Targeted DNA Methylation by a DNA Methyltransferase Coupled to a Triple Helix Forming Oligonucleotide To Down-Regulate the Epithelial Cell Adhesion Molecule

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The epithelial cell adhesion molecule (EpCAM) is a membrane glycoprotein that has been identified as a marker of cancer-initiating cells. EpCAM is highly expressed on most carcinomas, and transient silencing of EpCAM expression leads to reduced oncogenic potential. To silence the EpCAM gene in a persistent manner via targeted DNA methylation, a low activity mutant (C141S) of the CpG-specific DNA methyltransferase M.SssI was coupled to a triple-helix-forming oligonucleotide (TFO−C141S) specifically designed for the EpCAM gene. Reporter plasmids encoding the green fluorescent protein under control of different EpCAM promoter fragments were treated with the TFO−C141S conjugate to determine the specificity of targeted DNA methylation in the context of a functional EpCAM promoter. Treatment of the plasmids with TFO−C141S resulted in efficient and specific methylation of the targeted CpG located directly downstream of the triple helix forming site (TFS). No background DNA methylation was observed neither in a 700 bp region of the EpCAM promoter nor in a 400 bp region of the reporter gene downstream of the TFS. Methylation of the target CpG did not have a detectable effect on promoter activity. This study shows that the combination of a specific TFO and a reduced activity methyltransferase variant can be used to target DNA methylation to predetermined sites with high specificity, allowing determination of crucial CpGs for promoter activity.

INTRODUCTION

The epithelial cell adhesion molecule (EpCAM) is a membrane glycoprotein that is highly expressed on most carcinomas. Recently, EpCAM has gained renewed interest as a signal transducer in carcinogenesis. Its expression leads to reduced oncogenic potential. To silence the EpCAM gene in a persistent manner via targeted DNA methylation, a low activity mutant (C141S) of the CpG-specific DNA methyltransferase M.SssI was coupled to a triple-helix-forming oligonucleotide (TFO−C141S) specifically designed for the EpCAM gene. Reporter plasmids encoding the green fluorescent protein under control of different EpCAM promoter fragments were treated with the TFO−C141S conjugate to determine the specificity of targeted DNA methylation in the context of a functional EpCAM promoter. Treatment of the plasmids with TFO−C141S resulted in efficient and specific methylation of the targeted CpG located directly downstream of the triple helix forming site (TFS). No background DNA methylation was observed neither in a 700 bp region of the EpCAM promoter nor in a 400 bp region of the reporter gene downstream of the TFS. Methylation of the target CpG did not have a detectable effect on promoter activity. This study shows that the combination of a specific TFO and a reduced activity methyltransferase variant can be used to target DNA methylation to predetermined sites with high specificity, allowing determination of crucial CpGs for promoter activity.
oligopurine—oligopyrimidine stretches in promoter regions of human genes (23-25). Recently, we and others have observed that coupling of a TFO to the restriction enzymes scPvuII (20) and Mun-I is feasible (Geel et al., manuscript in preparation).

To explore the possibility of targeting methylation to specific DNA sequences using a TFO as targeting domain, we coupled a variant of the methyltransferase M.SssI to a TFO specifically designed for the EpCAM gene (26). This M.SssI variant, named C141S throughout this paper, carried the C141S and C368A substitutions and a C-terminal 6xHis-Cys tag. In this variant the internal cysteines were thus replaced and the C-terminal Cys was introduced to allow coupling of the methyltransferase to the TFO. Although one of the replaced cysteines (C141S) is the active site cysteine, the C141S mutation does not fully abolish the methyltransferase activity; the mutant enzyme has 2-5% of the wild type (WT) activity (27). DNA binding affinity of the C141S variant is similar to that of the WT enzyme (28).

Coupling of C141S to the TFO did not affect activity of the enzyme, and binding specificity of the TFO-C141S conjugate to the DNA was dominated by the TFO. Site-specific methylation by the TFO-C141S conjugate was demonstrated using a plasmid containing a 43 bp segment of the EpCAM promoter encompassing the TFS and a target CpG (26). To investigate targeted methylation in the context of a functional EpCAM promoter, reporter plasmids encoding the Green Fluorescent Protein (GFP) under control of different EpCAM promoter, reporter plasmids encoding the Green Fluorescent

EXPERIMENTAL PROCEDURES

Methylation of Plasmids with TFO-C141S. The p39E plasmid and its promoter deletion derivatives (29) are schematically depicted in Figure 1A. These plasmids encode GFP under control of a 3.4 kb fragment of the EpCAM promoter. Construction of the plasmids expressing WT M.SssI or C141S, purification of the enzymes (14) and coupling of the 5'-TTTTTTTTTTTTTTTTTTTTT-GFP fragment to M.SssI(C141S) were done as described (26). Plasmids were incubated with 5-fold molar excess of TFO-C141S, M.SssI, C141S, or TFO-C141S fragment containing the TFS and a target CpG sequence in a buffer containing 20 mM Tris, 50 mM NaCl, 10 mM MgCl₂, pH 7.9, with or without 640 μM S-adenosylmethionine (SAM) (New England Biolabs, Ipswich, MA) at 30 °C. The reaction was terminated after 20 h by heat inactivation at 65 °C for 20 min, and plasmids were purified by Qiagen PCR purification kit (Qiagen, Benelux B.V., Venlo, The Netherlands).

To test the involvement of enzyme activity in TFO-C141S-induced relaxation of supercoiled plasmid DNA, C141S and TFO-C141S were heat-inactivated by incubation at 65 °C for 20 min. Plasmid p39E was incubated with active or inactivated C141S or TFO-C141S for 15 or 30 min or 1, 2.5, 5, or 20 h at 30 °C. Then the reaction was stopped by 10% SDS and digestion with proteinase K.

Transfection. The SKOV3 (HTB-77) cell line was purchased from ATCC (Manasas, VA) and cultured according to ATCC recommendations. SKOV3 cells were seeded 100000/well in 24-wells plates, and transfection was performed at 60–80% confluency using SAIN-T-DOPE (SD, 0.75 mM) (Synvolyx Therapeutics, Groningen, The Netherlands) (30). Pretreated plasmid DNA (250 ng) in 25 μL of Hanks balanced salt solution was added to 5 μL of SD filled up with 20 μL of HBS. Within 15 min the complex was diluted in 200 μL of serum-free culture medium and added directly to the cells. After 3 h of incubation at 37 °C, in 5% CO₂, serum containing medium was added. After 48 h, cells were harvested and GFP expression was measured by flow cytometry (Beckton Dickinson Bioscience Calibr, San Jose, CA).

DNA Methylation Analysis. Plasmid DNA (250 ng) methylated in vitro by TFO-C141S was treated with sodium
RESULTS

Targeted DNA Methylation of the EpCAM Promoter in p39E. To test the targeting specificity of the TFO−C141S conjugate in the context of a functional EpCAM promoter, p39E containing the 3.4 kb EpCAM promoter was treated with TFO−C141S. Three independent TFO−C141S treatments resulted in 57% (n = 7), 89% (n = 9), and 75% (n = 8) methylation of CpG7 (Figure 1B) located directly downstream of the TFS. Of the 24 clones analyzed, 18 showed methylation of CpG7. Only three clones were devoid of methylation. One clone showed methylation only of CpG9, and two clones showed methylation only of CpG5 located ∼40 bp upstream of the TFS. Four of the 18 positive clones showed methylation of CpG5 and CpG7, and one of these clones also showed methylation of CpG6. CpG1, -2, -3, -4, and -8 within the TFS amplicon (Figure 1B) were not methylated in the clones sequenced. Importantly, apart from one sporadic methylation event, no methylation was observed downstream of the TFS neither in the EpCAM promoter (EpCAM first and second amplicon, Table 1A) nor in the part of the GFP gene (GFP amplicon). In the control samples, obtained by treating p39E with WT M.SssI, all CpGs analyzed were methylated (Table 1B), whereas treatment with the less active C141S variant resulted in random methylation.

GFP Expression from the EpCAM Promoter after Pretreatment with TFO−C141S. After observing that the TFO−C141S conjugate induced targeted DNA methylation, we tested if methylation of this single CpG (CpG7) is sufficient to inhibit EpCAM promoter activity. Hence, all treated plasmids were transfected in SKOV3 cells to determine GFP expression. The three independent treatments of p39E with TFO−C141S showed 46% ± 20 (n = 3), 48% ± 16 (n = 3), and 57% ± 6 (n = 2) GFP down-regulation (see Figure 2A for a representative experiment). Transfection of plasmids treated with untargeted M.SssI or untargeted C141S resulted in 91% ± 1 and 27% ± 1 GFP down-regulation, respectively, whereas treatment with just the TFO did not influence GFP expression. Transfection of p39E treated with a 100-fold excess of TFO in the presence of TFO−C141S (competition sample in Figure 2A) showed 24% ± 17 down-regulation of GFP, which is approximately 33% less than the value obtained with TFO−C141S only, indicating that targeting was dependent on the TFO. These observations suggested that methylation of a single CpG was sufficient to reduce EpCAM promoter activity.

Down-Regulation of GFP Gene Expression by TFO−C141S in the Absence of TFS or Methyl Donor. To investigate if the reduction in GFP expression was indeed due to targeted DNA methylation, deletion derivatives of p39E (p15-2, p7-2, p4-1, and p11-1) (Figure 1A) were treated with TFO−C141S and transfected in SKOV3 cells to determine GFP expression. Plasmids p15-2 and p7-2 contained the TFS, whereas plasmids p4-1 and p11-1 lacked this sequence. In addition to the targeted TFS, the EpCAM promoter region contains four sites, which differ from the targeted TFS only by three or four mismatches (3MM or 4MM, as indicated in Figure 1A). Plasmids p7-2 and p4-1 treated with TFO−C141S showed 51% ± 4 and 46% ± 8 GFP down-regulation, respectively (Figure 2B). The latter result was unexpected, as plasmid p4-1 lacked the TFS. To exclude the possibility that down-regulation was caused by binding of TFO−C141S to the TFS 4MM site (Figure 1A) present in p4-1, plasmids p15-2 and p11-1 were treated with TFO−C141S and transfected into SKOV3 cells. Plasmid p15-2 contains the TFS and two TFS-like sites, whereas from p11-1 all potential binding sites had been deleted (Figure 1A). Pretreatment of these plasmids with 100-fold excess of TFO−C141S did not influence GFP expression. Transfection of p15-2 and p11-1 all potential binding sites had been deleted (Figure 1A). Pretreatment of plasmids with 100-fold excess of TFO−C141S did not influence GFP expression. Transfection of p15-2 and p11-1 resulted in 24% ± 17 down-regulation of GFP, respectively (Figure 2B). The latter result was unexpected, as plasmid p4-1 lacked the TFS. To exclude the possibility that down-regulation was caused by binding of TFO−C141S to the TFS 4MM site (Figure 1A) present in p4-1, plasmids p15-2 and p11-1 were treated with TFO−C141S and transfected into SKOV3 cells. Plasmid p15-2 contains the TFS and two TFS-like sites, whereas from p11-1 all potential binding sites had been deleted (Figure 1A). Both pretreated plasmids showed approximately 45% reduction of GFP expression compared to their untreated controls (data not shown).

To exclude that the reduction in GFP expression was not caused by a specific DNA methylation, the methylation status of the deletion derivatives of p39E was analyzed. Treatment of p15-2 with TFO−C141S resulted in 36% methylation of CpG7 (5/14 clones), and one clone showed methylation of CpG5 (Table 2). Apart from one sporadic event, deletion derivatives p7-2 and p4-1 and p11-1 did not show methylation in the downstream amplicons. These results confirm the specificity of targeted methylation by TFO−C141S.

Table 1. Targeted DNA Methylation of p39E by TFO−C141S

| Treatment | n | TFS amplicon | EpCAM first amplicon | EpCAM second amplicon | GFP 33 CpGs |
|-----------|---|--------------|----------------------|----------------------|-------------|
| no        | 3 | 23           | 23                   | 23                   | 23          |
| no        | 2 | 23           | 23                   | 23                   | 23          |
| TFO-C141S-SAM | 3 | 23           | 23                   | 23                   | 23          |
| C141S     | 10| 14           | 14                   | 14                   | 14          |
| M.SssI    | 4 | 23           | 23                   | 23                   | 23          |
| M.Ssi     | 3 | 23           | 23                   | 23                   | 23          |
| M.Ssi     | 3 | 23           | 23                   | 23                   | 23          |

* Results of bisulfite sequencing.
To exclude that reduction in GFP expression was the result of the conjugate still being attached to the plasmid, p39E was also treated with TFO-C141S in the absence of S-adenosyl-methionine (SAM). No difference in reduction of GFP expression was observed between samples treated with or without the methyl donor (Figure 2C). To confirm that reduction in GFP expression was not caused by aspecific DNA methylation, plasmid p39C encoding GFP under control of the CMV promoter without a TFS-like sequence was subjected to TFO-C141S treatment. Also for p39C, GFP expression was reduced while no difference was observed between treatments in the presence or in the absence of SAM (Figure 2C).

Because it has been reported that M.SssI can display topoisomerase activity (31), TFO-C141S was heat-inactivated before treatment of p39E. Treatment of the plasmid p39E with heat-inactivated TFO-C141S did not result in conformation change, whereas treatment with active TFO-C141S led to

Figure 2. Effect of TFO-C141S treatment on GFP expression in EpCAM positive SKOV3 cells. (A) Relative GFP expression measured 48 h after transfection of pretreated p39E. Plasmid p39E was treated as indicated: p39E = treatment without TFO-C141S, treated with TFO only, with untargeted M.SssI or C141S, with the TFO-C141S conjugate or with 100-fold excess of TFO and TFO-C141S (=competition). The value obtained with p39E without TFO-C141S was taken as 100%. Shown is the average GFP expression (±SD) of one representative transfection performed in triplicate. (B) Relative GFP expression measured 48 h after transfection of pretreated deletion derivatives p7-2 and p4-1. For each derivative, the values obtained with samples treated without TFO-C141S were taken as 100%. Shown is the average GFP expression (±SEM) of the mean of three independent transfections performed in triplicate. (C) Relative GFP expression was measured 48 h after transfection of pretreated p39E or p39C. Treatments were as indicated: (+) or (−) indicates the presence or absence of the methyl donor (SAM). Shown is the average GFP expression (±SEM) of the mean of three independent transfections performed in triplicate.

Table 2. Targeted DNA Methylation of p39E Deletion Derivatives by TFO-C141S

Methylation status of bisulfite amplicons for the deletion derivatives p39E treated with TFO-C141S and the methyl donor SAM. Shown are the results of bisulfite sequencing. n = number of clones analyzed. Below the lollipop: the percentage of clones of which the CpG was methylated. Open lollipop: 0% methylated CpG. Black lollipop: 100% methylated CpG.

To exclude that reduction in GFP expression was the result of the conjugate still being attached to the plasmid, p39E was also treated with TFO-C141S in the absence of S-adenosyl-methionine (SAM). No difference in reduction of GFP expression was observed between samples treated with or without the methyl donor (Figure 2C). To confirm that reduction in GFP expression was not caused by aspecific DNA methylation, plasmid p39C encoding GFP under control of the CMV promoter without a TFS-like sequence was subjected to TFO-C141S treatment. Also for p39C, GFP expression was reduced while no difference was observed between treatments in the presence or in the absence of SAM (Figure 2C).

TFO-C141S Induced Relaxation of Supercoiled Plasmids. Because GFP expression from the transfected pretreated plasmids seemed to be independent of their methylation status, we examined the conformation of the pretreated plasmids. Treatment of p39E with TFO-C141S with or without methyl donor led to relaxation of the supercoiled plasmid (Figure 3A, lanes 6, 7). The deletion plasmid p11-1 behaved in the same way; treatment with TFO-C141S caused relaxation (lane 12), whereas treatment with only the TFO, M.SssI, or C141S did not affect conformation of the plasmid. Interestingly, the presence of excess TFO seemed to protect to some extent the plasmid from conversion into the relaxed form (lane 8).

The phenomenon of conformation change appeared to be general: treatment of p39C, p7-2, and p4-1 with TFO-C141S also caused a conversion to the relaxed form (data not shown).

To exclude the possibility that the slower electrophoretic mobility was caused by TFO-C141S still being attached to the plasmid, the TFO-C141S treated samples were digested with proteinase K. No difference in conformation was observed between the plasmids with and without proteinase K treatment (data not shown), indicating that C141S was not attached to the plasmid.
conversion from the supercoiled into the relaxed form (Figure 3B). This change in conformation was dependent on the duration of the treatment; i.e., longer treatment resulted in a less supercoiled and a more relaxed conformation. Treatment of p39E with C141S or heat-inactivated C141S showed no conformation change.

On the basis of the observation that excess TFO could protect the p39E plasmid from TFO-C141S mediated relaxation (Figure 3A, lanes 6, 7, 8), we investigated this protection in more detail. Treatment of p39E, as well as p39C with TFO-C141S only, showed efficient relaxation already after 2.5 h of treatment (Figure 3C). In the presence of excess TFO, again both plasmids showed relaxation although the efficiency was somewhat less (Figure 3C). To further prove that the presence of the TFS is not necessary for relaxation, plasmids with a different backbone were subjected to TFO-C141S treatment. Although the backbones did not contain any TFS-like sequences, we again observed an increase in the relaxed conformation when the incubation time with TFO-C141S was increasing (data not shown).

**DISCUSSION**

In this study, we demonstrated that a DNA methyltransferase can be directed to a predetermined site by a covalently attached TFO and that TFO-mediated targeting can be used to induce targeted methylation. Because the TFO binds antiparallel to the sense strand of the TFS in the EpCAM promoter and C141S is coupled to the 5’-end of the TFO, C141S should orient downstream the TFS. Indeed, treatment of reporter plasmids with TFO-C141S resulted in efficient (18/24 clones) and specific (14/21 clones) methylation of CpG7 located directly downstream the TFS. These results indicate that binding specificity of TFO-C141S is dominated by the TFO.

The 24 nucleotide long TFO used in this study contains three cytosines. Specific binding of the TFO requires protonation of the three cytosines to form the C+·GC triplets (pH < 6) (21). However, treatment of the plasmids with TFO-C141S was performed under conditions optimal for methylation (pH 7.9), i.e., at a pH higher than required for efficient formation of C+·GC triplets. Despite the suboptimal annealing conditions, efficient DNA methylation was observed at the target site.

The 3.4 kb EpCAM promoter encompasses four sites that are similar to the targeted TFS (i.e., differing only at three or four positions); hence, they would be expected to be potential sites of nontargeted binding and methylation. To test this possibility, deletion derivatives of p39E containing (p15-2, p7-2) or lacking the TFS (p4-1, p11-1) were treated with TFO-C141S. Despite the presence of a possible binding site in amplicon 1, CpG7 was preferentially methylated (18/24 clones), whereas no methylation was observed for amplicon 1 in all treated plasmids. Moreover, deletion derivatives lacking the TFS (p4-1, p11-1) displayed no methylation in the analyzed areas. We thus conclude that the TFO directs C141S only to its predetermined site.

Next, we investigated if this site-specific DNA methylation in the EpCAM promoter is sufficient to induce inhibition of gene expression. It has been shown that methylation of one or a few CpGs within a promoter might be sufficient to repress transcription. Transfection of a reporter plasmid under control of the p53 promoter in which a single CpG was methylated in vitro by HhaI showed 85% down-regulation of the reporter gene (32). Unexpectedly, in our experiments TFO-C141S treatment of all GFP reporter plasmids resulted in lower GFP expression,
and this phenomenon was independent of the methylation status of the transfected plasmid. Gel electrophoresis revealed that TFO–C141S treatment, in contrast to treatment with the enzyme or TFO only, led to relaxation of the supercoiled plasmid, explaining the observed GFP repression (33). Since plasmid treatment with heat-inactivated TFO–C141S did not cause a conformation change of the plasmid, we might conclude that the observed relaxation is probably caused by topoisomerase activity of C141S. Matsuo et al. described that M.SssI contains both methylase and topoisomerase activities (31). However, the observed topoisomerase activity was only displayed by the TFO–C141S conjugate and not after treatment with the TFO only or with C141S only. Independent of the presence of the TFS, all plasmids treated with the TFO–C141S conjugate showed relaxation of the plasmid: the observed ratio of supercoiled versus relaxed plasmid conformation was decreasing when the treatment time was prolonged. Somehow, the chemical coupling of the TFO with the enzyme seems to change the conformation of the enzyme, thereby uncovering the catalytic topoisomerase domain. It requires further research to determine which amino acid should be replaced to abolish this activity.

A puzzling observation of this study was that there was no significant difference in GFP down-regulation between plasmids treated in the presence or absence of the methyl donor. A possible explanation might be that the CpGs targeted in this study do not play a role in the epigenetic regulation of the EpCAM gene. Indeed, recent observations (11, 14, 15) suggest that the CpGs, which display differential methylation, are located further downstream (approximately −400 to +280 bps) of the TFS, which is located around −917. Currently, we are investigating which CpGs are crucial in regulating EpCAM gene expression. The important CpGs can then be targeted for induced DNA methylation.

In summary, the data obtained with the TFO–C141S conjugate offer a novel approach for targeted DNA methylation. The combination of a specific TFO and the reduced methyltransferase activity of the M.SssI mutant C141S allowed us to target methylation predominantly to a specific DNA sequence without significant background methylation. Because of the flexibility provided by the use of TFOs as targeting domain, this approach appears to be a promising tool in both research and therapeutic areas.

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