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 with monotherapy in vitro, in vivo (14, 15), and in clinical studies (16). Another application is through exploiting Her2/neu as a targeting device for antigenic targets. In this regard, TDM-1 (trastuzumab conjugated to a derivative of Maytansine 1, a potent antitumor agent) showed improved antitumor effect compared with 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 (ATF), composed of a DNA-binding domain fused to an effector domain (19). As DNA-targeting tools, engineered zinc finger proteins (ZFP) have shown great flexibility in gene targeting (20). Modulation of expression of various endogenous genes has been achieved with upregulation of CI30RF1B (21), Maspin (22), down/upregulation of EpCAM (23), and down-regulation 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, for example, super KRAB domain (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 some 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 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 4 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 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 2 mmol/L 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, 26, 31) and generously provided by Dr. Carlos Barbas (Department of Molecular Biology and Chemistry, The Scripps Research Institute, La Jolla, CA) 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 2 histone methyltransferases were cloned as reported previously (30). For G9a, the N-terminal domain (amino acid 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'-GCCAAAGATGCGGGCGGGGTC-CCATCTTGGC-3' and antisense: 5'-CGCCGGACCCCCGCCGC-CCATCTTGGC-3' primers. pMX vectors were cotransfected 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 2 consecutive days with the supernatant of transduced HEK293T cells supplemented with 6 μg/mL polybrene (Sigma) and 10% fetal calf serum.

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 cell sorting

Transduced host cells were washed and stained with the antibody against Her2/neu (APC anti-human-CD340, BioLegend). Calibruf flow cytometry (Beckton Dickinson Biosciences) was used to detect GFP expression and to determine mean fluorescence intensity.

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 on the basis of GFP and Her2/neu protein expression using a Beckman Coulter MoFlo XDP cell sorter (Becton Dickinson Biosciences).

Quantitative reverse transcription PCR

Total RNA was extracted from the transduced and nontransduced host cells using the Qiagen RNeasy plus mini kit (Qiagen), 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). 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 quantitative reverse transcription PCR reaction was conducted (ABIPrism 7900HT, Applied Biosystems) with 10 ng cDNA using ROX enzyme mixture (Abgene), 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’-CCACATCGCTCAGACACC-3’, Rv 5’-ACCAAACCCACCTTAAATACTC-3’), the medium was aspirated and treated with RNase (Roche) and high salt dilution (Invitrogen) during 15 minutes incubation, then unbound antibodies were washed-off and diluted sheared chromatin was added to the complex of magnetic Dynabeads (Invitrogen) and washed off unbound chromatin with PBS, chromatin was eluted with 2% SDS and 50 mmol/L NaHCO₃ and treated with RNase (Roche) and high salt concentrations at 65°C overnight. After reversing crosslinks and digestion of protein, DNA was purified as described previously (33).

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’-ACCTCTCCTCTTCTCTGTG-3’), region B (Fw 5’-GTTCGCACTCCAGATCCGTA-3’, Rv 5’-CTCTGTCCTACCAAACTCCTGTG-3’), and region C (Fw 5’-CGCGGCGCCGCGGCGCCGCC-3’, Rv 5’-GCA-CAAGGCCCAGCTC-3’). Quantitative PCR (qPCR) was conducted using Absolute 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^{(C_{Input} - C_{Input})} × dilution factor × 100.

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

To determine the specificity of the ZFP, we conducted chromatin immunoprecipitation (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 conducted using a Mondrian SP (NuGEN Technologies Inc.) 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 a HiSeq2000 (Illumina). Resulting reads were aligned to the human reference genome (NCBI37.2) using NextGENe V2.3.3 (SoftGenetics, LLC). 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. MTT reagent (Sigma) was added to the wells every 24 hours (for 24–120 hours or for 5 days as indicated). After 3 hours and 45 minutes of incubation at 37°C, the medium was aspirated and MTT crystals were dissolved in dimethyl sulfoxide (Merck). The optical 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 (3,000 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.

Cotreatment 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 μmol/L of lapatinib and transduced with pMX constructs. Cotreated 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 optical density was detected at a wavelength of 490 nm using Varioskan Flash (Thermo scientific, Fermentas).

Statistical analysis
Results were analyzed for significance using the Student t test or paired t test for sorted and cotreated 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-overexpressing cell lines, MDA-MB-231 and MCF-7 as Her2/low expressing cell lines. Her2/neu expression was measured at protein and RNA level (Fig. 1A and B). The 29 CpGs located in a 400 bp region (−143—+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 (−146—60 bp relative to the TSS) showed that this mark enriched in Her2/neu-positive cell lines (SKBR3, SKOV3) and not in Her2/neu-negative cell lines (MDA-MB-231, MCF7; data not shown).

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).

Figure 1. Her2/neu expression in the cancer cell line panel. A, measurement of Her2/neu expression at protein level was conducted by flow cytometry. The mean fluorescence intensity (MFI) of Her2/neu protein level in cell lines is the average (± SEM) of 3 independent experiments. B, measurement of Her2/neu expression at RNA level was conducted by qRT-PCR. The mean is the average (± SEM) of 3 independent experiments.

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 zinc finger 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 observed 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 with 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% ± 9.0% (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 SKOV3 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 (Supplementary Fig. S3A). 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 on the basis of 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 protein (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 with the sorted pMX empty (Supplementary Fig. S4B). Cells with very low expression of Her2/neu protein (7.3% of total viable cell population) showed 50- to 500-fold less expression of Her2/neu protein compared with GFP⁺ and GFP⁻ cell populations (Supplementary Fig. S4A). All 3 cell populations were subcultured but Her2/neu-low expressing cell population grew slowly and died. During subculturing, 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 for Her2/neu (P < 0.05) although, a slight increase was observed (Supplementary Fig. S4C). Interestingly, GFP expression of the GFP⁺ cells compared with the GFP⁻ cells maintained at a constant ratio during subculturing (Supplementary Fig. S4D). After 30 days 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 with the GFP⁻ cell population; also, there was more enrichment of H3K9me2 in GFP⁺ cells compared with GFP⁻ cells (Supplementary 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 with 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 with 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 super-transduced (retransduced) with pMX-E2C-G9a. Super-transduced 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
Her2/neu downregulation was associated with a significant decreased in metabolic activity at day 5 (42% ± 3% for -G9a, 40% ± 2% for -Suvdel76, and 35% ± 1% for SKD fused to pMX-E2C compared with 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 with 76 ± 8 for pMX empty–transduced cells ($P < 0.001$) and 55 ± 13 for pMX-E2C-SKD ($P < 0.05$; Fig. 5B).

**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. About 1 μmol/L lapatinib did not reduce growth of treated cells but there was an improvement in cell growth inhibition induced by 1 μmol/L lapatinib when combined with pMX-repressive constructs. When compared with 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 with pMX empty and lapatinib cotreatment (pMX-E2C-SKD: 43% ± 8.0% ($P < 0.05$); pMX-E2C-G9a: 49% ± 10%, ($P < 0.05$); and pMX-E2C-Suvdel76: 45% ± 11% ($P < 0.05$; Fig. 6).

**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 non-catalytic 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
Decrease of cell proliferation upon induction of H3K9 methylation. A, a 5-day cell proliferation assay was conducted on transduced SKOV3 cells with pMX empty, pMX-E2C, and pMX-E2C fused to transcription effector domains (SKD, VP64) and histone methyltransferases (G9a, Suv39-H1). Absorption is the average (± SD) of 3 independent experiments. B, transduced SKOV3 cells were replated and allowed to form colonies for 4 weeks. Number of colonies is the average (± SEM) of at least 3 independent experiments and representing colonies consisted of at least 50 cells. *, P < 0.05; **, P < 0.01; ***, P < 0.001).

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 transcription 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 pMX-E2C-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 this DNA 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 Suvedl76 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 position-dependent 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, pMX-E2C-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.
References


