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SHORT REPORT

Persistent downregulation of the pancarcinoma-associated epithelial cell adhesion molecule *via* active intranuclear methylation

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The epithelial cell adhesion molecule (EpCAM) is expressed at high levels on the surface of most carcinoma cells. SiRNA silencing of EpCAM expression leads to reduced metastatic potential of tumor cells demonstrating its importance in oncogenesis and tumor progression. However, siRNA therapy requires either sequential delivery or integration into the host cell genome. Hence we set out to explore a more definite form to influence EpCAM gene expression. The mechanisms underlying the transcriptional activation of the EpCAM gene, both in normal epithelial tissue as well as in carcinogenesis, are poorly understood. We show that DNA methylation plays a crucial role in EpCAM expression, and moreover, active silencing of endogenous EpCAM *via* methylation of the EpCAM promoter results in a persistent downregulation of EpCAM expression. In a panel of carcinoma derived cell lines, bisulfite analyses showed a correlation between the methylation status of the EpCAM promoter and EpCAM expression. Treatment of EpCAM-negative cell lines with a demethylating agent induced EpCAM expression, both on mRNA and protein level, and caused upregulation of EpCAM expression in an EpCAM-positive cell line. After delivery of the DNA methyltransferase M.SssI into EpCAM-positive ovarian carcinoma cells, methylation of the EpCAM promoter resulted in silencing of EpCAM expression. SiRNA-mediated silencing remained for 4 days, after which EpCAM re-expression increased in time, while M.SssI-mediated downregulation of EpCAM maintained through successive cell divisions as the repression persisted for at least 17 days. This is the first study showing that active DNA methylation leads to sustained silencing of endogenous EpCAM expression.

Key words: EGP-2 promoter; gene silencing; DNA methylation; siRNA; M.SssI; TROP1; 17-1A; CD326

Since its discovery the human pancarcinoma-associated Epithelial Cell Adhesion Molecule (EpCAM), also referred to as 17-1A, EGP-2, TROP1 or CD326, has become a major target for carcinoma-directed immunotherapy. However, evidence for its direct involvement in carcinogenesis has only been given recently. EpCAM expression has a direct impact on the cell cycle *via* c-myc and cyclin A/E, and inhibition of EpCAM expression with antisense mRNA reduces the proliferation and metabolism in human carcinoma cells.[1] Similarly, silencing of EpCAM expression with siRNA reduces the migration and invasive potential of breast cancer cells by 90%.[2] Moreover, EpCAM overexpression in breast, ovarian and gallbladder cancer correlates with a strong negative prognosis.[3–5] For human colorectal cancer it has been shown that the ability to engraft *in vivo* in immunodeficient mice, was restricted to a minority subpopulation of epithelial cells with high EpCAM expression.[6] This direct involvement of EpCAM in the development of carcinomas qualifies EpCAM as an important target for therapy.

The EpCAM regulatory sequences have been cloned and characterized,[7,8] and the basic proximal promoter region still able to confer epithelial-specific expression was defined.[8] It has been described that DNA methylation prevents the amplification of the EpCAM gene.[9] Furthermore, recent studies provide evidence that DNA methylation is involved in the regulation of the EpCAM gene.[10,11] Although currently siRNA is most commonly used to down-regulate gene expression, a major drawback of siRNA is that downregulation is transient. SiRNA treatment requires either sequential deliveries or integration of shRNA (small hairpin RNA) expressing plasmid DNA into the target cell's genome, encompassing the same limitations as encountered with gene therapy. Hence, we set out to explore active DNA methylation as a tool to silence EpCAM gene expression. One major advantage of gene silencing by DNA methylation compared to siRNA-mediated silencing is that the cellular DNA methylating system will maintain the new methylation pattern in the absence of the methyltransferase and long-term presence of the methylating agent is not required.[12] Moreover, DNA methylation affects the initiation of transcription, whereas siRNA acts in general on the mRNA level, where the target pool is much larger. In principle only one initial event is required for DNA methyltransferases as the DNA methylation pattern is epigenetically imprinted[13] and inherited to the daughter cells. To actively silence endogenous EpCAM expression we used the prokaryotic DNA (cytosine-5) methyltransferase (MTase) M.SssI, which methylates cytosines in CpG dinucleotides.[14] Since M.SssI has the same base and sequence specificity as mammalian DNA MTases, this enzyme appears to be an excellent tool to study the role of DNA methylation in healthy and diseased eukaryotic cells provided that it can be delivered into the cell nucleus.

Material and methods

Cell culture and 5-AZAC treatment

The HEK293A/T (CRL-1573), U373MG (HTB-17), SKOV3 (HTB-77) and SW948 (CCL-237) cell lines were purchased from ATCC (Manassas, VA) and cultured according to ATTC recommendations. The HEKOGM cell line was kindly provided by Dr. O. Gires (Ludwig-Maximilians-University, Munich, Germany). The lung carcinoma cell lines GLC8 and GLC1 were maintained in RPMI-1640 medium (BioWhittaker, Walkersville, MD). The

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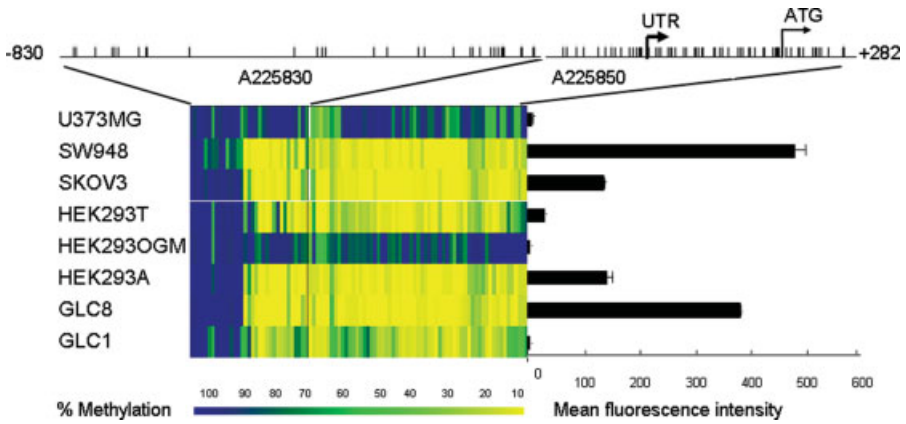

FIGURE 1 – Analysis of methylation status of part of the EpCAM promoter and exon 1 in relation to EpCAM expression. CpGs in the analyzed region are depicted by vertical bars, the transcription initiation site corresponds to position +1, the untranslated (UTR) as well as translated (ATG) region of exon 1 are shown by arrrows. A CpG-dense region (A225850) and a less dense region (A225830) spanning together about 1100 bp of the EpCAM gene are covered by 3 amplicons. Each row corresponds to one cell line and each rectangle represents one CpG, of which the methylation status is indicated as a color code (blue: methylated to yellow: unmethylated). White areas indicate CpGs for which no reliable data were retrieved. EpCAM expression was measured by flow cytometry.

fetal lung fibroblasts (FLF) were isolated in 1992 under informed consent and cultured in DMEM (BioWhittaker, Walkersville, MD). Cells were cultured at 37°C and 5% CO_2.

For methylation inhibition studies, U373MG, SKOV3, FLF and GLC1 cells were cultured in their appropriate media with a final concentration of 2 μg/ml 5-aza-2′-deoxycytidine (5-AZAC; Sigma, St Louis, MO) during days 1, 3 and 5. At day 2 and 4 medium was refreshed and on day 6 cells were harvested for extraction of total mRNA and EpCAM expression.

Protein expression

EpCAM detection was performed with 1 μg/ml mouse Mab MOC31 (protein A purified) or supernatant, followed by RαM-PO or RαM-F(ab)$_2$-FITC (DAKO, Glostrup, Denmark). The Mean Fluorescence Intensity (MFI) was measured on a BD FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). For Western blotting cells were lyzed in 200 μl buffer, 10 μg total protein separated and blotted as previously described.[15] As loading control GAPDH (Abcam, Cambridge, UK) was used, detection was accomplished with GαR-AF (Jackson ImmunoResearch, Suffolk, England) and BCIP/NBT substrate.

Reverse-transcriptase PCR

RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer recommendations. Prior to cDNA synthesis on 2 μg of purified total RNA with an oligo(dT$_{18}$) primer and M-MuLV Reverse Trancriptase (Fermentas, Hanover, MD), RNA samples were treated with rDNaseI (Ambion, Cambridgeshire, UK). cDNA was amplified using primers for EpCAM: exon 3 5′-GAACAATGATGGGCTTTATG-3′ (sense), exon 7 5′-TGAGAATTCAGGTGCTTTTT-3′ (antisense), β-actin 5′-TCAC CAACTGGGACGACATG-3′ (sense), 5′-ACCGGAGTCCAT CACGATG-3′ (antisense), purchased from Biolegio (Malden, The Netherlands). The predicted size of the PCR product was 500 for EpCAM and 242 bp for β-actin.

Quantitative gene expression analysis by real-time RT-PCR

RT-PCR was performed as previously described.[16] In short, 1 μg RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). Quantitatieve PCR amplifications were performed according to manufacturer's protocol on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Applera Nederland, Nieuwekerk a/d Ijssel, The Netherlands). Primers and probes for EpCAM (Hs00158980_m1) and the housekeeping gene GAPDH (Hs99999905_m1) were purchased as customized assays from Applied Biosystems. All PCR reactions were carried out in triplicate on duplicate profections. Relative quantitation of gene expression was calculated based on the comparative cycle treshold (C_t) method ($\Delta C_t = C_t$ EpCAM - C_t GAPDH). Comparison of EpCAM expressions in different samples was performed based on the differences in ΔC_t of individual samples ($\Delta\Delta C_t$).

Methylation analysis

DNA extracted from the cells was subjected to bisulfite treatment as previously described.[17] Bisulfite specific primers void of any CpG were used in order to obtain amplification products unbiased for the methylation status. Two overlapping amplicons were selected to cover a 700 bp region (A225830, Fig. 1). Primer sequences for the first amplicon were 5′-ACCTCCCCAATAAC TAAAATTAC-3′ (forward), 5′-TTGAAGATTTTGTGTTGAG ATTT-3′ (reverse), and for the second amplicon 5′-AGT GTTTTGGAAGGTTTTTTGT-3′ (forward), 5′-AAATTAAAA AAATAAATAAACTCCC-3′ (reverse). A neighboring region extending into the CpG island (A225850, Fig. 1) was covered with an amplicon of 441 bp. Primers were 5′-GGAGGGGAGTTTATT-TATTTTT-3′ (forward) and 5′-CACAACTCTACTCCAATC-3′ (reverse). PCR conditions: 95°C for 15 min, followed by 40 cycles of 95°C for 60 sec, 55°C for 45 sec and 72°C for 60 sec and finished with 72°C for 10 min. Purified PCR products were used directly for cycle sequencing on an ABI3730-capillary sequencer using the ABI Prism BigDye Terminator V3.1 sequencing chemistry. The obtained trace files were subsequently analyzed using the ESME software as previously described.[18]

Plasmids

The plasmids pBHNC-MSssI and pBHNC-MSssI(C141S) were described previously.[19] The variant encoded by pBHNC-MSssI has MTase activity comparable to the wild-type enzyme, and will be referred to as M.SssI. Its mutant derivative, M.SssI(C141S) encoded by pBHNC-MSssI(C141S), in which the active site cysteine is replaced by serine, has a greatly reduced (2–5%) activity relative to the wild-type enzyme.[19]

DNA MTases

E. coli ER1821 cells, harbouring pBHNC-MSssI or pBHNC-MSssI(C141S), were grown at 37°C in LB containing 100 μg/ml ampicillin. At OD^{600} ~0.6, M.SssI or M.SssI(C141S) production was induced by adding 1.0% arabinose. After 4-hr incubation at 30°C, cells were harvested by centrifugation, resuspended in breaking buffer (50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl, 1 mM imidazole), sonicated and cell debris removed by centrifugation. For purification a His-Select Nickel Affinity gel column (1 ml, Sigma) was used according to the manufacturers instructions. The eluate was diluted with cation exchange buffer (6.7 mM MES, 6.7

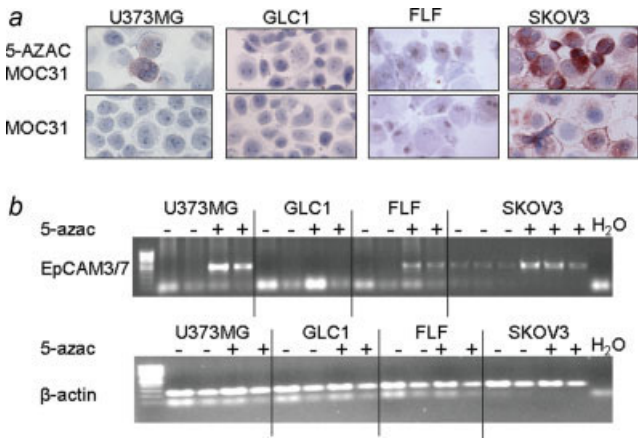

FIGURE 2 – EpCAM expression on protein and mRNA level before and after 5-AZAC treatment. (*a*) Immunohistochemical staining with the EpCAM-specific antibody MOC31: after AZAC treatment de novo induction of EpCAM was observed in the EpCAM negative U373MG cells, but not in the EpCAM negative FLF and GLC1 cells. SKOV3 cells showed upregulation of EpCAM compared with non-treated cells (magnification: ×40). (*b*) Reverse-transcriptase PCR analysis displayed induction of EpCAM mRNA (exon 3 and 7) in EpCAM-negative FLF and U373MG cells and upregulation in SKOV3 cells after AZAC treatment.The gel has been loaded with 15 and 5 μl of each PCR-product obtained from the U373MG, FLF and GLC1 cells. For SKOV3 cells, due to the high expression found on these cells 3, 2 and 1 μl PCR product has been loaded For the loading control β-actin 5 and 2 μl PCR product has been loaded (− = without AZAC, + = with AZAC).

mM Hepes, 6.7 mM NaOAc, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol) and applied to a HS POROS 50 column (Applied Biosystems, Fostercity, CA). After washing with 100 ml cation exchange buffer containing 0.2 M NaCl, proteins were eluted with a linear NaCl gradient (0.2–1 M) in cation exhange buffer. DNA MTase containing fractions were pooled, concentrated by ultrafiltration, mixed with an equal volume of glycerol and stored at −20°C. All purification steps were performed at 4°C.

DNA MTase and siRNA delivery

SAINT-2:DOPE (SD; 0.75 mM) was purchased from Synvolux Therapeutics (Groningen, The Netherlands).[20] SKOV3 cells were seeded 0.5×10^6/6 well or 12.5×10^4/chamber slide well. MTase or siRNA delivery was performed at 50–80% confluency. Ten micrograms MTase, 1 μg siRNA-EpCAM (sense 5′-GGAGAUCACAACGCGUUAUUU and antisense 5′-AUAACGCGUUGUGAUCUCCUU) (Qiagen) or 1 μg irrelevant siRNA (AM4611, Applied Biosystems) in 100 μl PBS was complexed with 20 μl SD in an equal volume of PBS and the SD-MTase or SD-siRNA complex was pipeted directly onto the cells. In one chamber slide well 0.625 μg M.SssI/C141S was complexed with 2.5 μl SD. As controls, MTase, siRNA or SD alone were added. Cells were split, and EpCAM expression was measured at day 2, 6, 10, 14 and 17.

Results

To investigate the relation between EpCAM expression and DNA methylation, we assessed the methylation status of the EpCAM promoter in a panel of cell lines with different EpCAM expression levels by bisulfite sequencing (Fig. 1). In the EpCAM-negative cell lines U373MG and HEK293OGM, the promoter

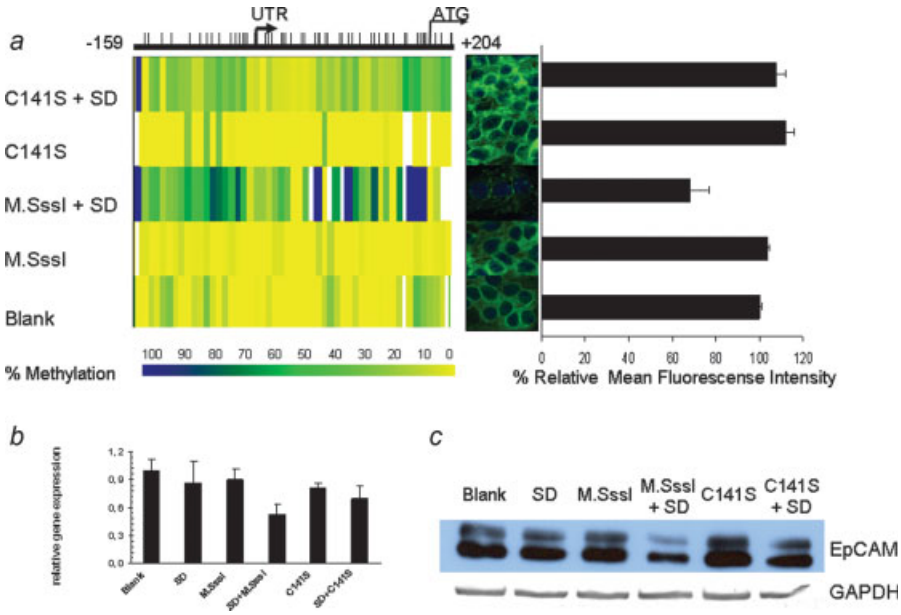

FIGURE 3 – Active silencing of EpCAM expression in SKOV3 cells by induced methylation of the EpCAM promoter *via* profection with M.SssI (48 hr after profection). (*a*) Bisulfite sequencing data obtained from the 441 bp fragment (A225850) within the CpG island spanning part of the promoter and exon 1 of the EpCAM gene. Each row corresponds to one experimental treatment of the cell line (SD: SAINT-2:DOPE, C141S: low-activity mutant of M.SssI) and each rectangle represents one CpG, of which the methylation status is indicated as a color code (blue: methylated to yellow: unmethylated). White areas indicate CpGs for which no reliable data were retrieved. Profection of SKOV3 cells with M.SssI resulted in increased methylation levels, delivery of its less active mutant C141S showed an intermediate methylation status. Immunohistochemical staining of EpCAM (*a*, middle panel) displayed a reduced EpCAM expression after profection with M.SssI compared to the controls, which was confirmed by flow cytometric analysis (*a*, right panel). (*b*) Quantitative Real-Time PCR analysis showed a reduced EpCAM mRNA level compared to the controls, expression levels of mRNA in untreated SKOV3 (blank) cells were arbitrarily set at 1. (*c*), Western blot analysis with the EpCAM-specific antibody MOC31 demonstrated a clear reduction of EpCAM expression after profection with M.SssI compared to the controls. The two bands are due to differential glycolysation of EpCAM, GAPDH is shown as loading control.

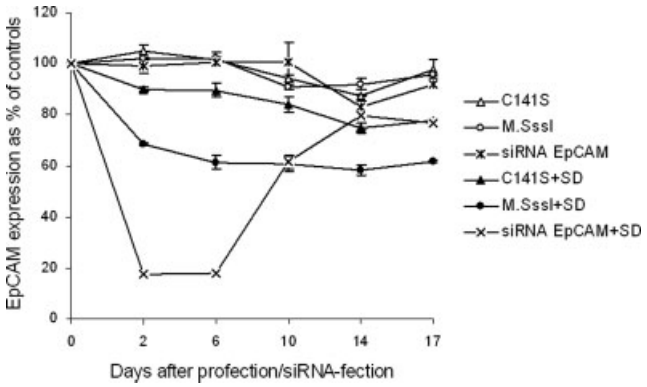

FIGURE 4 – Persistent downregulation of EpCAM *via* profection with M.SssI as compared to transient downregulation of EpCAM after siRNA-fection. At day 0, SKOV3 cells were profected with M.SssI or transfected with EpCAM-specific siRNA, cells were cultured for 17 days and EpCAM expression was measured by flow cytometry at the days indicated (SD: SAINT-2:DOPE, C141S: low-activity mutant of M.SssI.) Because of auto-fluorescence of SD, the EpCAM expression after profection or siRNA-fection was expressed as percentage of the SD control. For MTases or siRNA without SD, the blank was set as 100%. The reduction in EpCAM expression at day 2 after siRNA-fection remains up to day 6 after which re-expression is increasing. The 40% reduction in EpCAM expression at day 6 after profection with M.SssI persisted up to day 17.

region was extensively methylated. Whereas the most upstream portion of the analyzed region was methylated in all cell lines analyzed, hypomethylation in the promoter region adjacent to the coding region was characteristic for those cell lines that do express EpCAM. In this latter region, more CpGs were methylated in the low EpCAM expressing HEK293T cell line, compared to the higher expressing HEK293A. The EpCAM-negative GLC1 cell line, displayed an intermediate methylation status.

The observed correlation between EpCAM expression and the methylation status of the EpCAM promoter suggests that the EpCAM gene is regulated by DNA methylation. Indeed, after addition of the demethylating agent 5-AZAC for 3 nonconsecutive days, *de novo* induction of EpCAM expression was observed in U373MG and upregulation in SKOV3 cells (Fig. 2*a*). RT-PCR confirmed the presence of mRNA in the 5-AZAC treated EpCAM-negative U373MG and FLF cells (Fig. 2*b*), although in the latter cells no EpCAM protein was detected. The 5-AZAC treatment of the GLC1 cell line did not result in EpCAM expression on both protein and mRNA level.

As EpCAM expression is clearly associated with promoter methylation, we investigated whether we could actively silence EpCAM expression by induced methylation of the EpCAM promoter. To this end, we delivered M.SssI[19] directly as protein *via* a cationic amphiphilic compound SAINT-2:DOPE (SD)[20] into SKOV3 cells (profection). As a control, cells were profected with the mutant M.SssI protein C141S, which has ~2–5% catalytic activity of the wild-type enzyme.[19] Analysis of genomic DNA, obtained from SKOV3 cells 48 hr after profection with M.SssI, demonstrated increased methylation of CpGs located in the EpCAM promoter and the first exon of the gene, whereas the cells treated with the MTases without SD, were not, or much less methylated (Fig. 3*a*). Cells profected with C141S showed an intermediate methylation status, which is in agreement with the residual activity observed *in vitro*.[19]

Next, we assessed whether the induced methylation was associated with repression of gene and protein expression. Quantitative Real-Time PCR displayed reduced EpCAM mRNA levels after profection with M.SssI (Fig. 3*b*). Moreover, immunohistochemical staining (Fig. 3*a* middle panel), flow cytometric analysis (Fig. 3*a* right panel) and Western blot analysis (Fig. 3*c*) showed reduced EpCAM protein expression after profection with M.SssI compared to the cells profected with C141S or treated with the enzymes without SD. Altogether, these results demonstrate that methylation is involved in the regulation of EpCAM expression, and even more important, EpCAM expression can be actively downregulated by DNA methylation.

In contrast to siRNA-mediated silencing, which generally requires sequential deliveries to maintain silencing, only one initial exposition of the genome to the MTase is required as the resultant DNA methylation pattern is inherited through successive cell divisions.[12,13] To investigate if the downregulation of EpCAM *via* methylation is lasting, we delivered, only once, the protein M.SssI or siRNA directed against EpCAM, into SKOV3 cells on day 0, and cultured them for 17 days (Fig. 4). Within 2 days after siRNA-fection, EpCAM expression was reduced to 20% of the expression levels observed in nontransfected EpCAM expressing SKOV3 cells. This downregulation remained up to day 6, where after EpCAM re-expression increased with time. Irrelevant siRNA had no effect on EpCAM expression (data not shown). In contrast, profection with M.SssI resulted in a 40% reduction of the EpCAM expression, which persisted at least up to 17 days after profection. Although the EpCAM specific siRNA-mediated downregulation of EpCAM was initially more effective than profection with the nontargeted M.SssI, downregulation *via* profection was enduring. Profection with the mutant C141S showed a gradually decrease in EpCAM expression, which stabilized at day 14 at 75% of the expression levels observed in nonprofected cells (Fig. 4). This residual activity of C141S, as also shown by the intermediate methylation status (Fig. 3*a*), was not detectable on the level of protein expression at day 2 after profection (Fig. 3*a* middle and right panel, 3C), but this gradually decrease does correlate with the observed slight decrease in mRNA level (Fig. 3*b*).

Discussion

This study demonstrates for the first time that endogenous EpCAM expression can be actively downregulated in a *persistent* manner *via* induced DNA methylation. Previously Tai *et al* reported inhibition of EpCAM promoter activity by *ex vivo* DNA methylation of the promoter. Indeed, a significant association was demonstrated between EpCAM expression and methylation status of the EpCAM promoter (−265 to −100) in microdissected tumor tissue.[11] Also Spizzo *et al* found part of the promoter and exon 1 (−156 to +361) to be methylated to a higher degree in an EpCAM negative breast cancer cell line as compared to an EpCAM positive cell line. Interestingly, in this study no correlation was observed between methylation status and EpCAM expression in primary breast cancer tissue.[10] These paradoxical observations concerning the methylation status of the EpCAM gene and its expression in clinical tumor specimens might be due to the different tumor types analyzed, but also to differences in the region examined.

In this study, we therefore investigated a larger region of the EpCAM promoter (−830 to +282), and showed a correlation between EpCAM expression and the methylation status of the promoter region in EpCAM expressing and nonexpressing cell lines. As previously demonstrated,[8] this region includes part of the promoter (−687 to +93) which is sufficient to confer epithelial specificity. In the EpCAM-negative cell lines U373MG and HEK293OGM this promoter region was extensively methylated, whereas in EpCAM expressing cells this was not the case. The importance of methylation in regulating EpCAM promoter activity is further demonstrated by 5-AZAC treatment, which indeed led to de novo induction of EpCAM expression in U373MG and FLF and a further upregulation in SKOV3 cells. Only the EpCAM-negative GLC1 cell line, which displayed an intermediate methylation status, showed no induction of EpCAM expression upon 5-AZAC treatment. This finding might be due to genomic deletions or mutations in the EpCAM gene in this particular cell line.

Controversial results regarding EpCAM expression in correlation with cancer invasiveness and tumor progression have been reported. Several studies showed that EpCAM overexpression correlates with a bad patient survival.[3–5] Futhermore, inhibition of EpCAM expression with antisense mRNA or siRNA reduces the oncogenic potential of carcinoma cells.[1,2] Moreover, the capacity to form tumors out of human colorectal xenograft lines has been shown to be restricted to EpCAM high expressing stem cells, whereas EpCAM low expressing cells failed to form tumors.[6] These findings supported our aim to develop a potential strategy to downregulate EpCAM expression in a persistent manner. However, an other study found an inverse correlation between EpCAM expression and cancer invasiveness in cancer cell lines.[11] Dalerba *et al*. demonstrated that tumors grown from EpCAM high expressing colon carcinoma cells in immunodefficient mice contained both EpCAM high and low expressing populations in proportions similar to those of their parent lesions.[6] This heterogeneity in EpCAM might explain the contrary results concerning the prognostic value of EpCAM.

Tools including active and sustained modulation of endogenous EpCAM expression should be developed to provide insights in the precise role of EpCAM in tumorigenesis and tumor progression of different origin and might eventually lead to potent therapeutics. Therefore we set out to explore active DNA methylation as a tool to silence EpCAM gene expression. DNA MTase- and siRNA-mediated downregulation of EpCAM expression showed different kinetics (Fig. 4). The initial downregulation *via* siRNA was higher compared to DNA methylation,. This difference in efficiency can be explained by the fact that the siRNA used is specific for EpCAM, whereas M.SssI is not. The resulting overall increase of methylated CpGs in the genome is toxic. Targeting of M.SssI to the EpCAM promoter will allow to optimize the dose of M.SssI thereby increasing the reduction of EpCAM expression. Nevertheless, in this study even by using nontargeting M.SssI, we could show efficient downregulation of EpCAM which was enduring and more pronounced than siRNA after 17 days.

Now that we have shown that active methylation of the EpCAM promoter results in sustained silencing of gene expression, the next step is to target M.SssI to the EpCAM promoter specifically to reduce its toxicity. Targeting of the EpCAM promoter by engineered zinc finger protein transcription factors (ZFPs) has already been demonstrated by us.[21] Methyltransferases fused to zinc finger proteins targeting predetermined sites in the DNA to repress gene expression have been reported.[12,22,23] Engineering ZFPs targeted to the EpCAM promoter[21] fused to M.SssI as an effector domain, provides a powerful tool to achieve targeted methylation. The ZFP binds specific to the promoter where after the enzyme will methylate only the CpGs close to the ZFP target sequence. Another approach is the use of a Triple helix-Forming Oligonucleotide (TFO) targeted to the EpCAM promoter. Such targeting devices will enable efficient and sustained gene silencing which has potent applications for basic research and therapy. Considering the dynamic change of EpCAM expression in different tumor stages, active regulation of the EpCAM gene is a powerful tool to explore the function of EpCAM. Because of the contributory role of cancer-linked genomic hypomethylation of oncogenes to tumorigenesis or tumor progression[24] active silencing of specific genes *via* DNA methylation can provide a novel approach in anti-cancer treatment.

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