNA (Fermentas, Leon-Rot, Germany). 20 ng cDNA was used for qRT-PCR for the quantification of EPB41L3, VP64, CDKN2A, p21, p53 and GAPDH, as previously described [19]. Samples without amplification curves were assigned a Ct value of 40. Sequences of primers and probes are listed in Table I. RNA levels were determined by the following formula: $2^{-\Delta\Delta Ct}$.

Bisulfite sequencing

DNA of untreated cells was bisulfite converted (EZ DNA Methylation-GoldTM Kit, Zymo research, Irvine, CA) and amplified with primers specific for the EPB41L3 promoter (Fw 5'-GTAATAGGGGGYGGGGGGAATAG-3', Rev 5'-AACCCCCTCRCAATCCCCCACTC-3') as previously described [14]. PCR products were cloned into the pCR 2.1-TOPO Vector (Invitrogen, Leusden, the Netherlands) and subjected to sequencing.

Chromatin ImmunoPrecipitation (ChIP)

A ChIP for the detection of HA tag, acetylation of histone 3 (H3Ac) and trimethylation of Lys9 of histone H3 (H3K9me3) was performed 72 hour after transduction. ChIP was performed as previously described [19] with the following antibodies: normal rabbit IgG (ab46540) (Abcam, Cambridge, UK), HA tag (101P-200) (Covance, Uden, the Netherlands), H3Ac (06-599) and H3K9me3 (07-442) (Millipore, Billerica, MA). DNA specific for the EPB41L3 promoter was amplified with the following primers: Fw 5-'CCCGGGCTCCCTGCT-GATCC-3' and Rv 5-'CCTCGGGCTCTTCCTCCGCA-3'.

Cell growth/apoptosis assay

To quantify the fraction of apoptotic cells, cells were incubated in 1,1',3,3,3',3'-Hexamethylindodicarbocyanine iodide (DilC) (Enzo Life Sciences, Farmingdale, NY) containing medium (50 nM, 20 min), and analyzed by flow cytometry

using a FACS Calibur cytometer and CellQuest software (BD Biosciences, San Jose, CA). The fraction of living cells with decreased DiLC signal was considered apoptotic, as exemplified in Fig. S1. A MTT assay (Sigma) was performed to examine metabolic activity representing cell growth of transduced cells as previously described [19].

Statistical analysis

Statistical significance was determined using GraphPad Prism 5 software (Student's t-test). A p-value of less than 0.05 was considered statistical significant (*$p<0.05$ and **$p<0.01$).

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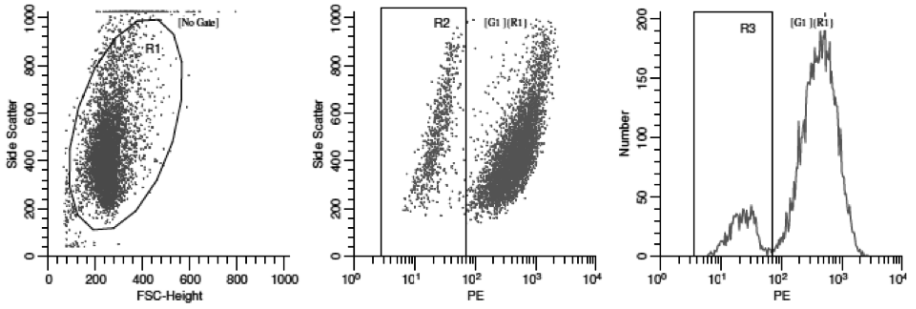
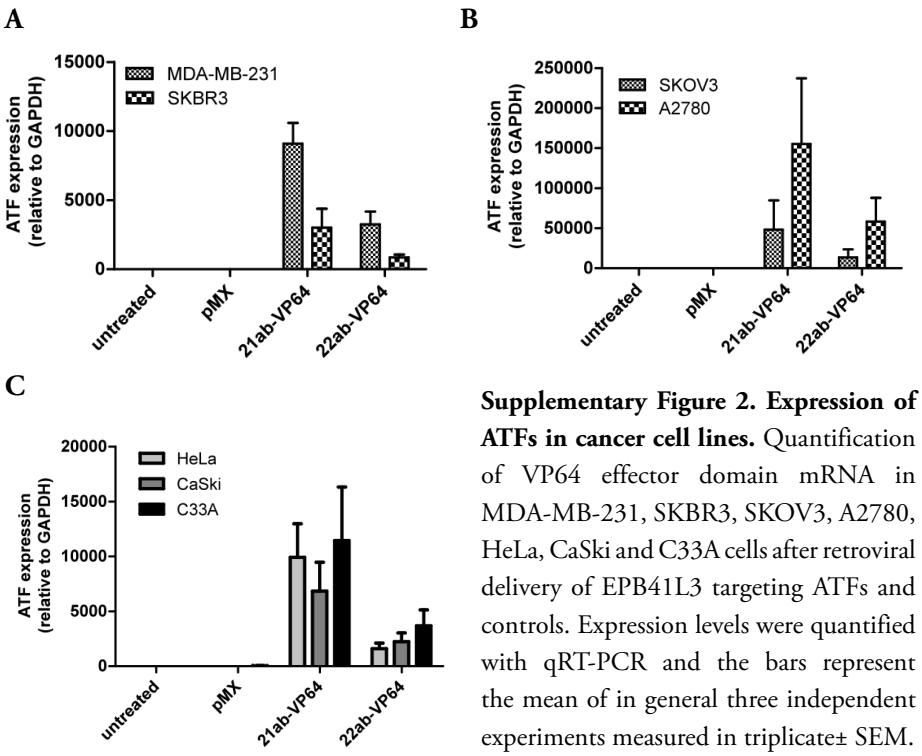


Supplementary Figure 1. Fraction of apoptotic cells determined with a DilC assay. From the living cell population (R1), the fraction of apoptotic cells was determined as the number of cells with a decreased DilC intensity (R2, R3).



Supplementary Figure 2. Expression of ATFs in cancer cell lines. Quantification of VP64 effector domain mRNA in MDA-MB-231, SKBR3, SKOV3, A2780, HeLa, CaSki and C33A cells after retroviral delivery of EPB41L3 targeting ATFs and controls. Expression levels were quantified with qRT-PCR and the bars represent the mean of in general three independent experiments measured in triplicate± SEM.

Chapter 6

General discussion and future perspectives

In this thesis, we modulated the expression of target genes using artificial transcription factors (ATFs). We also successfully induced epigenetic modifications on two target genes in a targeted manner (Epigenetic Editing), and thereby we set the stage to permanently modulate expression of target genes. Epigenetic modifications are aberrantly altered in several diseases including cancer. The genes we selected, were reported to be genetically or epigenetically mutated in cancer. Our first target, Her2/neu gene, is overexpressed/amplified in cancer. According to previous studies, HER2/neu expression is associated with the absence of H3K9me2. So, we demonstrated the induction of H3K9me2 mark on HER2/neu gene using H3K9 methyltransferase G9a fused to the zinc finger protein (ZFP) which target Her2/neu. Interestingly, this Epigenetic Editing resulted in downregulation of this gene [Chapter 3], which, in turn, was associated with less cell growth. Our next target was ESR1/ER-a gene, another key gene in breast cancer. We demonstrated up- and downregulation of this gene using ATFs composed of ZFPs fused to transient effector domains (VP64 or SKD). Subsequently, we induced DNA methylation on this gene using a DNA methyltransferase fused to an ESR1-targeting ZFP which was associated with its downregulation [Chapter 4].

Another target gene in this thesis was EPB41L3, a tumor suppressor gene silenced in several types of cancer including breast. We upregulated EPB41L3 using ATFs composed of EPB41L3-ZFPs fused to VP64 which resulted in apoptosis. Using an inducible expression system, we showed a decline in upregulation of EPB41L3 overtime. Epi-drugs in combination with ATFs resulted in an improvement in duration of EPB41L3 upregulation which implies that epigenetic mechanisms affect its expression and makes it an attractive target in epigenetic therapy of cancer [Chapter 5].

Although feasibility of Epigenetic Editing of genes is shown in this thesis and by other studies, it requires further investigation to become a straightforward approach. The efficacy of both epigenetic effector domains and DNA binding domains, the two minimal components of Epigenetic Editing, is very important in the ultimate outcome of Epigenetic Editing. The dominant functionality of written or erased epigenetic modifications in different microchromatin contexts as the result of epigenetic effector domains determines efficiency of Epigenetic Editing approach. There are various known and unknown factors influencing

the function of epigenetic modifications e.g. the genomic locations of epigenetic modifications (the target site), the crosstalk between epigenetic modifications, and the higher order chromatin context of cells. The current Epigenetic Editing approach can be used as a research tool to interrogate epigenetic regulation mechanisms. Moreover, to fully exploit this promising approach as a therapeutic option, first, the factors which influence the ultimate effect of epigenetic modifications need to be addressed. In addition, to write or erase epigenetic modifications in the right position, the efficiency and specificity of DNA binding domains (e.g. ZFPs) are factors to be taken into account for designing the Epigenetic Editing tools and ATFs. Here we briefly discuss some of this factors.

The most suitable position within a gene to control its expression

Gene expression is controlled and regulated in several levels including the transcriptional level. At the transcriptional level, the regulation of gene expression is a result of complex interactions between the DNA sequence and the transcription machinery, as well as epigenetic modifications such as DNA methylation and histone modifications. The position of epigenetic modifications can influence their correlation with gene expression [1] . So, to exploit Epigenetic Editing for regulating genes, it is necessary to write or erase epigenetic modifications at the most suitable and relevant position of the target gene. In general, enhancers, and regions around the transcription start sites (TSSs) play essential roles in gene expression regulation. It has been shown that epigenetic modifications of the promoter region are correlated with gene transcription [2-4]. Correlation of epigenetic modifications with gene expression has been intensively investigated. In this regard, an international project called ENCODE [5] provides the field of epigenetics and genetics with a wealth of data including epigenetic features which are correlated with gene expression. Indeed, it has been demonstrated that epigenetic modifications can be used to predict gene expression [6].

DNA methylation of the promoter and/or in the close proximity to the TSS is known to be associated with gene repression [1]. For instance, DNA methylation of the promoter of imprinted genes is responsible for allele-specific expression [7]. It is commonly known that several silent (tumor suppressor) genes in different types of cancer including breast cancer are aberrantly hypermethylated [Chapter 2, Chapter 4].

Although it is not completely clear whether DNA methylation directly causes the gene silencing, induction of DNA methylation on target genes was associated with their repression [8, 9]. In this thesis, we also demonstrated that induction of DNA methylation on ESR1 was correlated to gene downregulation [Chapter 4]. Moreover, targeted DNA demethylation was correlated to gene expression [10-13].
As well as in promoters, CpG islands are commonly found in the first exon of genes [14]. DNA methylation in the first exon of some genes has been reported to coincide with their repression in cancer [15]. As an example, ESR1 gene has been reported to be methylated in the first exon and its proximal promoter in ER-negative breast cancer cells [16, 17] which is in line with our observation [Chapter 4]. In contrast, occurrence of DNA methylation was shown in gene bodies of transcribed genes [18]. Such observations suggest that DNA methylation of the gene body is not linked to gene repression [19], instead, it might have roles in the process of splicing of RNA [20, 21].
Besides DNA methylation, histone modifications are thought to be involved in the regulation of gene expression and indeed these modifications have also been successfully investigated for predicting gene expression [4, 6]. Histone modifications are classified as active or repressive histone modifications; some histone modifications like acetylation of histones H3/H4 and methylation of histone H3 lysine 4 (H3K4me3), are classified as euchromatin-related marks and are commonly associated with active transcription; whereas modifications like methylation of lysine 9 or lysine 27 of histone H3 (H3K9me2/3 or H3K27me3), are considered as heterochromatin-related marks which are often related to gene repression [22].
It is under debate whether histone modifications are causative in gene expression regulation [23, 24]. However, modulation of the target gene by induction of histone marks in the chromatin context had been shown only once in 2002 [25] before we demonstrated, in 2013, that the induction of repressive histone mark H3K9me2 on Her2/neu gene was enough for gene downregulation [Chapter3],[26]. Recently, there are two more studies validating our observations of targeted histone modifications resulting in gene repression association of induced histone marks with gene expression [27, 28].
The position of histone modifications is important for their effect on gene expression. Histone modifications at the promoter and the TSS seem to be

associated with gene expression. For instance in actively expressing genes, H3K4me3 is found on nucleosomes flanking the nucleosome depleted TSSs [1] and the histone acetylation of the promoter is correlated with gene expression[29,30]. Inactive histone marks such as H3K9me3 and H3K27me3 are often located at the promoters of inactive genes. However, H3K9me3 mark was found to be co-located with H3K36me3, an indicator of transcribed genes, in the gene body[31].

So far, Epigenetic Editing which target either promoter, around TSS, enhancer, the first exon of genes led to successful results [8-12, 26-28], [Chapter 4]. However, the importance of the target position needs to be taken into account, it is possible that some positions are more crucial in gene regulation. In this respect, DNA methylation of a particular CpG, without the need for methylation of the entire CpG island, can be enough for gene downregulation [32].

In our research, we could induce repressive histone marks and DNA methylation downstream of TSSs of two different genes in different cell types. Importantly, the induced modifications were correlated with downregulation of our target genes which suggest that downstream of TSSs can be considered as a suitable position for induction of H3K9me2 and DNA methylation of HER2/neu and ESR1, respectively. Studies targeting different positions and comparing the outcome address this question [11, 27, 28, 33]. Taken together, the efficiency and effect of Epigenetic Editing can be dependent on the importance of the target site in gene regulation besides the fact that it can be gene-type dependent.

Epigenetic modifications crosstalk

Epigenetic modifications such as DNA methylation and histone modifications interact and influence each other. Such interactions (crosstalk) including (i) co-localization of epigenetic modifications, (ii) recruitment of modification-binding proteins/epigenetic enzymes by epigenetic modifications, and (iii) recruitment of epigenetic enzymes by other epigenetic enzymes seem to be necessary for maintaining the gene expression status.

(i) DNA methylation and repressive histone marks at promoters and TSSs were found to co-localize. For instance, in inactive genes, DNA methylation of the promoter is accompanied with H3K9me3 mark on nucleosomes at the TSS [31].

(ii) Alternatively, methylated DNA binding domain proteins were found to recruit

histone deacetylases which, altogether, stabilize the repressive state of chromatin. Interestingly, the H3K9me3 writers can be recruited by DNA methylation, since DNA methylation was found to direct H3K9me3 or H3K9me2 [34].

(iii) Such crosstalk can also occur through the direct interaction between epigenetic enzymes; for instance, DNA methyltransferases (DNMTs) recruit histone modifier enzymes [35, 36]. However it can be the other way round, when assessment of silenced genes in the embryo cells showed that histone methyltransferases such as G9a which induce repressive histone mark H3K9me2 can recruit DNMTs, so they co-silence the gene [37, 38]. Indeed, such interactions are involved in spreading of epigenetic modifications along the genome. In this respect, H3K9me3 is able to recruit HP1, heterochromatin binding protein, which interacts with SUV39H1 (H3K9me3 writer), and is involved in spreading heterochromatin marks [39]. HP1 can also interact with HDACs which further enhance the repressive state of chromatin. We also observed that induction of a repressive histone mark was associated with reduction of active H3 histone marks [26][Chapter 3] which further confirms the interactions between histone modifications and /or the epigenetic enzymes.

Importantly, DNA methylation and active histone marks like H3K4me3 also prevent each other. There is evidence that the presence of H3K4me3 prevents DNA methylation occurrence [40]. H3K4me3 is found on the nucleosomes which are flanking the nucleosome-free region of TSSs of actively transcribed genes [41,42], and it is of interest to know that de novo DNA methylation (using DNMT3L) require nucleosomes that contain unmethylated H3 lysine 4 to recruit the related DNMT enzymes [43]. So both presence of H3K4me3 and absence of nucleosomes around TSS can cause absence of DNA methylation.

Investigations on crosstalk between epigenetic modifications has shown a complex picture [1, 22] and Epigenetic Editing as a research tool is uniquely suited to address the order of events. Understanding interactions between epigenetic modifications will be helpful in improving Epigenetic Editing as a research tool and also for unraveling the epigenetic mechanisms which underlie the gene expression for future therapeutic purposes. Since the impact of epigenetic modifications on gene expression might be influenced by their interactions, Epigenetic Editing of one epigenetic modification may be not stable as interactions of native signature recruit epigenetic enzymes to re-establish the original epigenetic signature.

Epigenome context

It is well known that the expression of a single gene may vary in different cell types as well as in different levels of differentiation. This is due to the fact that although cells share identical genomes, but they have different epigenetic patterns [44]. Interestingly, cells inherit their epigenetic patterns during cell divisions, thereby maintain their gene-specific gene expression profiles [45]. The human embryonic and differentiated cells have distinguishable epigenomes. One important epigenetic feature of embryonic cells is the high rate of bivalent promoters which have both active (H3K4me3) and inactive histone marks (H3K27me3) [4].

Differentiated cell types have their own distinct epigenetic patterns. Comparing two differentiated cell types (human mammary epithelial cells and human mammary fibroblasts) derived from breast tissues of three women identified nearly 3000 cell-type specific differentially methylated regions (ctDMRs) of which 1236 ctDMRs were methylated in human mammary fibroblasts and 1572 other ctDMRs were methylated in human mammary epithelial cells [46]. DNA methylation patterns were found different among human individuals, and large scale studies showed that the inter-individual variation of DNA methylation patterns are more apparent in CpG poor regions than in CpG rich regions. Assessing differentially methylated regions of imprinted genes in the same tissue but between different individual showed a high degree of inter-individual variability and different patterns [47].

We observed different efficiency levels of ATFs for upregulation of ESR1 in two different ER-negative breast cancer cell lines [Chapter 4]. This difference might be due to different epigenome contexts of these ER-negative cell lines as we observed that ESR1 gene in one of this cell lines is highly methylated, whereas it is less methylated in the other one [Chapter 4]. We also observed that changes in histone marks upon upregulation of EBP41L3 is cell line dependent [Chapter 5]. Our observations and studies investigating epigenetic context variations suggest that the function of epigenetic modifications is dependent on the microchromatin context they are located in. Understanding epigenetic features/signatures of different cell types in normal situation is required and helpful for unraveling epigenetic modifications underling diseases including cancer and for restoring the normal epigenetic feature/signature.

Specificity and efficiency of DNA binding domains

The DNA binding domain of Epigenetic Editing plays a critical role in the specificity of the Epigenetic Editing approach. In this thesis, we used ZFPs which are engineered to target and regulate endogenous genes for almost two decades [48]. ZFPs are designed commonly as proteins with 3-finger or 6-finger modules, each finger is designated to be specific to 3 nucleotides of DNA, therefore 6-finger ZFPs target 18-bases, which is expected to be a unique site in the genome. Specificity of 6-finger ZFPs was determined in a study that showed ErbB2-ZFP (HER2-ZFP) is specific to HER2/neu and not to another member of HER-family (ErbB3) with highly similar DNA sequence [49]. Moreover, specificity of the ZFP was confirmed when the effect of the ZFP was assessed on 16000 genes and the ZFP effect was found specific to the target gene [50].

However, specificity of ZFPs is under debate. We showed that although HER2-ZFP preferentially binds to the HER2/neu gene it has genome-wide and off-target effects [Chapter 3]. Mapping the binding sites of different ZFPs and comparing them will be interesting and it, indeed, provides us with useful information for designing more specific ZFPs.

Towards enhancing specificity and efficiency of DNA binding domains, two recent domains have emerged and are used as alternatives for ZFPs mainly in genome engineering but also in Epigenetic Editing: TALEs (transcription activator like effectors) and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) [51].

TALEs derived from a genus of plant pathogen bacteria were expected to be more specific compared to the ZFPs, as fingers of ZFPs can influence each other specificity dependent on their context [52]. However, TALEs have their limitations including the difficulties for cloning of repeat TALE arrays due to very identical repeat sequences (DNA rearrangements), and their big size which makes TALEs delivery into the cells difficult, whereas ZFPs are much smaller and easier for delivery, and interestingly, they entered the clinical trials [51, 53, 54]. CRISPR technology seems to be groundbreaking and it shows to be promising as a research tool, however, sequence requirement for "conserved dinucleotide-containing protospacer adjacent motif" which is involved in target recognition by CRISPRs may limit some of their applications [51]. However, systematic comparison of these three DNA binding domains need to be performed to validate their specificity.

Future perspectives

In conclusion, Epigenetic Editing as a research tool is useful to regulate expression of genes and to understand the role of epigenetic modifications in gene regulation. However, there are important factors which should be considered and controlled to develop robust Epigenetic Editing as a therapeutic option. Factors including microchromatin context and epigenetic modifications interactions which influence function of epigenetic modifications should be further addressed. Although, it is under debate whether epigenetic modifications are causes or consequences with respect to gene expression, in this thesis we could show that H3K9me2 and DNA methylation are causative in gene expression. Addressing this question for the role of different epigenetic modifications on gene expression is necessary for developing a therapeutic approach which can be used to mimic and reverse epi-mutations.

Although, our studies and other Epigenetic Editing studies demonstrated the efficacy of Epigenetic Editing in writing of epigenetic modifications, sustainability and heritability of these marks as well as the level of specificity of Epigenetic Editing technology remain to be addressed. The inducible expression system seems promising for studying gene expression regulation [Chapter 5], therefore a long–term study exploiting the inducible condition is necessary to validate the heritability and sustainability of edited epigenetic modifications.

It is getting more apparent that epigenetic modifications interact, and their interactions are essential for their sustained effect on gene expression. It implies that efficiency of Epigenetic Editing approach on gene expression can be improved via co-targeting multiple key epigenetic modifications. A sustained gene repression is expected to be achieved, for example, by simultaneously erasing H3K4me3 and writing of DNA methylation, however, it should be kept in mind that erasing H3K4me3 might be not enough for inducing DNA methylation[55]. In this respect, combination of epigenetic therapies which inhibit DNA methylation and histone deacetylation was shown to have a better effect than monotherapy in breast cancer patients [Chapter 2]. However, because of the genome-wide function of epigenetic drugs [Chapter 2], Epigenetic Editing, in future, will be a good candidate to be used as an alternative or in combination with other treatments for cancer and other epigenetic-related diseases.

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Chapter 7

Summary

Epigenetic modifications such as histone modifications and DNA methylation are found to be correlated to gene expression. Besides genetic mutations, aberrant epigenetic modifications (epigenetic mutations) are linked to several diseases including cancer. In contrast to most genetic mutations, epigenetic modifications are reversible; this feature makes epigenetic modifications attractive targets for novel therapeutic approaches. The aim of this thesis was to re-write epigenetic modifications on target genes (Epigenetic Editing) in order to permanently modulate their expression.

After a general introduction on this thesis in chapter 1, we have reviewed the role of epigenetic modifications specifically in breast cancer and the current and possible future epigenetic therapies for breast cancer in chapter 2. Indeed, global changes of epigenetic modifications, including genome-wide DNA hypomethylation and histone hypoacetylation, occur in breast cancer. DNA hypermethylation of several key genes in breast cancer is associated with silencing of these genes. Several epigenetic drugs (epi-drugs) have shown efficient suppression of breast cancer growth in preclinical studies. Mono-treatment of breast cancer patients with epi-drugs has very modest anti-tumor effects, so current clinical trials focus mainly on combination therapies. However, the genome-wide function of epi-drugs is a disadvantage of these drugs, and the recent Epigenetic Editing technology seems promising to provide a more specific approach as an alternative or in synergy with other approaches.

In chapter 3, using Epigenetic Editing, repressive histone H3K9me2 marks were induced on the HER2/neu gene. HER2/neu is overexpressed in several types of cancer and its overexpression is associated with the increased cell growth. We fused a ZFP targeting HER2/neu gene to a catalytic domain of a histone methyltransferase (G9a or SUV39-H1). We demonstrated that the ZFP binds preferentially to HER2/neu gene. Upon transduction of cancer cells to express the HER2-ZFP fused to G9a, H3K9 methylation marks were efficiently induced on the HER2/neu gene, resulting in reduced HER2/neu expression and less cell growth. In addition, we demonstrated that co-treatment of ovarian cancer cells with lapatinib and targeted downregulation of HER2/neu was associated with an enhanced response of ovarian cancer cells to lapatinib.

In chapter 4, we targeted the estrogen receptor gene (ESR1) by engineering artificial transcriptional factors (ATFs) and Epigenetic Editing tools. Expression

of ESR1 is dysregulated in cancer. We up- and downregulated expression of this gene using ATFs which are composed of the ZFP fused to transient transcription effector domains (VP64, SKD) in ER-negative and ER-positive cancer cells. We also induced DNA methylation on exon 1 of this gene using the ZFP fused to DNA methyltransferase M.SssI. We showed that induced DNA methylation through Epigenetic Editing, was associated with downregulation of ESR1 gene. DNA methylation-mediated downregulation of ESR1 as well as SKD-mediated downregulation of ESR1 was linked with less colony formation capacity of cancer cells.

In chapter 5, our target was EPB43L1 gene which functions as a tumor suppressor gene and is silent in cancer. We upregulated this gene using ATFs composed of the ZFP fused to VP64. Upregulation of EPB43L1 was associated with increased apoptosis. Interestingly, upregulation of EPB43L1 by VP64, a non-catalytic activator of gene expression, was correlated with reduced histone repressive marks and increased histone active marks. Sustainability of upregulation of EPB43L1 was assessed overtime via the inducible expression system. It was shown that the upregulation level of EPB43L1 is reduced overtime, but co-treatment of cells with epi-drugs resulted in stable elevated expression of EPB43L1.

In chapter 6 a general discussion on the research in this thesis is provided; we also describe some important factors which influence the efficacy of Epigenetic Editing technology.

Appendices

Nederlandse Samenvatting, List of publications and biography, Acknowledgments

Nederlandse Samenvatting

Epigenetische markeringen zoals histonmarkeringen en DNA methylatie zijn geassocieerd met genexpressies. Naast genetische mutaties, zijn ook epigenetische dysregulaties (epigenetische mutaties) geassocieerd met verschillende ziekten, zoals kanker. In tegenstelling tot de meeste genetische mutaties, zijn epigenetische veranderingen omkeerbaar. Bij verschillende ziekten wordt remming van epigenetische enzymen onderzocht als mogelijke nieuwe drugtarget. Het doel van dit proefschrift was om aan tonen dat gen-specifiek de epigenetische markeringen kunnen worden overschreven (Epigenetische Editing) en dat een dergelijke interventie resulteert in blijvende veranderingen in de expressie van het gen van interesse.

Na de algemene introductie in hoofdstuk 1, wordt een overzicht gegeven van de rol van epigenetische veranderingen in borstkanker en van de huidige en mogelijke epigenetische therapieën voor borstkanker in hoofdstuk 2. Globale veranderingen in epigenetische markeringen, zoals genoom-brede DNA hypomethylatie en histon hypoacetylatie, worden vaak gevonden in borstkanker. DNA hypermethylatie van verschillende belangrijke genen in borstkanker is geassocieerd met inactivatie van deze genen. In preklinische studies resulteren verschillende epigenetische geneesmiddelen (epi-drugs) in efficiënte remming van borstkankergroei. In klinisch onderzoek met epi-drugs bij borstkankerpatienten worden nog geen overtuigende anti-tumor effecten waargenomen, in de huidige klinische studies worden daarom epi-drugs nu beoordeeld in combinatie met standaardbehandelingen. De genoom-brede effecten van behandeling met epi-drugs is een nadeel van deze geneesmiddelen. Epigenetische Editing lijkt daarom meer belovend door de specifiekere aanpak.

In hoofdstuk 3 zijn remmende specifieke histonmarkeringen aangebracht (H3K9me2) op het promotergebied van het oncogen HER2/neu. HER2/neu komt hoog tot expressie op verschillende typen kankercellen en deze overexpressie resulteert in de toegenomen celdeling. Om de expressie van HER2/neu te remmen, fuseerden wij enzymen die een remmende epigenetische markering aanbrengen aan een DNA bindend domain (gebaseerd op zogenaamde Zink Vinger Eiwitten), welke gemaakt is om het HER2/neu gen te binden. We hebben eerst laten zien dat het ZFP domain preferentieel aan het HER2/neu gen bindt.

In cellen die dergelijke fusie eiwitten (zgn Epigenetische editors) tot expressie brengen, werd inderdaad de remmende epigenetische markering gevonden op het HER2/neu gen. Deze epigenetische editing resulteerde in een verminderde HER2/neu expressie en in een remming van de celgroei. Verder lieten we zien dat combinatie-behandeling van een Epigenetische Editor met Lapatinib op eierstokkankercellen met een hoge HER2/neu expressie meer tumorceldood induceerde dan met Lapatinib alleen.

In hoofdstuk 4 hebben we het gen coderend voor de estrogeen receptor gene (ESR1) gekozen voor genexpressiemodulatie. We hebben hiertoe eerst Artificiële Transcriptie Factoren (ATFs) gemaakt (DNA bindende domeinen gefuseerd aan niet-enzymatische transcriptie modulatoren) en daarna de DNA bindende domeinen gefuseerd aan epigenetische enzymen (schriivers van DNA methylatie of histonmethylatie): de Epigenetische Editing benadering. Ook expressie van ESR1 is vaak verstoord in kanker. De ATFs konden de expressie van dit gen aanzetten in ER-negative kankercellen en de expressie remmen in ER-positieve cellen. Vervolgens is DNA methylatie aangebracht op exon 1 van het ESR1 gen door een DNA bindend domein te fuseren aan DNA methyltransferase M.SssI. De DNA methylatie door middel van deze Epigenetische Editing resulteerde in remming van expressie van het ESR1 gene. De ATF-gemedieerde en de DNA methylatie gemedieerde remming van ER expressie resulteerde in verminderde groeicapaciteit van de kankercellen.

In hoofdstuk 5 was het EPB43L1 gen ons doelgen, dit gen functioneert als een zogenaamd tumor suppressorgen en is veelal uitgeschakeld in kanker. We hebben de expressie van dit gen geïnduceerd mbv ATFs, en deze gen re-expressie resulteerde in toegenomen celdood. Door middel van een induceerbaar systeem hebben we laten zien dat upregulatie van EPB43L1 afnam in de tijd. Deze afname konden we tegengaan door de cellen te behandelen met een remmer van epigenetische enzymen.

In hoofdstuk 6 volgt een algemene discussie en beschrijven we belangrijke factoren die de efficiëntie van Epigenetische Editing verder kunnen verbeteren.

List of publications/ in preparations

Fahimeh Falahi, Christian Huisman, Hinke G. Kazemier, van der Vlies Pieter, Klaas Kok, Geke A. P. Hospers, Marianne G. Rots. Towards sustained silencing of HER2/neu in cancer by epigenetic editing. Molecular Cancer Research journal, 2013;11: 1029-1039.

Fahimeh Falahi, Christian Huisman, Elisa Garcia Diaz, Hinke G Kazemier, Geke AP Hospers, Marianne G Rots. Gene specific overwriting of epigenetic signatures to modulate the expression of selected tumor-promoting genes in cancer. Epigenetics & Chromatin. 03/2013; 6(1). DOI:10.1186/1756-8935-6-S1-P15.

Fahimeh Falahi, Michel van Kruchten, Nadine Martinet, Geke A P Hospers and Marianne G Rots. Current and upcoming approaches to exploit the reversibility of epigenetic mutations in breast cancer. submitted to Breast Cancer Research.

Fahimeh Falahi, Geke AP Hospers, Marianne G Rots. Epigenetic Editing of Estrogen Receptor-Alpha Gene in Breast Cancer as an innovative tool to modulate its expression level (to be submitted).

Christian Huisman, Fahimeh Falahi, Juul Overkamp, Gellert Karsten, Ate G.J. van der Zee,, Ed Schuuring, G. Bea A. Wisman and Marianne G. Rots. Epigenetic sustained re-expression of EPB41L3 in cancer using ATFs and epigenetic drugs (in preparation).

Fernando J Bustos, Fahimeh Falahi, Martín Montecino, Brigitte van Zundert, Marianne Rots. Targeted epigenetic silencing of PSD95 gene expression rescues dendritogenesis in hippocampal neurons (in preparation).

Biography

Fahimeh Falahi was born in Iran. She obtained her high school and pre-university degrees at the high school for honors students where she was selected as the top student. Then she could successfully pass the entrance exam of the university and started her 4 year-bachelor study of Genetics at the Shahid Chamaran University, she performed her bachelor project under supervision of Prof. Galehdari to detect the mutations on the essential genes in hereditary nonpolyposis colorectal cancer. She graduated her bachelor as the top student. In 2003, she could successfully pass the entrance exam for the Master of Science studies and started the master of Molecular and Cellular Biology at the Kermanshah University and did her master project at the National Institute for Genetic Engineering and Biotechnology. During her 3 year-master study, she performed her research under supervision of Prof. Zamani and Prof. Motallebi and she engineered the artificial constructs carrying beans polygalacturonase inhibitor to inhibit polygalacturonase activity in Sclerotinia sclerotiorum. In 2006, after obtaining her master of science degree and being selected as the top student, she worked as the research assistant at the Royan Institute for the Stem Cell Research and worked as a part time teacher at 2 universities. In 2009, she moved to the Netherlands and started her PhD project at the department of Pathology and Medical Biology at the UMCG. Under the supervision of Prof. Marianne Rots and Prof. Geke Hospers, she performed her research on the gene-targeted silencing of overexpressed genes in cancer using epigenetic editing. The results of this research are presented in this thesis. Since February 2014, Fahimeh is working at the UWA, Perth, Australia as the research associate and she continues her research in the epigenetic editing field.

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