Oncomodulatory properties of the human cytomegalovirus-encoded receptors US28 and UL33

Langemeijer, E.V.

2012

document version
Publisher's PDF, also known as Version of record

chapter 5

Viral Chemokine Receptor US28 modulates cyclin D1 expression by DNA hypomethylation

Ellen V. Langemeijer, Azra Delic, Wim Zoutman, Kees Tensen,
Marco Siderius and Martine J. Smit

Abstract

Human cytomegalovirus (HCMV), a widely spread herpesvirus, has been shown to play a role in tumor progression. During the last decade the viral-encoded chemokine receptor US28 has been studied intensively and has been shown to induce proliferative anti-apoptotic and pro-angiogenic behaviour via activation of COX-2, STAT3-IL-6 and β-catenin. Activated signaling pathways may directly stimulate the aforementioned cellular responses e.g. by alteration of transcription factor activity to change gene expression patterns. Alternatively cellular responses can be mediated via enzymes that control epigenetic properties. A clear link between cancer and epigenetic changes has been reported [275]. Epigenetic modifications can be described as modifications of DNA and chromatin that are stable over rounds of cell division but do not involve changes in the underlying DNA sequence of the organism [276]. The epigenetic modifications of the DNA include methylation by methyltransferases in CpG-rich regions of genes and histone modifications. When CpG islands are in the unmethylated state, a particular gene can be transcribed. In a cancerous setting, CpG islands of the same genes can be hypermethylated [277], leading to disrupted (blocked) transcription. *Vice versa*, hypomethylation of a gene that is normally not transcribed due to presence of methylation, may result in increased transcription.

In this study we describe US28-mediated epigenetic changes in the third CpG island of the cyclin D1 gene encoding cell cycle regulator cyclin D1. US28-induced changes in methylation of the cyclin D1 gene were studied using methylation-specific melting curve analysis (MS MCA), inhibitors of methyltransferase activity

and bisulfite sequencing. Sequence analyses suggested specific CG-pairs in the cyclin D1 promoter region to be hypo-methylated in US28-expressing NIH-3T3 cells.

Demethylation in the third CpG island of the cyclin D1 gene by Dnmt Inhibitor Decitabine (DAC) yields induced expression of cyclin D1 mRNA. Additionally, the Cyclin D1 protein expression is enhanced upon treatment in these cells. Treatment with HDAC inhibitor Trichostatin A (TSA), which targets the histones, did not alter cyclin D1 mRNA and protein levels, indicating histone modifications most likely are not involved in this epigenetic regulation.

The US28-induced changes in cyclin D1 gene methylation described here might play a role in tumor onset. This is the first report in which a virally encoded GPCR is linked to the specific epigenetic alterations of a cyclin D1 CpG island, resulting in upregulation of the cyclin D1 cell cycle protein.

5.1 Introduction

In the past decades it has become clear that infection with human cytomegalovirus (HCMV), a β-herpesvirus, is associated with several diseases. It has been suggested that HCMV contributes to either onset or progression of chronic inflammation, vascular diseases and infection with human immunodeficiency virus (HIV) [1]. Furthermore, presence of the HCMV genome and - antigens has been observed in malignancies, such as colon cancer [136], prostate cancer [278], Hodgkin's lymphoma [279], glioblastoma [80], medulloblastoma [234] and in mucoepidermal carcinomas [4]. In these studies, the HCMV virus is present in tumor cells, and cells from surrounding tissue appear to be HCMV negative. Until recently, HCMV was not considered to be oncogenic, as it fails to transform healthy human cells [137]. However, in the case of mucoepidermoid carcinoma of the salivary glands, HCMV is now considered to be a causing agent [4].

US28 is a well-studied, constitutively active viral chemokine receptor encoded by HCMV [7]. Chemokine receptors belong to the G protein coupled receptor (GPCR) family, large family of 7 transmembrane receptors that process extracellular signals into intracellular responses. The viral receptor US28 shows homology to the human CCR1 [151] and CX3CR1 [256] chemokine receptors. Moreover, it can bind several CC-chemokines (i.e. CCL: 2, 3, 4, 5, 7, 11, 13, 26 and 28) and CX3CL1

(fractalkine) [280]. The receptor is likely to be expressed on the virion [234] as well as on the host cell membrane in early - and latent stage of infection [281]. Besides acting as a chemokine sink, US28 is constitutively active towards multiple signaling pathways like NF-κB, CRE and NFAT [82]. US28-expressing NIH-3T3 cells display a pro-angiogenic phenotype via up-regulation of vascular endothelial growth factor (VEGF) protein) and transformed phenotype via enhanced cell growth and cell cycle progression. This is *e.g.* exemplified by increased levels of cyclin D1 [6], a gene known to have promoter elements responsive to the pathways triggered by US28 [282]. US28-expressing NIH-3T3 cells promote tumorigenesis when injected into nude mice in a xenograft model.

We have shown that US28-expressing NIH-3T3 cells display enhanced transcription of COX-2, a key mediator of inflammatory diseases and a major determinant in many types of cancer, driving the production of vascular endothelial growth factor VEFG [81]. Targeting COX-2 *in vivo* with Celecoxib resulted in a marked delay in onset and growth rate of tumor formation. Additionally, in medulloblastoma, the most malignant brain tumors in children, a large proportion of primary medulloblastomas is HCMV positive and displays high levels of COX-2 and PGE2 [234]. Thus, proliferative diseases linked to HCMV can partially be attributed to the US28-induced activation of COX-2. Another major contribution in US28-induced proliferative signaling involves the IL-6/Stat3 axis [80]. US28 activates the IL-6/JAK1/STAT3 signaling axis through a positive feedback loop, which is initiated by activation of the NF-κB transcription factor. NF-κB activation results in increased levels of IL-6 that upon secretion induces activation of STAT3. Moreover, activation of the oncogenesis-related β-catenin by US28 has also been observed in US28 transfected cells as well as virus-infected cells (**Chapter 3**, or Langemeijer *et al.*, manuscript submitted) and in transgenic animals [87]. These observations underline US28's extensive potential to alter cellular signal transduction, resulting in proliferative responses [7].

Recently, epigenetic changes induced by HCMV were investigated in the context of oncogenic development [283]. Epigenetic modifications can be described as modifications in DNA and chromatin that are stable over rounds of cell division but do not involve changes in the underlying DNA sequence of the organism [276]. These include DNA methylation by DNA methyltransferases (DNMTs) and histone modifications by histone deacetylases (HDACs) and histone acetyltransferases (HAT).

The cytosines from CpG rich regions, or CpG islands in the promoter region of genes, can be modified by methylation, by DNA methyltransferases (DNMT). Unmethylated CpG islands enable transcription of that particular gene. In a cancerous setting, the cytosines of this same CpG island can be hypermethylated [277],

leading to disrupted (blocked) transcription (e.g. tumor supproessor genes). Other genes, involved in proliferation, appear hypomethylated, resulting in increased transcription. For histone modifications, HDAC proteins regulate gene transcription by modulating the chromatin architecture via deacetylation of histones, the core protein around which DNA is wrapped [284]. The pattern of these histone modifications encodes information, reflecting an active or silent DNA transcription state.

It is known that herpesviruses use epigenetic mechanisms in the viral life cycle or virus-induced pathogenesis. For instance, the switch between latency and lytic replication in Kaposi's sarcoma herpesvirus (KSHV) is controlled by epigenetic factors [285]. DNA packaging, or chromatinisation of herpes simplex virus and HCMV genomes, is induced via epigenetic regulation [286]. Recently, MRC-5 cells infected with the clinical HCMV-VR1814 strain were assayed for global methylation level. Analysis of DNA extracted from these MRC-5 cells infected with VR1814 revealed a profound hypomethylation compared with uninfected cells [283].

In this study we have explored potential epigenetic modifications of the cyclin D1 gene induced by constitutive activity of US28 the HCMV-encoded vGPCR. Using different techniques we have shown the cyclin D1 promoter region is hypomethylated in US28-expressing NIH-3T3 cells. The observed epigenetic modulation of cyclin D1 gene expression is reflected in increased levels of the cyclin D1 protein that may contribute to US28-induced proliferative responses.

5.2 Results

Cyclin D1 gene is hypomethylated in US28-expressing cells

One of the key players in cancer is the cell cycle regulator cyclin D1 gene (Ccnd1) [287]. The promoter region of this cell cycle regulating gene is known to be subject to (de-) methylation in rat leukemic cell lines [288] as well as in human B cell malignancies [289]. We have previously shown cyclin D1 levels to be upregulated in US28-expressing cells [6]. Therefore, we set out to examine the cyclin D1 promoter region in detail. Genomic DNA (gDNA) from NIH-3T3 cells transfected

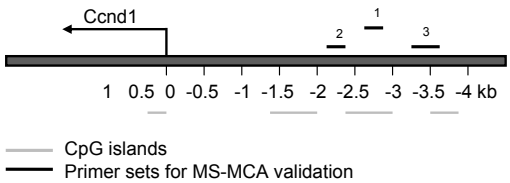


Figure 5.1: Cyclin D1 promoter region. Schematic representation of the genomic map of the cyclin D1 promoter region. The transcription start site of the antisense oriented cyclin D1 gene is indicated by the left-pointing arrow. The four CpG islands are indicated by grey bars, MS MCA validation primer sets 1 to 3 by black bars.

with an empty vector (mock) was compared to gDNA from cells expressing US28, or the US28-R^{129}A, the G protein-uncoupled receptor mutant. The cyclin D1 gene (located on Chr 7, 72.3 cM) contains 4 CpG islands close to the transcription start site (TSS) (Figure 5.1). In the schematic representation of the genomic map of this gene the 4 CpG islands (grey lines) are positioned around the TSS of the antisense orientated cyclin D1 primary transcript. Methylation specific melting curve analysis (MS MCA) was performed using three primer sets (black lines) covering 2.0 - 3.7 kb downstream of the TSS, to detect possible differences in DNA methylation. Sequence of MS MCA primers are based on bisulfite converted (BC) gDNA. Therefore, areas without cytosines were selected for primer design, to amplify areas with potentially differentially methylated CpG sites.

Figure 5.2 shows the melting curves for three MS MCA primer sets for the cyclin D1 gene. On the x-axis the temperature and on the y-axis the amount of Sybr green fluorescence (amount of DNA) is indicated. In each panel the left peak (=lower temperature) represents the amount of unmethylated DNA and the right peak represents the amount of methylated DNA. The control samples, being 100% unmethylated DNA (U) and 100% methylated DNA (M) are shown in blue. The melting temperature curves representing 3T3-mock (green) and 3T3-US28 (pink) using primer set 1 show two peaks, indicating a difference in methylation status of this CpG site. With this primer set a part of the third CpG island, which is located around 2.3 to 3 kb downstream of the transcription start site of the gene, was checked. The US28-expressing cells showed a larger peak overlapping the unmethylated control DNA, indicating hypo-methylation, which might lead to increased mRNA levels of cyclin D1 in these cells. Comparable peak intensities and overlapping peaks, as observed with primer set 2 and 3 for the two neighbouring CpG islands respectively, indicate there is no difference in methylation state in these areas of the cyclin D1 promoter region.

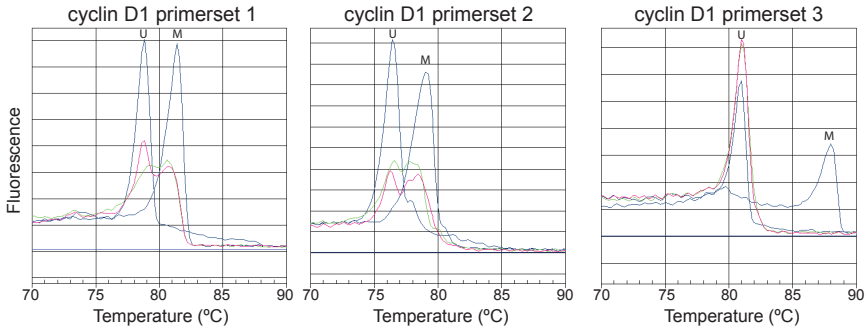


Figure 5.2: MS MCA of three regions of cyclin D1 promoter region. Melting curves obtained after the MS MCA PCR reactions. X-axis shows the temperature, Y-axis indicates the amount of fluorescence. In each graph blue peaks indicate 100% methylated DNA (M) and 100% unmethylated DNA (U). The curves representing 3T3-mock (green) and 3T3-US28 (pink) bisulphite converted DNA samples are shown in the same panels.

DNMT inhibitor treatment confirms hypomethylation of cyclin D1 gene in US28-expressing cells

To characterize the functional consequence of hypomethylation of the cyclin D1 promoter region as shown with primer set 1, the effects of DNMT and HDAC inhibitors were examined. Cells were treated with the DNMT inhibitor DAC and HDAC inhibitor TSA. MS MCA melting curves of PCR products derived from 3T3-mock, 3T3-US28 and 3T3-US28-R^{129}A cells are shown in the top columns from left to right respectively (Figure 5.3A). Melting curves of cells treated with TSA (second row graphs) and cells treated with DAC (bottom row graphs) are shown. As shown in Figure 5.2, US28 samples showed the highest peaks of unmethylated DNA. In the mock as well as the US28-R^{129}A cells, DAC treatment increased the left, unmethylated DNA peak, indicating that expression of US28 removes (part of) the methylation of the third CpG island of the cyclin D1 gene.

Validation of the sequence of this part of the 3T3-mock and 3T3-US28 cyclin D1 promoter region was performed by using gDNA that was bisulfite-treated in MS MCA with the cyclin D1 primer set 1 (Figure 5.3B). The obtained amplicon was isolated from gel and sequenced with the forward primer. The reverse primer from primer set 1 used for amplicon production is underlined in the alignment. All cytosines found in the sequenced area are derived from methylated CG pairs. Three sites have partially shifted from a CG to a TG (bold and highlighted in grey)

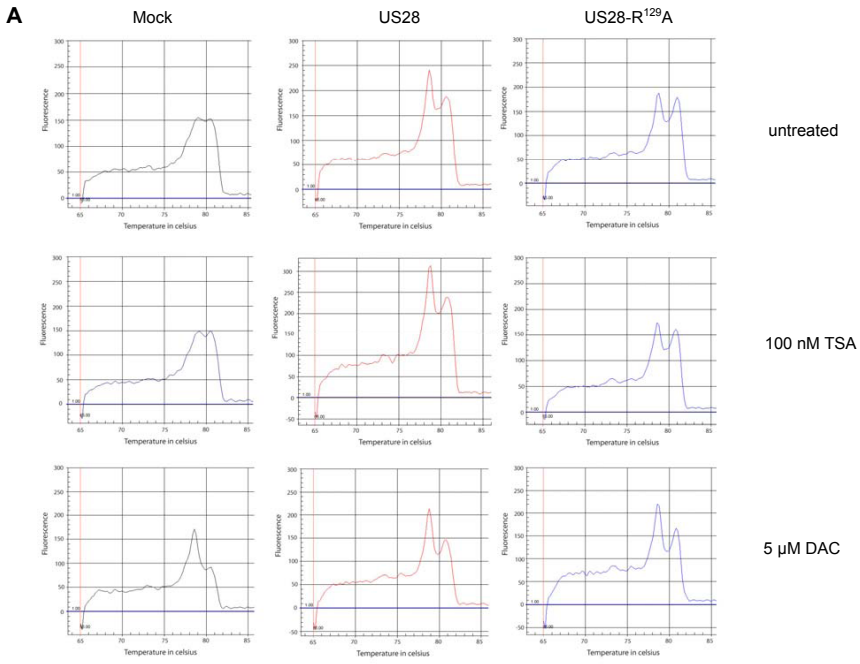
in US28-expressing 3T3 cells in comparison to mock cells, indicating these three sites are not, or to a lesser extent, protected from bisulfite conversion and therefore not methylated.

Cyclin D1 expression and protein level are controlled by US28-mediated epigenetic changes

As we have previously shown, US28-expressing cells display an increase of cyclin D1 mRNA and protein levels [6, 81]. Increased mRNA level for cyclin D1 was observed in US28-expressing NIH-3T3 cells compared to mock and US28-$R^{129}A$ cells (Figure 5.4A). Demethylation by means of DAC treatment increased the cyclin D1 mRNA in both mock and US28-$R^{129}A$ cells (Figure 5.4B). Additionally, the cyclin D1 protein expression was enhanced upon treatment in these cells. Western blot of cyclin D1 in mock transfected, US28- and US28-$R^{129}A$-expressing NIH-3T3 cells showed the highest cyclin D1 protein level in the US28-expressing cells. However, DAC treatment resulted in increased cyclin D1 protein level in mock cells (Figure 5.4C). In US28-$R^{129}A$ cells this enhanced protein expression appears less clear. These data demonstrate that the observed hypomethylation of the cyclin D1 promoter region in US28-expressing NIH-3T3 cells results in increased cyclin D1 mRNA and protein expression, thus indicating a potential role for HCMV-encoded receptor US28 in epigenetic regulation of the host cell genome. Treatment with HDAC inhibitor TSA alone did not result in altered cyclin D1 mRNA and protein levels, indicating histone acetylations are most likely not playing a role in the US28-mediated epigenetic control of the cyclin D1 gene.

5.3 Discussion

Infection with herpesviruses is known to alter gene expression and cellular function of the host cell. Accumulating evidence indicates that in HCMV-induced pathogenesis, hijacking of signal transduction pathways of the host cell is of crucial importance. The HCMV-encoded GPCRs such as the constitutively active



B

```
3T3-US28        CGTTTGGGAGATAGGATGCGACGGTTTTAGGTGTGTATAGGGGATGAGTTGAGTATATAT 60
3T3-Mock        CGTTTGGGAGATAGGATGCGACGGTTTTAGGTGTGTATAGGGGATGAGTTGAGTATATAT 60
                ************************************************************

3T3-US28        ATTTTAGTTGAAGGGTGTTTGAAATTTGTTTAGGGTAATTTAGGCGGAGTAGTCGTGTAG 120
3T3-Mock        ATTTTAGTCGAAGGGTGTTTGAAATTCGTTTAGGGTAATTTAGGCGGAGTAGTCGTGTAG 120
                ******** ********** ********** ********************************

3T3-US28        TATTGTGGGTTGTTACGCGCGTTTTAAAACGTTTTTTGGGTGAGGAGAGGGAA 172
3T3-Mock        TACGTGGGTTGTTACGCGCGTTTTAAAACGTTTTTTGGGTGAGGAGAGGGAA 172
                ** ************************************************
```

Figure 5.3: MS MCA of cyclin D1 Primerset 1 treated with DNMT and HDAC inhibitors. (A) MS MCA melting curves of PCR products derived from 3T3-mock, 3T3-US28 and 3T3-US28-R^{129}A DNA are shown in the columns from left to right. Melting curves shown are from untreated cells (top row), cells treated with HDAC inhibitor (100 nM TSA, middle row) and cells treated with DNMT inhibitor (5 µM DAC, bottom row). X-axis shows the temperature, Y-axis indicates the amount of fluorescence. In each panel the left peak (=lower temperature) represents the amount of unmethylated DNA, the right peak the amount of methylated DNA. US28 samples show the highest peaks of unmethylated DNA, indicating a higher expression of cyclin D1 protein. In the mock as well as the US28-R^{129}A cells DAC treatment increases the left, unmethylated DNA peak, indicating that the cyclin D1 gene is blocked for transcription by DNMTs in these cells. The presence of US28 overcomes this active hypermethylation and cyclin D1 protein expression is induced by hypomethylation. **(B)** Sequence validation of the 3T3-mock and 3T3-US28 cyclin D1 promoter region. Bisulfite converted gDNA is used in MS MCA with cyclin D1 primerset 1. The amplicon is isolated from gel and sequenced using the forward primer. All cytosines found in the sequenced area are derived from methylated CG pairs. Three sites have shifted from a CG to a TG (bold and highlighted) in US28-expressing 3T3 cells in comparison to 3T3-mock. Therefore these hypomethylation sites appear important in cyclin D1 protein expression. Hypomethylation of this cyclin D1 promoter region causes upregulation of the cyclin D1 protein in 3T3-US28. MS MCA on bisulfite converted gDNA shows the positions of the hypomethylated cytosines (TG instead of CG in the alignment shown in Figure 5.3B). The reverse primer from primerset1 is indicated (underlined).

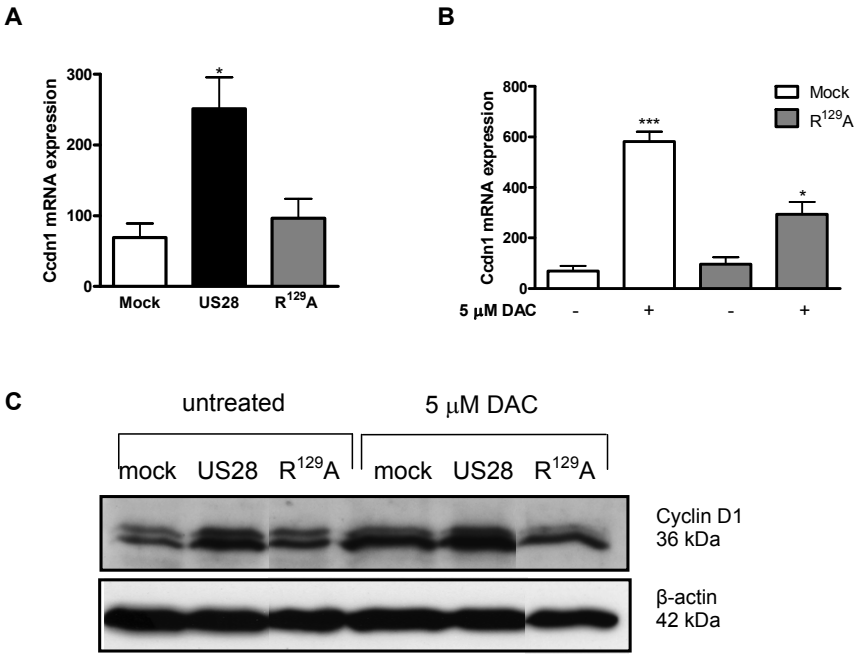


Figure 5.4: Validation of cyclin D1 mRNA and protein levels. (A) qPCR validation of cyclin D1 mRNA expression. US28-expressing cells show increased mRNA level for cyclin D1 compared to mock and US28-$R^{129}A$ cells. **(B)** DAC treatment (5 µM) increases the cyclin D1 mRNA in both mock and $R^{129}A$ cells. **(C)** Western blot of cyclin D1 protein in 3T3-mock, 3T3-US28 and 3T3-US28-$R^{129}A$ cells, shows highest cyclin D1 protein expression in US28-expressing cells. After treatment with the Dnmt inhibitor DAC Ccdn1 protein expression in mock cells (and US28-$R^{129}A$ cells) is upregulated.

chemokine receptor US28 seem to play a determining role herein [6, 80-82, 234]. Recently, HCMV infection was shown to induce global demethylation of the DNA in MRC-5 cells [283]. It was postulated, epigenetic mechanisms might also play a crucial role in HCMV-induced pathogenesis. Previously, we have shown that levels of the cell cycle regulating protein cyclin D1 are upregulated in US28-expressing NIH-3T3 cells [6]. In this study, we demonstrate that hypomethylation of CpGs in the cyclin D1 promoter may be the underlying cause of the increased cyclin D1 mRNA and protein levels in US28-expressing NIH-3T3 cells. The methylation status of three CpG islands in the vicinity of the cyclin D1 promoter region was investigated using bisulfite conversion in combination with MS MCA (Figure 5.2) and DNA sequence analysis (Figure 5.3). We found that US28-expressing NIH-3T3

cells display an altered methylation pattern of a region in the cyclin D1 promoter, with fewer CpG sites methylated compared to mock transfected and US28-R^{129}A-expressing 3T3 cells (Figure 5.2). Since the US28-R^{129}A-expressing cells, which are devoid of G protein signaling, do not diplay hypomethylation of the third CpG island in the Cyclin D1 promoter region, we conclude that G protein-mediated signaling by the viral receptor is necessary to achieve demethylation. Using the demethylating agent DAC, the melting curves of cyclin D1 PCR products derived from 3T3-mock and 3T3-US28-R^{129}A cells (Figure 5.3A) were demethylated, causing an enhancement of transcription. Expression of US28 caused hypomethylation of a CpG island methylated in mock-transfected 3T3 cells, resulting in increased cyclin D1 mRNA. Sequence determination of the 3T3-mock and 3T3-US28 cyclin D1 promoter region showed that three cytosines partially shifted from a CG to a TG (Figure 5.3B) indicating these three sites are not protected from bisulfite conversion and therefore not (or less often) methylated. Additionally, DAC treatment resulted in enhanced Cyclin D1 protein expression level in mock and US28-R^{129}A cells. Hypomethylation of these three cyclin D1 promoter region CpG dinucleotides caused, besides upregulation of the cyclin D1 mRNA, also upregulation of the Cyclin D1 protein. And even higher mRNA and protein levels are reached in US28-expressing cells upon DAC treatment (Figure 5.4).

In the studies of Liu *et al.*, the cyclin D1 promoter in Cyclin D1 over-expressing cell lines and in samples from patient with B cell malignancies was found to be hypomethylated and lysine residues in histone proteins were hyperacetylated versus methylated and hypoacetylated in control cell lines [289]. Therefore we think the observed phenomenon might play a role in the proliferative phenotype seen in 3T3-US28 cells.

Besides changes in methylation status in US28-expressing cells, the mRNA for histone deacetylase 9, Hdac9, was found to be highly upregulated in these cells [81]. Likewise, Hdac5 mRNA levels were increased to a lower extent, whereas mRNAs coding for other known Hdac and Dnmt proteins (DNA methyltransferases) were found to be unchanged. Since Hdac proteins are key players involved in the development of many cancers [284], this findings further indicate that US28 might rewire cellular signaling by means of epigenetic changes, causing alterations involved in cancer development. However, since TSA treatment does not show changes in mRNA or protein levels, this is most likely solely regulated by methyltransferases and not by changes in chromatin structure.

Additionally, transcription of cyclin D1 can also be controlled via multiple transcription factors including TCF-4 [226]. This transcription factor is regulated via β-catenin, which is constitutively activated by US28 (Chapter 3). Therefore, US28-expression might lead to upregulation of the cyclin D1 protein in multiple

ways. Which part of the US28-mediated cyclin D1 protein upregulation is mediated via transcriptional activation through β-catenin or other transcription factors, the hypomethylation of the CpG island or involves histone modifications, remains to be studied.

Taken together, in this study we showed that expression of US28 (partially) removes the methylation of the third CpG island of the cyclin D1 gene, and mRNA as well as protein expression are induced after this hypomethylation. Therefore, we propose hypo-methylation of these three sites within the cyclin D1 promoter region CpG dinucleotides can be important for regulating cyclin D1 gene expression through US28. Our data suggest that US28 might contribute to an HCMV-induced hypomethylation status of infected cells. Further research on HCMV infection and epigenetic perturbations in (pre)cancerous settings will help to elucidate the role of viral (chemokine receptor) proteins like US28 in cancer development. Besides potential US28-mediated transcriptional upregulation via activation of transcription factors targeting the cyclin D1 gene, we demonstrated US28 is involved in epigenetic regulation via demethylation of cyclin D1 CpG islands.

5.4 Materials and Methods

Cell culture + inhibitor treatment. Mouse NIH-3T3 cell lines stably expressing US28, mutant US28-R^{129}A or no viral chemokine receptor (referred to as Mock in this paper), were maintained in DMEM supplemented with 10% bovine serum, penicillin/streptomycin and 300 µg/ml G418 as previously described [6]. In 6 well plate format 100.000 cells/well were incubated with Dnmt Inhibitor Decitabine (DAC, a.k.a. 5'-azadecitabine or 5-aza Sigma, 5 µM) which was replaced every 24 hours for 72 hrs, and/or Hdac inhibitor Trichostatin A (TSA, Sigma, 100 nM), which was added during the final 24 hrs of 72 hrs incubation [290]. The next day total cell lysates were harvested for protein analysis (WB) and cell pellets for RNA isolation (qPCR) or gDNA isolation for bisulfite conversion.

Methylation specific melting curve analysis (MS MCA) and Bisulfite Sequencing. Cyclin D1 DNA promoter methylation in US28-expressing NIH-3T3 cells was evaluated by methylation specific melting curve analysis (MS MCA, [291]) and bisulfite sequencing (BS). Therefore, genomic DNA was extracted from cells

using QIAamp DNA Mini kit (QIAgen, Inc.). Unmethylated and methylated genomic DNA from mice (Chemicon International, Inc.) was used as controls in MS MCA. After extraction 2 µg of DNA from cells and control DNA was used for bisulfite conversion treatment (EZ DNA Methylation kit, Zymo Research). MS MCA was performed to analyze the change in the methylation status of the CpG islands of the cyclin D1 gene during DAC treatment in cells. Primers used for MS MCA were manually designed and checked with Primer 3 software for annealing temperature and possible dimer formation. Primers were blasted in MethBLAST, available at http://medgen.ugent.be/methblast, and checked for specificity. MS MCA primers were selected for 3 specific promoter regions around the third CpG island (Primerset 1 forward, 5'-GGTGGGAATTAGGGATTGAAA-3', and reverse, 5'-TTCCCTCTCCTCACCCAAA-3'; 210 bp, Primerset 2 forward, 5'-GGAGGGGTTAGAGTTAGAGGTTT-3', and reverse, 5'-CCCTACCCCCACCTAACTAAA-3'; 202 bp, Primerset 3 forward, 5'-GGGTTTGGTGTTTTTATTGAAG-3', and reverse, 5'- TACTAAACCCTCCATAAAACCCCTA -3'; 318 bp,). PCR was performed in a reaction mix containing 50 ng bisulfite-converted genomic DNA. Amplifications were run on the MyiQ (BioRad), hot started with an initial denaturation step at 95°C for 3 minutes; the PCR profile were 35 cycles at 95°C for 20 seconds, 58°C for 30 seconds, and 72°C for 30 seconds; final extension was performed at 72°C for 2 minutes. Melting curves were obtained by increasing temperature with 0.2°C per 10 seconds from 65°C to 95°C and were analysed with the Bio-Rad iQ5 2.0 software. For BS PCR products were isolated from 1% agarose gel. To determine the methylation pattern of part of the CpG islands of the cyclin D1 gene promoter in US28-expressing cells, bisulfite treated DNA was sequenced using the same Primerset1 PCR primers.

qPCR. RNA isolation was performed with RNeasy kit (QIAgen), followed by Nanodrop measurement of RNA concentration and quality. 1 µg RNA was DNase (Fermentas) treated for 30 minute at 37°C and cDNA was synthesized (iScript kit Bio-Rad) 60 minute at 42°C. Primers used for qPCR validation were designed with Beacon Designer Software and validated on dilutions of cDNA. PCR was performed using specific primers for the cyclin D1 gene (forward, 5'-GGGATGTGAGGGAAGAGGTGAAG-3', and reverse, 5'-GATCCTGGGAGTCATCGGTAGC-3'; 87 bp) (5 µl) using SYBRGreen (10 µl), qPCR primers and 10-40x diluted cDNA (5 µl). Standard 2 step PCR + melting curve analysis was performed in the BioRad MyiQ. PCR cycles involved incubation at 95°C for 3 minutes; then 95°C for 10 seconds and 56°C for 30 seconds for 40 cycles; Melting curves were obtained by increasing temperature with 0.5°C from 65°C to 95°C for 10 seconds and were analysed with the Bio-Rad iQ5 2.0 software. Reference transcripts were

used as internal control as previously described [81]. Data were analysed using the ddCt method [292]. Statistical analysis of fold induction values was performed using GraphPad Prism software (San Diego, CA). One-way ANOVA was used for the comparison of more than two groups, followed by multiple testing correction (Dunnett's in Figure 4A, Bonferroni in Figure 4B).

Western Blot Analysis. NIH-3T3 mock, -US28 or -US28-R^{129}A-expressing cells were lysed in RIPA containing protease inhibitor cocktail α-complete (Roche), 1 mM PMSF, 1 mM $NaVO_4$ and 1 mM NaF. A BCA protein determination was performed for equal protein loading (Thermo Fisher Scientific, Rockford lL, USA). Standard SDS/PAGE on 12% gels was performed using BioRad minigel and electroblot systems (Hercules CA USA). Anti-CyclinD1 (Cell Signalling #2926 1:2000) and β-actin (1:10000) were used for protein detection.