Targeted DNA Methylation Using Modified DNA Probes: A Potential Therapeutic Tool for Depression and Stress-Related Disorders

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Posted Date: 1 April 2025

doi: 10.20944/preprints202504.0034.v1

Keywords: Epigenetics; DNA methylation (DNAm); gene expression; cortisol; FK506 Binding Protein 5 (FKBP5)

 doi:10.20944/preprints202504.0034.v1

Article

Targeted DNA Methylation Using Modified DNA Probes: A Potential Therapeutic Tool for Depression and Stress-Related Disorders

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Abstract: Epigenetic modifications play a crucial role in gene regulation and have been implicated in various physiological processes and disease conditions. DNA methylation (DNAm) has been implicated in the etiology and progression of many stress-related psychiatric behaviors such as depression. The ability to manipulate DNAm may provide a means to reverse and treat such disorders. Although CRISPR-based technologies have enabled locus-specific DNAm editing, their clinical applicability may be limited due to immunogenicity concerns and off-target effects. In this study, we introduce a novel approach for targeted DNAm manipulation using single-stranded, methylated DNA probes. We designed probes targeting the glucocorticoid response element (GRE) within the FKBP5 (FK506 binding protein 5) gene, a key regulator of stress response and depressive symptoms, and the promoter region of the MAOA (monoamine oxidase A) gene. In both HEK293 human embryonic kidney and mouse pituitary AtT-20 cells, transfection with their respective methylated probes significantly increased DNAm at targeted CpG sites in a persistent and dose-dependent manner. Importantly, the induced methylation effectively attenuated glucocorticoid-induced upregulation of FKBP5 gene expression. Alteration of methylation was specific to single-stranded probes, as double-stranded methylated probes and unmethylated probes showed no significant effects. Our findings suggest that methylated DNA probes have the potential to function as a simple tool for targeted epigenetic manipulation and serve as a safer alternative to CRISPR-based epigenome editing tools for the treatment of stress-related disorders such as depression.

Keywords: epigenetics; DNA methylation (DNAm); gene expression; cortisol; FK506 binding protein 5 (FKBP5)

Introduction

Epigenetic modifications, such as DNA methylation (DNAm), can lead to alterations in nuclear architecture and landscape, which can in turn affect the accessibility of transcription and regulatory factors to genes. DNAm typically occurs at 5′ end cytosine residues within CpG dinucleotides and is generally associated with transcriptional repression, which is mediated by the binding of methyl-CpG binding proteins and recruitment of histone-modifying enzymes [1]. Aberrant DNAm has been linked to many diseases from cancer to neurodegenerative disorders, but also psychiatric diseases such as depression, anxiety, and PTSD [2–7].

Epigenetic mechanisms can also mediate the impact of adverse environmental conditions on gene function. For instance, environmental stressors can cause epigenetic changes in the brain [8,9]. In such instances, it has been shown that the glucocorticoid (GC) receptor that binds to cortisol can directly alter DNAm of genes that are targets of GC signaling, thus, raising the possibility of mitigating disease symptoms by potentially reversing these epigenetic marks.

Targeted manipulation of DNAm at specific gene loci can modulate gene function and provide new therapeutic strategies for disorders associated with aberrant epigenetic regulation. In plants,

 doi:10.20944/preprints202504.0034.v1

small RNAs have been shown to direct DNAm and gene silencing [10]. Studies have shown the possibility of using pharmacological methods, such as DNMT inhibitors, to impact DNAm in mammalian systems. However, these drugs target the epigenetic machinery and are not locus-specific [11]. Although recent advances in CRISPR-based technologies have enabled locus-specific DNAm editing in mammalian systems [12–14], these methods may have limited clinical applicability due to their immunogenicity [15–17] and off targeting [18,19]. However, CRISPR-based therapies are now on the horizon for the treatment of debilitating diseases such as sickle cell-disease [20] and cancer [21]. Until these technologies are further refined to minimize their own disease burden and gain traction for the treatment of more common disorders, safer methods need to be explored.

The ability of the glucocorticoid receptor to induce loss of DNAm provides a useful model for testing alternative epigenetic tools. In particular, the FK506 binding protein 5 (FKBP5) gene encodes a co-chaperone of the glucocorticoid receptor and has been identified as a key regulator of the stress response [22]. It is thought that GC-induced increase in *FKBP5* levels leads to attenuated intracellular signaling and GC resistance, which are comorbid in more than 50% of cases of depression [23]. As such, genetic and epigenetic variations have been linked to depressive symptoms [24,25]. At the molecular levels, chronic exposure to stress or excess glucocorticoids can induce the persistent demethylation of intronic glucocorticoid response elements (GREs) in the FKBP5 gene [26]. This demethylation allows for increased binding of the GR to the GREs and a more robust transcription of *FKBP5*, which in turn leads to decreased sensitivity to GCs and GC resistance [27,28]. Epigenetic alterations in *FKBP5* have been linked to several stress-related psychiatric disorders such as PTSD, anxiety, and alcohol abuse [5,25,29,30].

In this study, we sought to develop a simpler tool for site-specifically altering DNAm. We asked whether a single-stranded, methylated DNA probe can induce DNAm of its complementary target. Specifically, we sought to reverse the persistent loss of DNAm caused by chronic exposure to GCs. Our findings suggest that methylated DNA probes may serve as a promising tool for targeted epigenetic manipulation and have potential therapeutic applications for mitigating the impact of excess stress or GC exposure in psychiatric disorders such as depression and non-psychiatric disorders associated with aberrant DNAm patterns. Further, it may be refined to target other genes in a simpler and safer way that can circumvent some of the limitations posed by the CRIPR technology.

Methods

Probe Design and Amplification

Methylated DNA probes targeting the conserved glucocorticoid response element (GRE) in intron 5 of the human (chr6:35,601,961 – 35,602,194; GRCh38/hg38, 256 bp) and mouse (chr17: 28,639,321 – 28,639,560; GRCm39/mm39, 239 bp) of *FKBP5* were designed. A separate DNA probe designed against an intronic, regulatory region of the human *MAOA* gene (chrX:43,656,383 – 43,656,553; GRCh38/hg38, 170 bp) was also tested [31]. Additional tests for non-specific effects of the human *FKBP5* probe investigated another adjacent region in intron 5 (chr6:35,610,962 – 35,611,313), intron 1 (chr6:35,687,767-35,688,045), and intron 7 (chr6:35,590,524-35,591,014). For both human and mouse probes, PCR primers targeted smaller regions than those analyzed by bisulfite sequencing to preclude the amplification of probe DNA during methylation analysis. Primers used for generating the probes are shown in **Table 1**. The genomic organization of the human *FKBP5* locus is shown in **Figure 1.**

Table 1. Sequence of primers for Probe Design and Pyrosequencing.

Probe Primers	Sequence	Size
Human FKBP5 Probe – Forward	5′- AAAGTCAAACCAAACCAAATTACC -3′	256 bp
Human FKBP5 Probe – Reverse	5′- TTTGTTACTGCTGTGCACTCTCT -3′	
Human MAOA Probe – Forward	5′- TCGACGTAGTCGTGATCGG -3′	170 bp
Human MAOA Probe – Reverse	5′- GCAGGATATGGGGCCAAG -3′	

 doi:10.20944/preprints202504.0034.v1

Mouse Fkbp5 Probe – Forward	5′- CAGACACCAGCTACTATAATTAG -3′	239 bp
Mouse Fkbp5 Probe – Reverse	5′- GCACATGAACTCGATGTGCTGACA -3′	
Pyrosequencing Primers	Sequence	Size
Human FKBP5 Intron 5 Outside – A	GGTAGAGAAAGAAATAAATAAGTTA	
Human FKBP5 Intron 5 Outside – B	TTCTTACATTTCATTTTTATTACTACTA	
Human FKBP5 Intron 5 Inside – A*	AAGATTATGTAATTTAAAGGGGGAGGG	
Human FKBP5 Intron 5 Inside – B	CTCTCTTTCCTTTTTTCCCCCCTAT	286 bp
Human FKBP5 Intron 5 Pyro 1	TCTTTCCTTTTTTCCCCCCTATT	
Human FKBP5 Intron 5 Pyro 2	CAATTTAAATAATATTTTACAACT	
Human FKBP5 Intron 5_2 Out – A	ATTTAATTGGTTTGGGTGTTAGAA	
Human FKBP5 Intron 5_2 Out – B	CCTCTCAATACTTTCAACCACA	
Human FKBP5 Intron 5_2 In – A*	GAGAATTATTGTATTGGAGGTT	
Human FKBP5 Intron 5_2 In – B	ATTCTACAAATTCCAATTATTAAC	
Human FKBP5 Intron 5_2 Pyro 1	GTATTGGAGGTTTATTGGTT	
Human FKBP5 Intron 5_2 Pyro 2	TAGATGATTATGAGTTTGGAGTT	406 bp
Human FKBP5 Intron 5_2 Pyro 3	GTTTAAGTTTTTTTTATATTTGTT	
Human FKBP5 Intron 5_2 Pyro 4	GATTTGGAGAGGGAAAGGAGGT	
Human FKBP5 Intron 1 Out – A	AGTTTAAATTGTTTTATGTAGAATTTATTGA	
Human FKBP5 Intron 1 Out – B	TCACTCCCAAACCATACC	
Human FKBP5 Intron 1 Inside – A	GTTTTGAATTATATTGAAGGGTATTT	
Human FKBP5 Intron 1 Inside – B*	CAAAACTCCTTATACTCTTCTATTCTAA	350 bp
Human FKBP5 Intron 1 Pyro 1	GTAGAATTYGATTTTAGAGA	
Human FKBP5 Intron 7 Outside – A	AGAGTGAAATTGAGATGGAAATATGT	
Human FKBP5 Intron 7 Outside – B	AATTTCTTCTCCATCCACTTCCTATA	
Human FKBP5 Intron 7 Inside – A	AGGAGGTATGTTGTTTTTGGAATTTAAG	
Human FKBP5 Intron 7 Inside – B*	AATTTATCTCTTACCTCCAACACT	
Human FKBP5 Intron 7 Pyro 1	GGAGAAGTATAAAAAAAAAATGG	
Human FKBP5 Intron 7 Pyro 2	GTTATAGAGTTTAGTGGTTT	
Human FKBP5 Intron 7 Pyro 3	GGAGTTATAGTGTAGGTTTT	503 bp
Human FKBP5 Intron 7 Pyro 4	TTAAGGAGTTATTTGGTAGA	
Human FKBP5 Intron 7 Pyro 5	TGATATATAGGAATAAAATAAGAAT	
Human MAOA Outside – A	GATTTAGGAGYGTGTTAGTTAAAGT	
Human MAOA Outside – B	TTATTATATCTACCTCCCCCAATC	
Human MAOA Inside – A	AGTTAAAGTATGGAGAATTAAG	
Human MAOA Inside – B*	ATCTACCTCCCCCAATCACACCACCAAC	
Human MAOA Pyro 1	AAAGTATGGAGAATTAAGAGAAGG	278 bp
Human MAOA Pyro 2	GAGTATYGYGGGTTATATG	
Human MAOA Pyro 3	AGGTGGTATTTTAGGTTAGTGTGGA	
Mouse Fkbp5 Intron 5 Outside – A	GATGATTAGTTTTTTTTAGTAGTGATGT	
Mouse Fkbp5 Intron 5 Outside – B	CTTATTATTCTCTTACTACCCTAA	
Mouse Fkbp5 Intron 5 Inside – A	TAGTTTTTGGGGAAGAGTGTAGAGTTAT	
Mouse Fkbp5 Intron 5 Inside – B*	ATTTTAAAAAACACAAAACACCCTATT	308 bp
Mouse Fkbp5 Intron 5 Pyro 1	AGAAAAGGGAAAGTAGG	
Mouse Fkbp5 Intron 5 Pyro 2	TAGTTTTTGTTATTGTTGTATG	

*These primers have been biotinylated and HPLC-purified for pyrosequencing.

In Vitro Methylation of DNA Probes

Purified *FKBP5* probes were subjected to in vitro methylation using the bacterial CpG methyltransferase (M.SssI, New England Biolabs) [32]. One μg of the probe was incubated with 4 units of M.SssI, 160 μM S-adenosylmethionine (SAM), and 1X NEBuffer 2 in a total reaction volume of 100 μL at 37°C for two 1-hour cycles followed by 20 minutes at 65°C for enzyme inactivation. A negative control reaction (*FKBP5* SssI-) was performed in parallel, where the M.SssI enzyme was replaced with an equal volume of water. Following in vitro methylation, probes were purified again and eluted in 20 μL of EB buffer. The concentrations of the methylated (FK SssI+) and unmethylated (FK SssI-) probes were measured using Qubit 4 Thermo Fisher Scientific, Waltham, MA). Similar reactions were performed for the mouse *Fkbp5* and human *MAOA* probes.

Cell Culture and Transfection

 doi:10.20944/preprints202504.0034.v1

To induce demethylation at the GRE of the endogenous *FKBP5* gene, we chose cell lines that we have previously shown to undergo DEX or glucocorticoid-induced loss of methylation [33,34]. Human embryonic kidney 293 (293HEK) and mouse pituitary AtT-20 cells were purchased from Atcc.org and were treated with 1 μM dexamethasone (DEX) for 5 days and cultured for an additional 5 days without any DEX [27]. This would allow CpGs to undergo persistent loss of DNA methylation which would then be restored by introducing methylated probes. Separate wells of cells were left untreated as negative controls or transfection reagent only controls. Prior to transfection, cells were seeded in 24-well plates at a density of 2×10^5 cells per well and allowed to adhere overnight in DMEM free of antibiotics. The transfection of the methylated and unmethylated probes was performed in triplicate (500 ng of probe per well) using X-tremeGENE 360 (MilliporeSigma, Burlington, MA) according to the manufacturer's instructions. Cells were fed fresh DMEM media one day after transfection and harvested on the second day for collecting gDNA. For the assessment of glucocorticoid-induced gene expression, a subset of the transfected 293HEK cells was treated with 1 μM DEX for 4 hours prior to harvesting. Cells were harvested for total RNA extraction using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

DNA Extraction and Methylation Analysis by Bisulfite Pyrosequencing

Genomic DNA was extracted from 293HEK and AtT-20 cells using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The extracted DNA was bisulfite-converted using the EZ DNAm-Gold Kit (Zymo Research, Irvine CA) according to the manufacturer's instructions. Conditions for bisulfite pyrosequencing have been published elsewhere [35]. Primers used for assessing DNAm levels of *FKBP5* and *MAOA* genes are shown in **Table 1**.

Gene Expression Analysis

Reverse Transcription Quantitative Real-Time PCR (RT-qPCR) was performed to assess the impact of targeted DNAm on gene expression. Complementary DNA (cDNA) was synthesized from 293HEK or AtT-20 RNA samples using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Procedures for gene expression analysis using the Taqman probe and QuantStudio5 platform (Thermo Fisher Scientific) have been published elsewhere [36].

Results

The Use of Methylated, Single-Stranded Probe to Induce Target-Specific DNAm

To test whether a simple DNA probe can target-specifically alter DNAm, we generated a probe against specific CpG sites within the glucocorticoid response element (GRE) of the human FKBP5 gene. In vitro-methylated, single- or double-stranded DNA probes were generated and transfected into human HEK293 cells. Probes were designed with sufficiently short lengths to avoid amplification by subsequent epigenetic assays (**Figure 1**). Two days post-transfection, cells were analyzed for DNAm levels of the *FKBP5* GRE by bisulfite pyrosequencing. Pyrosequencing analysis showed an increase of 20.5% increase in DNAm at CpG-1 ($P=7.0\times10^{-4}$) and an increase of 15.0% at CpG-2 (P= $P=4.9\times10^{-4}$) in 500 ng of methylated, single-stranded probe compared to untransfected samples (**Figure 2A**). We also observed a dose-response when we performed transfections with only 250 ng of probe, with CpG-1 showing a more modest increase of 8.6% (P=0.0066) and CpG-2 showing an increase of 7.6% ($P=1.1\times10^{-4}$) compared to untransfected samples. Samples transfected with unmethylated, single-stranded or methylated, double-stranded probes did not lead to an appreciable increase in DNAm (P>0.05). Representative pyrosequencing tracing is shown in **Figure 2B**.

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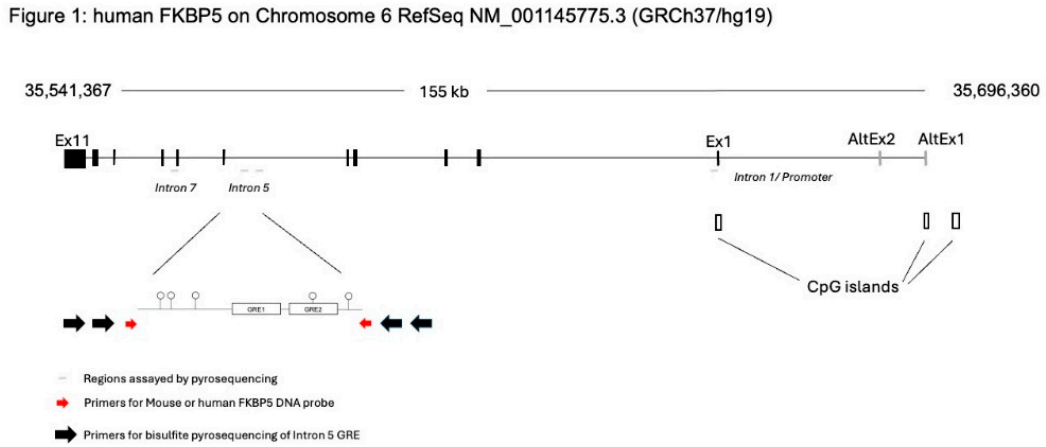

Figure 1. Genomic organization of *FKBP5*. The human FKBP5 locus is located on the negative strand of chromosome 6. For this project, three intronic regions have been tested, and these are indicated by thin horizontal gray lines. Two sets of large black arrows represent the outside and nested PCR primers used for bisulfite pyrosequencing. These black arrows flank the primers used for generating the probe (red arrows) which cannot be amplified by the pyrosequencing primers. The intron 5 GREs are composed of the main GRE formed by GRE1 and GRE2 for which the DNA probe was designed and an adjacent GRE that was tested as a negative control region.

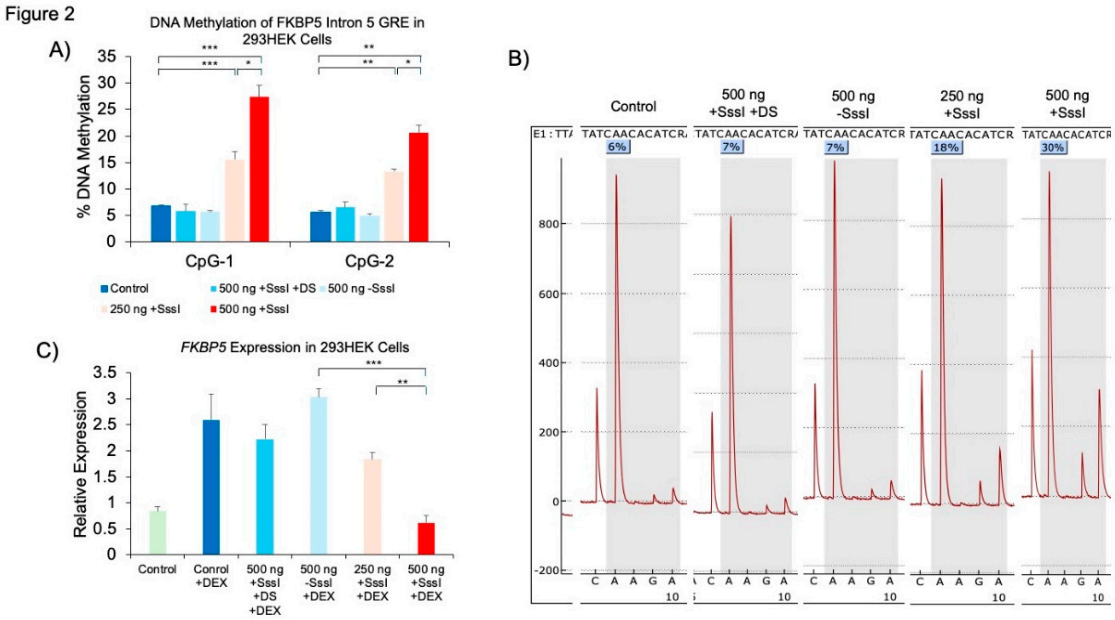

Figure 2. Dose-dependent DNAm and gene expression changes following transfection of methylated DNA probes against *FKBP5*. (A) 293HEK cells were transfected with single-stranded unmethylated probe (500 ng -SssI), single-stranded methylated probe at two different concentrations (250 ng and 500 ng +SssI), or double-stranded methylated probe (500 ng +SssI +DS). Untransfected cells served as controls (Control). DNAm levels of five CpG sites at the conserved glucocorticoid response element (GRE) of human *FKBP5* intron 5 were analyzed by bisulfite pyrosequencing. Data for the first two CpGs are shown. (B) Typical pyrograms obtained from bisulfite pyrosequencing is shown for each group at CpG-1. The percent DNAm determination occurs when R (or A/G) is dispensed, and it corresponds to the reverse complement of T/C (T for unmethylated and C for methylation CpG, respectively). (C) *FKBP5* expression was measured by qRT-PCR in the same groups of 293HEK cells as in (A) treated with 1 µM dexamethasone (DEX) for four hours prior to collection. Bar graphs represent mean ± SEM from samples processed in triplicate. ***P<0.001, **P<0.01, and *P<0.05.

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The Effect of Probe-Induced DNAm on Gene Expression

We also investigated whether the increase in DNAm was associated with differential gene expression. Previous studies have shown that while DNAm changes may not immediately result in changes in gene expression, they can modulate the level to which a gene can respond to a stimulus. In this case, the GREs of *FKBP5* are responsive to glucocorticoids in a methylation-sensitive way [34]. Treatment of the 293HEK cells with dexamethasone (DEX) caused a significant increase in FKBP5 expression (2.5-fold, P=0.036). While transfection with unmethylated, single-stranded or methylated, double-stranded probes did not lead to an appreciable attenuation of DEX-induced increase in expression, methylated, single-stranded probes significantly reduced the DEX-induced increase in *FKBP5*. Specifically, cells treated with methylated, single-stranded 500 ng of probe DNA showed 76.2% reduction in expression compared to that of unmethylated, single-stranded 500 ng of probe DNA treated with DEX. Furthermore, we observed a dose-response with methylated, single-stranded 250 ng of probe DNA, where 29.2% reduction in expression was observed compared to unmethylated, single-stranded 500 ng of probe DNA treated with DEX (**Figure 2C**).

Persistence of Probe-Induced DNAm Patterns and Accumulation of DNAm Following Multiple Probe Transfections

Previously, we demonstrated that glucocorticoid-induced DNA methylation persisted throughout development [26]. This persistence was replicated in a simpler mouse neuronal cell line treated with DEX [34]. Therefore, we tested whether DNA probe-induced increase in DNA methylation can persist through several weeks. Cells transfected once with 500 ng of methylated DNA probe showed appreciable increase in DNAm across all 5 CpGs compared to unmethylated DNA probe (P<0.038) at Week 1 (W1). Further culturing the transfected cells for four weeks showed that those transfected once with methylated DNA probe retained their DNA methylation patterns at CpG-1, CpG-3, and CpG-5 (P<0.0054) compared to cells transfected with unmethylated DNA probe at Week 1 or Week 4 (**Figure 3A**). We observed no significant increase in DNAm at CpG-2 at Week 1 and hence no significant increase at Week 4. At CpG-4, we observed significant increase in DNAm by 10.4% but this probe-induced increase in DNAm showed a decay of 5.2% by Week 4 (P=0.003). We also tested whether repeated transfections can have a cumulative effect on DNA methylation. 293HEK cells were transfected once, twice, three-times, and four-times with the same amount of DNA probes, each transfection taking place three days following the previous transfection. Results showed a cumulative effect on CpGs 3,4, and 5, with a more profound increase in DNAm at CpGs 1 and 2 following third and fourth transfections (**Figure 3B**).

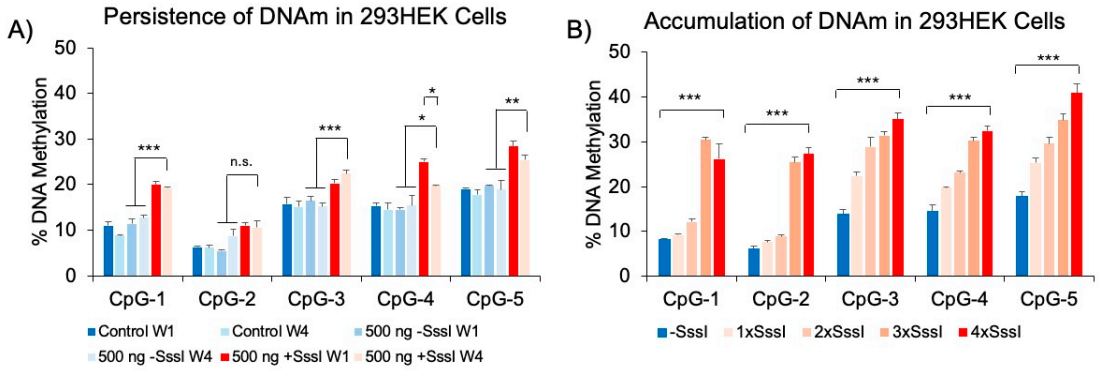

Figure 3. Persistence and accumulation of DNA methylation. (A) 293HEK cells were transfected with the unmethylated (-SssI) and methylated (+SssI) *FKBP5* DNA probe at Week 1 (W1), after which the cells were cultured for an additional four weeks (W4) before analysis by bisulfite pyrosequencing. (B) 293HEK cells were transfected with unmethylated (-SssI) and methylated (+SssI) *FKBP5* DNA probe consecutively every three days and expanded, while 50% of the cells were collected for analysis. ***P<0.001, **P<0.01, and *P<0.05.

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Effect of DNA Probes in Non-Targeted Regions

We also tested the effect of our targeted probe on non-targeted regions. Chromosome conformation capture experiments have demonstrated physical interactions between GREs and the promoter [5,34], and it has been speculated that GREs that are scattered across several intronic regions of a glucocorticoid-responsive gene interact in the 3-D space of the nucleus to coordinate glucocorticoid-induced gene expression. It may be possible that other genomic regions in proximity with the region targeted by the probe may be exposed to the DNA methylation machinery. Therefore, we assayed the DNAm levels of a GRE immediately adjacent to the probe-targeted GRE in intron 5, a GRE in intron 7, and a GRE immediately downstream of the first exon in intron 1 (**Figure 4A**,**B**). DNAm analysis showed that most of the CpGs tested in introns 5 and 7 were too hypermethylated to undergo probe-induced DNA methylation. CpG-6 in intron 7, which was strangely hypomethylated compared to other CpGs (<2% for all treatment groups), did not show any increase in DNAm. Further, hypomethylated intron 1 CpGs showed no differences among untransfected cells, cells treated with the unmethylated probe, and cells treated with the methylated probe. Only exception was at CpG-1, where cells transfected with the methylated probe showed a small 1.3% increase in DNAm compared to the other groups (P=0.004, **Figure 4C**).

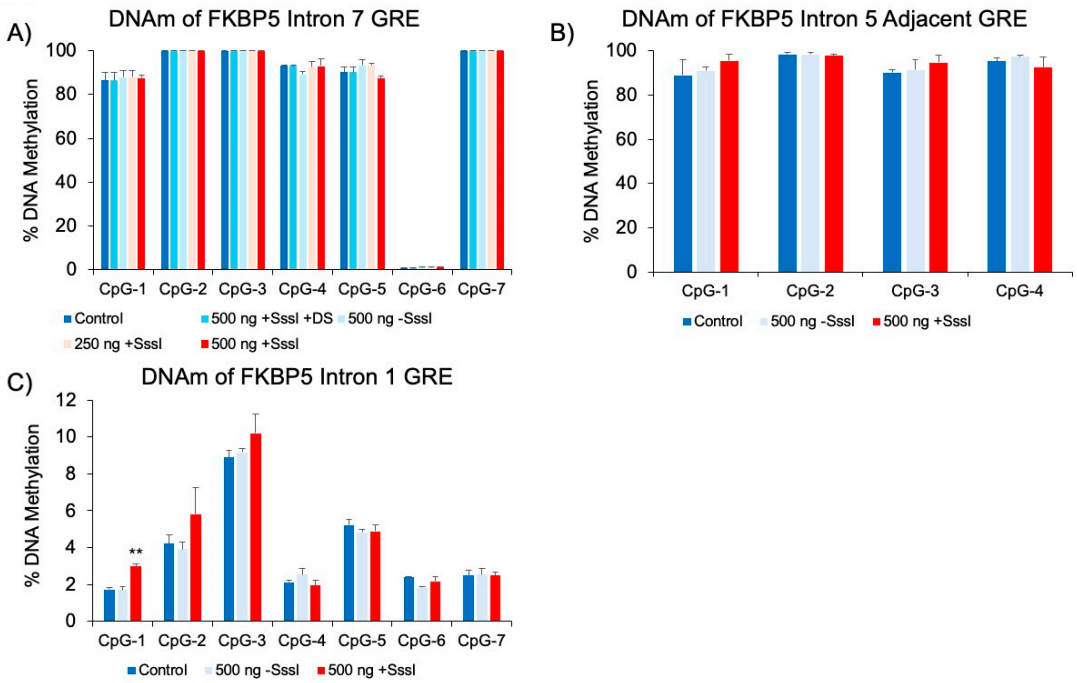

Figure 4. DNAm analysis of human *FKBP5* GREs at additional intronic regions. Experimentally verified glucocorticoid response elements (GREs) at introns 1, 5, and 7 were evaluated in probe-transfected 293 HEK samples for non-specific epigenetic effects. There were no significant differences in cells transfected with unmethylated vs. methylated DNA probes. **P<0.01.

Epigenetic and Transcriptional Effects of Methylated, Single-Stranded Probe in a Mouse Pituitary Cell Line

We also tested mouse pituitary cells to determine whether the findings in 293HEK can be recapitulated in a different cell-type and species. Mouse AtT-20 cells were transfected with the mouse version of the probe against the conserved *Fkbp5* intron 5 GRE. Results showed that the use of methylated, single-stranded probes increased DNAm at three of the four CpGs when compared to unmethylated single-stranded probes: CpG-2 (9.5%, P=0.003), CpG-3 (6.1%, P=0.003), and CpG-4 (7.2%, P=0.03). There were no appreciable differences in DNAm between unmethylated, single-stranded probes and methylated, double-stranded probes (**Figure 5A**). We then tested whether transfected DNA probes can modulate DEX-inducibility as the human probes had in the 293HEK cells. *Fkbp5* expression analysis showed that samples treated with no DNA probe, methylated,

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double-stranded probe, or unmethylated, single-stranded probe had similar levels of gene induction by DEX treatment (7.8-, 6.3-, 6.7-fold induction, respectively, and $P<1.3x10^{-4}$). However, samples transfected with methylated, single-stranded probe showed a significant reduction in DEX-induced expression compared to samples transfected with unmethylated, single-stranded probe (42.5% reduction, $P=6.4x10^{-5}$, **Figure 5B**).

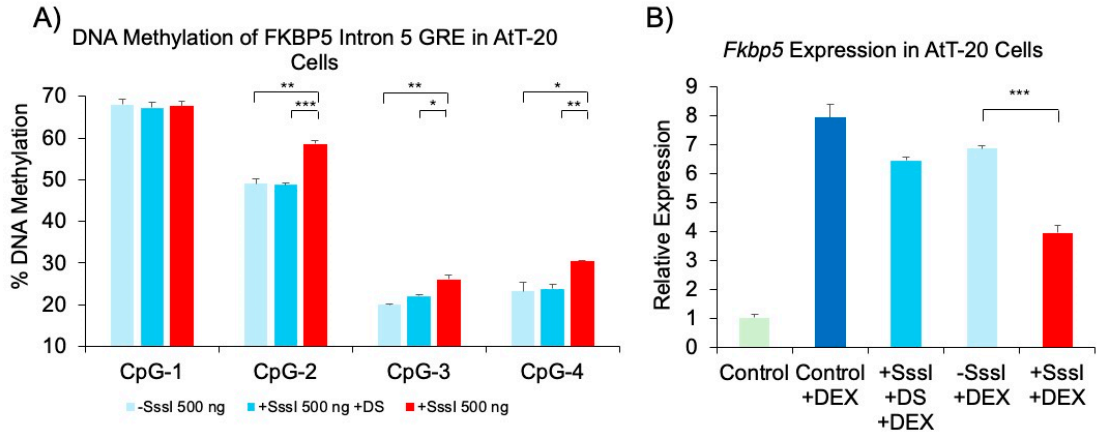

Figure 5. DNAm and gene expression analysis following transfection of methylated DNA probes against mouse *Fkbp5*. (A) AtT-20 cells were transfected with single-stranded unmethylated probe (500 ng -SssI), single-stranded methylated probe (500 ng +SssI), or double-stranded methylated probe (500 ng +SssI +DS). Untransfected cells served as controls (Control). DNAm levels of four CpG sites at the conserved glucocorticoid response element (GRE) of human *FKBP5* intron 5 were analyzed by bisulfite pyrosequencing. Data for all four GRE CpGs are shown. (B) *Fkbp5* expression was measured by qRT-PCR in the same groups of AtT-20 cells as in (A) treated with 1 µM dexamethasone (DEX) for four hours prior to collection. Bar graphs represent mean ± SEM from samples processed in triplicate. ***P<0.001, **P<0.01, and *P<0.05.

Additional Genomic Target of DNAm Probe: MAOA.

To demonstrate the broader applicability of our methylated probe approach beyond the *FKBP5* gene, we tested the regulatory intronic region of Monoamine Oxidase A (*MAOA*) [31], a gene whose encoded protein metabolizes monoamine neurotransmitters such as dopamine, serotonin, and norepinephrine. Following the transfection of methylated single-stranded probes targeting the CpG-dense *MAOA* intronic region into 293HEK cells, we observed significant increases in DNAm across multiple CpG sites compared to cells transfected with unmethylated probes: CpG-1 (10.1%, P=0.01), CpG-5 (12.7%, P=0.04), CpG-6 (11.2%, P=0.02), CpG-8 (9.3%, P=0.006), CpG-10 (11.8%, P=0.006), and CpG-12 (14.3%, P=0.01) (**Figure 6A**). A one-way ANOVA revealed a significant difference between the two treatments ($F(1, 26) = 6.25$, $P = 0.019$), where methylation levels were higher in the methylated probe-treated group ($M = 58.68$, $SD = 7.52$) compared to the unmethylated probe-treated group ($M = 52.31$, $SD = 10.25$). Consistent with the increased methylation, we observed a corresponding decrease in MAOA gene expression. qRT-PCR analysis revealed that cells transfected with methylated probes showed a 27.3% reduction in *MAOA* expression compared to cells transfected with unmethylated probes (P=0.041) (**Figure 6B**).

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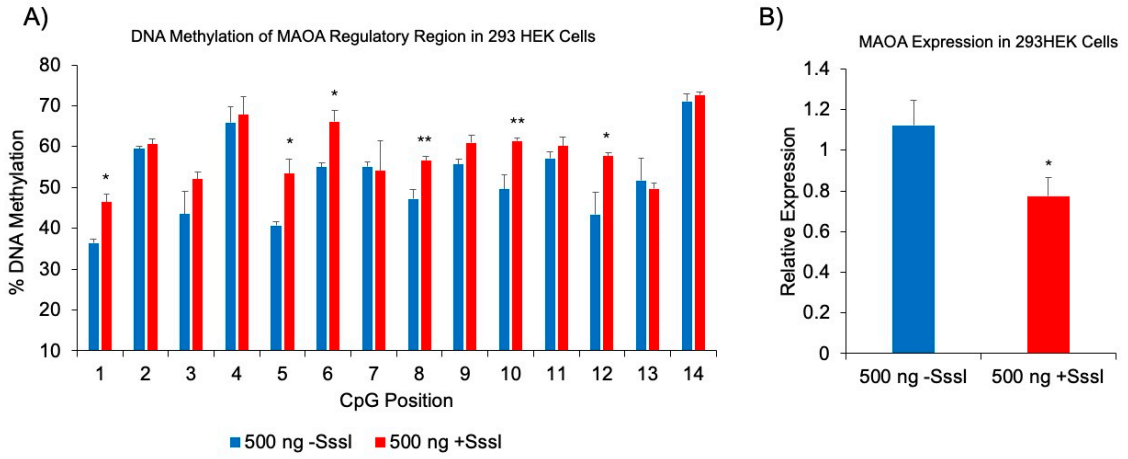

Figure 6. DNAm and gene expression analysis following transfection of methylated DNA probes against MAOA. (A) 293HEK cells were transfected with single-stranded unmethylated probe (-SssI) or single-stranded methylated probe (+SssI). DNAm levels of 14 CpG sites at a regulatory region of human MAOA were analyzed by bisulfite pyrosequencing. (B) MAOA expression was measured by qRT-PCR in the same groups of 293HEK cells. Bar graphs represent mean ± SEM from samples processed in triplicate. *P<0.05 and **P<0.01.

Discussion

In this study, we introduce a simple approach for inducing targeted DNAm using methylated DNA probes. Our approach consists of the PCR amplification against a region of interest, which in this case was a methylation-sensitive, glucocorticoid response element in the intronic region of the *FKBP5* gene [27]. The PCR product was first in vitro-methylated using the bacterial methyltransferase M.SssI and denatured to yield single-stranded DNA probes. Using the DNA probes against the *FKBP5* GRE, we observed appreciable increases in DNAm across at least two of the five CpGs across the GRE in the human 293HEK cells. The observed increase in DNAm was specific to the single-stranded, methylated probe, as double-stranded, methylated probe and single-stranded unmethylated probe did not induce an increase in DNAm. Importantly, increase in DNAm at these CpGs was able to attenuate DEX-induced increase in *FKBP5* levels. Further, gene regulation by the DNA probe was dose-dependent, as the introduction of half the amount of DNA probe resulted in a reduced addition of DNAm and reduced attenuation of *FKBP5* induction by DEX compared to the full amount of DNA probe.

Compared to the other two epigenetic mechanisms, namely histone modification and RNA-mediated gene silencing, DNA methylation tends to be more stable. As such, a single administration of the DNA probe lasted through at least a four-week period, suggesting robustness of the probe-induced DNA methylation. This phenomenon is reminiscent of glucocorticoid loss of DNAm, which persisted for at least four weeks in the mouse brain and in a DEX-treated neuronal cell line [26,34]. It should be noted that the reversal of DNAm induction observed at CpG-4 in **Figure 3A** is not new, as some of the CpGs that lost DNAm by glucocorticoid treatment also showed a similar reversion to baseline by the fourth week [34]. In addition, repeated introduction of the probe had a cumulative effect on DNA methylation, although the increase in DNAm with successive transfections was not at the same magnitude as following the first transfection.

Investigation of other GREs in intronic regions, which have been thought to physically interact in 3D-space with the intronic GRE (intron 5) targeted by the probe, did not show any significant changes in DNAm, emphasizing the specificity of probe targeting. One exception to the negative results at other GREs is the increase in DNAm at one CpG in intron 1 (**Figure 4C**). However, the magnitude of the difference was less than 2%, which is unlikely to be functionally relevant.

We then tested whether probe-induced DNAm was unique to human cells by testing the mouse version of the *Fkbp5* probe in the AtT-20 mouse pituitary cell line. Although more subtle than those observed in the human cell lines, increase in DNAm was significant. Similarly, this increase in DNAm

 doi:10.20944/preprints202504.0034.v1

was able to thwart DEX-induction of *Fkbp5*. There are a few factors that can potentially explain the difference in the magnitude of probe-induced increase in DNAm, including cell-type and species-specific differences, growth rate differences of cells, and different levels of DNAm-modifying enzymes. Growth rate may play an important role, as some methylation-altering events, such as glucocorticoid-induced loss of methylation, depends on cell proliferation [27].

An additional genomic region was tested to further generalize the probe-induced increase in DNAm. This time we designed a probe against an important regulatory region of *MAOA*, whose methylation levels correlated with enzymes levels determined by PET brain imaging [31]. In addition, the MAOA enzyme metabolizes the neurotransmitter serotonin and is thus targeted by a class of antidepressants called monoamine inhibitors (MAOI). Although significant increases in DNAm were observed across many CpGs, many CpGs were impervious to the probe. The sub-optimal increase in DNAm at these CpGs may be due to the sequence of the regulatory region in MAOA. It is also possible that CpG-dense regions may be harder to modify, as the MAOA probe covers approximately the same base pairs of DNA as that for *FKBP5* but contains almost three times more CpGs.

Our current study highlights the potential of this method to modulate stress response pathways, as demonstrated by the targeted methylation of the *FKBP5* gene. *FKBP5* is known to be influenced by glucocorticoid exposure and methylation status, with demethylation of the glucocorticoid response element leading to increased gene expression upon subsequent glucocorticoid exposure [27]. By inducing targeted methylation of this region, we were able to attenuate the glucocorticoid-induced upregulation of FKBP5 expression, suggesting that our approach could be used to fine-tune stress response pathways and potentially mitigate the detrimental effects of chronic stress or glucocorticoid exposure.

The success of our method in both human and mouse cell lines indicates its potential for translation to in vivo models and eventual clinical applications. However, several challenges need to be addressed before this approach can be fully realized. First, the efficiency of probe delivery and cellular uptake may vary depending on the cell type and target tissue, requiring optimization of transfection methods and probe design. Second, the long-term stability and persistence of the induced methylation changes need to be evaluated in vivo to determine the durability of the therapeutic effects. The safety and potential off-target effects of the methylated probes must also be thoroughly assessed in animal models before considering human applications. Finally, we will need a suitable delivery device that can tissue-specifically deliver DNA probes for in vivo studies. Small extracellular vesicles (EVs) such as exosomes hold immense therapeutic value as delivery devices, as their vesicle cargo space can easily accommodate a ~200 bp DNA fragment and their surface proteins profiles can be modified to alter tissue targeting [37].

Despite these challenges, our study provides a proof-of-concept for the use of methylated DNA probes as a targeted epigenetic therapy. The ability to induce site-specific DNAm changes can open new avenues for the treatment of a wide range of diseases characterized by aberrant methylation patterns. As our understanding of the epigenetic basis of disease continues to grow, the development of targeted epigenetic therapies, such as the one presented here, will become increasingly important. In conclusion, we have developed a novel method for targeted induction of DNAm using methylated DNA probes. This approach offers a promising tool for modulating gene expression and function, with potential therapeutic applications in various diseases characterized by aberrant methylation patterns.

Author Contributions: Nishtaa Modi – conceptualization, investigation, funding acquisition, and original draft preparation; Jeffrey Guo – investigation and validation; Ryan Lee – investigation and validation; Alisha Greenstein – investigation and validation; and Richard Lee - conceptualization, investigation, funding acquisition, and original draft preparation.

Funding: This work was supported by the Woodrow Wilson Fellowship (Johns Hopkins University) granted to N.M. and George Browne Genetics Cell Laboratory Fund granted to R.S.L.

 doi:10.20944/preprints202504.0034.v1

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