University of Cyprus
Department of Biological Sciences

"Investigating the role of Methyl CpG Binding proteins in Cervical Cancer"

Eleftherios Patera

A Research-Based Master's Thesis

for the

Degree of Master of Science in Biomedical Sciences

Eleftherios Patera

Abstract:

DNA methylation (5mC) is one of the main epigenetic modifications that drive gene regulation. Methyl CpG binding proteins (MBDs) bind methylated DNA and recruit the nucleosome remodelling and deacetylase complex (Nurd) that promotes transcriptional repression. Exogenous and endogenous factors can disturb the Nurd mediated epigenetic regulation and lead to silencing of tumour suppressor genes or reactivation of oncogenes, which can give rise to carcinogenesis. Cervical cancer (CC) is the fourth most common cancer type in women and maintains a respectable mortality rate in developing countries. Human papilloma viruses (HPVs) have been identified as the main risk factor for development of CC through the expression of their E6 and E7 oncogenes. Recent studies suggest that E7 can affect the MBD subunit composition of the Nurd complex resulting in a switch from a MBD2-Nurd to MBD3-Nurd. Yet, MBD2 and MBD3 have not been characterized in the context of CC development. To this end, in the current study we aimed to knock down MBD2 and MBD3 in C33A, a cervical cancer cell line and investigate their role and impact in CC. Our results suggest that MBD3 is important for CC cells to maintain high proliferation. More than that, core reprogramming transcription factors Oct4 and Sox2 are under MBD regulation in C33A cells. Furthermore, MBD2 knock down gave significant rise to chromatin hydroxy methylation (5hmC). Finally, we conclude that MBD3 is essential for CC growth and loss of MBD2 impacts genomic hydroxy methylation status, which could lead to transcriptional deregulation.

Eleftherios Patera

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Eleftherios Patera

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Eleftherios Patera

Seminar Announcement

University of Cyprus
Department of Biological Sciences

Master Research Dissertation in Biomedical Sciences (BIO 830/600)

Student Presentation

Monday, 22 May 2023 at 10:30

Building Library, Room LRC014, Panepistimioupoli Campus

This seminar is open to the public

Eleftherios Patera

Thesis Supervisor: Assoc. Prof. Katerina Strati

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DNA methylation (5mC) is one of the main epigenetic modifications that drive gene regulation. Methyl CpG binding proteins (MBDs), bind methylated DNA and recruit the nucleosome remodelling and deacetylase complex (Nurd) that promotes transcriptional repression. Exogenous and endogenous factors can disturb the Nurd mediated epigenetic regulation and lead to silencing of tumour suppressor genes or reactivation of oncogenes, which can give rise to carcinogenesis. Cervical cancer (CC) is the fourth most common cancer type in women and maintains a respectable mortality rate in developing countries. Human papilloma viruses (HPVs) have been identified as the main risk factor for development of CC through the expression of their E6 and E7 oncogenes. Recent studies suggest that E7 can affect the MBD subunit composition of the Nurd complex resulting a switch from a MBD2-Nurd to MBD3-Nurd. Yet, MBD2 and MBD3 have not been characterized in the context of CC development. To this end, in this study we aimed to knock down MBD2 and MBD3 in C33A, a cervical cancer cell line and investigate their role in CC. Our results suggest that MBD3 is important for CC cells to maintain high proliferation. More than that, core reprogramming transcription factors Oct4 and Sox2 are under MBD regulation in C33A cells. Furthermore, MBD2 knock down gave significant rise to chromatin hydroxy methylation (5hmC). Finally, we conclude that MBD3 is essential for CC growth and loss of MBD2 impacts genomic hydroxy methylation status which could lead to transcriptional deregulation.

Eleftherios Patera

Table of Contents

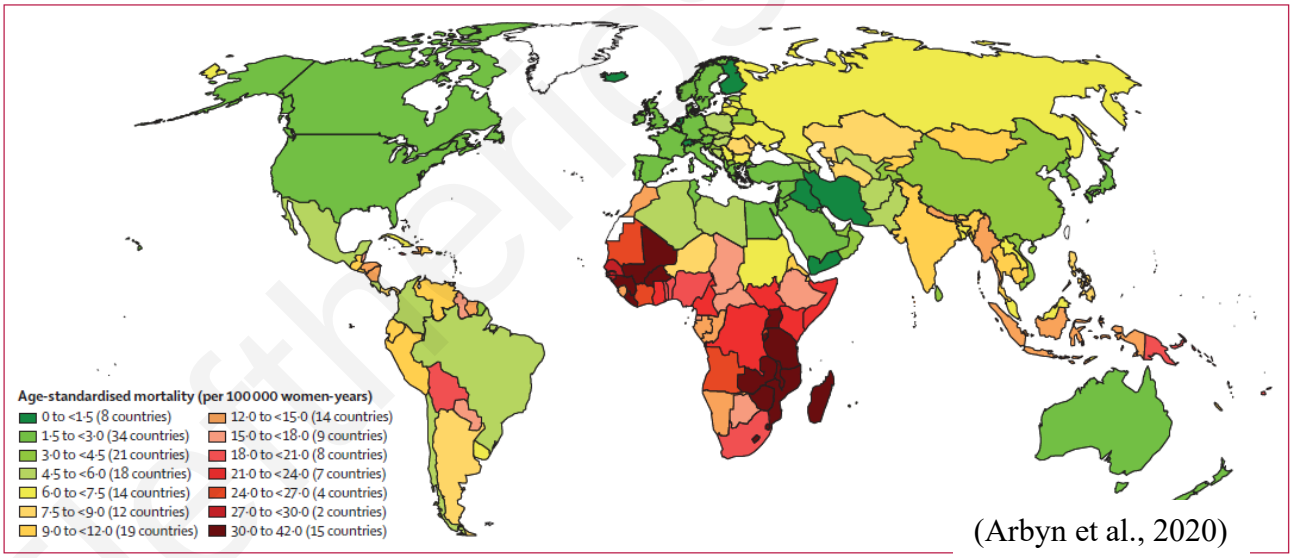
Eleftherios Patera

Eleftherios Patera

Introduction:

Cervical Cancer.

Cervical Cancer (CC) has the fourth largest incident rate in women globally, affecting an average of 13 out of 100,000 women worldwide (Arbyn et al., 2020). The majority of cervical cancers are squamous cell carcinomas (74.8%), although adenocarcinomas (18.3%) and adenosquamous carcinomas (1.7%) can also develop in the cervix (Lee et al., 2020). Cervical carcinomas are developed from initial cervical intraepithelial neoplasia (CIN) lesions, which progress gradually in the span of 10-20 years from a low-grade lesion (CIN1 & CIN2) to high grade lesion (CIN3) and invasive carcinoma (Martin & O'Leary, 2011). Human papillomavirus (HPV) has been identified as the main causative agent of cervical cancer and it is present in 99.7% of cervical cancer cases globally (Walboomers et al., 1999). The link of cervical cancer to an infectious agent such as HPV, as well as the ease of access to the cervix, has given rise to a variety of prevention strategies that include HPV vaccinations



(Arbyn et al., 2020)

Figure 1: Global map of cervical cancer mortality rates in 2018.

Cervical cancer mortality rates of 2018 were retrieved from World health organization (WHO) mortality database and represented in a colour coded Global map. On the bottom left the legend indicates the correlation of colours to mortality rates from low mortality rates designated to green colour to high mortality rates designated to dark red. Developed countries reported 0-4.5 deaths per 100,000 women, whereas developing countries reach as high as 42 deaths per 100000 women.

Elisthenis Patera

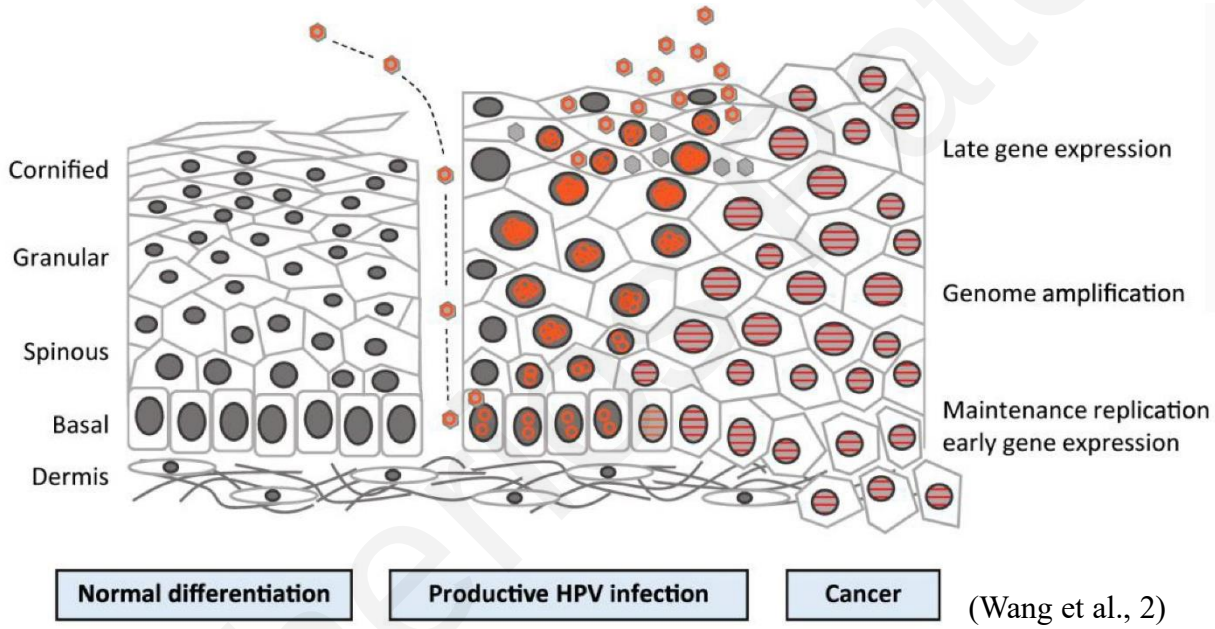
and screening methods. Early detection of cervical cancer with Pap smear screening, since 1940s has helped to reduce the mortality rate by approximately 80% (Shingleton et al., 1995). Additionally, with the development of the first HPV vaccine in 2006, young women can become immunized against various types of HPVs which can limit approximately 90% of cervical cancer formation (Muñoz et al., 2010, de Sanjosé et al., 2018). Yet, in 2018 over 300,000 cervical cancer caused deaths were reported globally, and mortality rates remain high (15-42 per 100,000 women) in developing countries (Figure 1) (Arbyn et al., 2020). Therefore, further understanding of cervical cancer is needed, with the aim to limit incident rates and develop successful treatments.

Oncogenic Human Papilloma Viruses:

Human Papillomaviruses (HPVs) are small viruses with approximately 8kbp long double stranded circular DNA concealed in a non-enveloped capsid structured by L1 and L2 viral proteins (Buck et al., 2013, Wang & Roden, 2013). Based on the L1 gene sequence, HPVs are divided into Alpha, Beta, Gamma, Mu, and Nu genera (de Villiers et al., 2004). Alpha HPVs infect mucosal and cutaneous epithelia and are further categorised into high risk and low risk HPVs according to their oncogenicity. High risk HPV16 and HPV18 alone are linked to at least 70% of all cervical cancer cases (Castellsagué, 2008). HPV genes are classified into early and late genes depending on their expression pattern during HPVs infectious cycle. Early genes E1 and E2 are used for viral genome replication, E4, E5, E6 and E7 are responsible for both immune evasion and promotion of cellular proliferation, and last, late proteins L1 and L2 are the main components of virion formation (McBride, 2017). Their life cycle starts by infecting cells of the basal layer of the cervical epithelium that was exposed through micro-wounds. Then HPV utilizes the differentiation of the initially infected cells to move to the upper layer of the epithelia changing its gene expression profile accordingly (Figure 2). On the lower layers HPV expresses the early genes necessary to replicate its genome while in the upper layers late genes are expressed to achieve virion assembly and release for re-infection (Figure 2). During its infectious cycle, utilizing two oncoproteins, E6 and E7, HPV actively induces increased cell proliferation to gain access to the molecular machinery of the cell for DNA replication. E7 binds and inhibits pRb, thus promoting proliferation (Syrjänen & Syrjänen, 1999), while E6 binds and promotes the degradation of p53 and therefore eliminates from the already over-proliferative cells the option of apoptosis

Eleftherios Patera

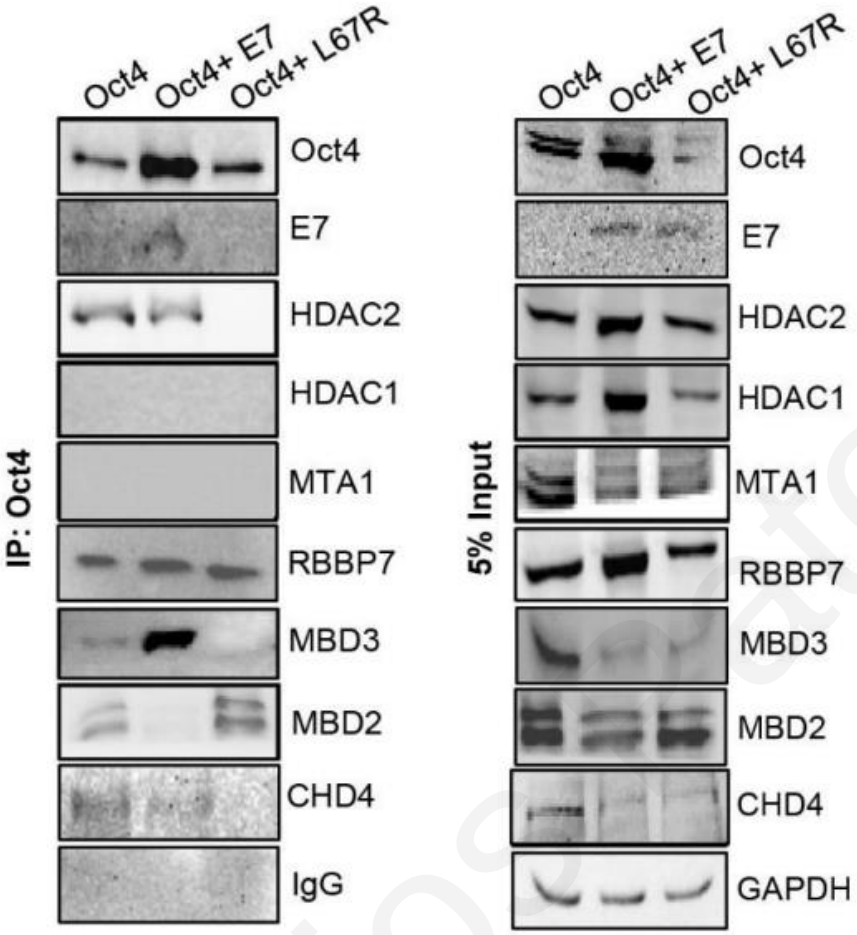
(Thomas et al., 1999). The collective effect of E6 and E7 in persistent infection results in the initiation, progression and maintenance of carcinogenesis (Riley et al., 2003, Jabbar et al., 2012). A novel function of E7 was uncovered in the last years inducing the expression of pluripotency marker Oct4 (Panayiotou et al., 2020). Additionally, unpublished data from our laboratory reveal that Oct4 interacts with MBD2, a component of the NuRD complex, in the absence of E7, and interacts with MBD3, an alternative component of NuRD complex, in the presence of E7 (Figure 3) (Panayiotou et al., submitted). This could potentially unravel a new role of E7 in cervical cancer through NuRD variant complex.



(Wang et al., 2)

Figure 2: Human Papilloma Virus infection cycle in stratified epithelia.

Schematic representation of Human Papilloma Virus (HPV) infection cycle. Cycle progression in time is visualised horizontally starting with a healthy stratified epithelia on the far left and ending in invasive cervical carcinoma on the far right. Horizontally the different epithelial layers are labelled along with associated viral expression pattern. HPV virions find access to the basal cells, through micro wounds, which they infect. The infected cells (labelled in the figure with red colorised nuclei) replicate and differentiate into cells of higher epithelial layers. HPV utilizes the cell differentiation to drive different viral gene expression patterns (Early gene expression, Genome amplification, Late gene expression) and once in the outer layers, virion particles formed of L1 and L2 are ready for assembly. Released virions are then ready to establish infection in neighbouring tissues or in different individual.



(Panayiotou et al., submitted)

Figure 3: E7 alters the NuRD subunits interactions with Oct4 in C33A.

Unpublished immunoprecipitation (IP) data from Dr Panayiotou. Lysates from C33A cells with Oct4 alone, Oct4 + Wild type E7 or Oct4 + Mutant E7 (L67R) were used. IP was performed using an Oct4 antibody to pull down Oct4 and its interactors. On the left panels the IP blots and on the right panels blots form 5% of input lysates are presented with GAPDH as loading control. Oct4 with the absence of wild type E7 interacts with an MBD2-NuRD while Oct4 in the presence of E7 interacts with MBD3-NuRD.

NuRD: Histone Deacetylase and Remodelling Complex

Nucleosome remodelling and deacetylase (NuRD) complex was first characterised in the late 90s and was associated with transcriptional repression (Tong et al., 1998, Wade et al., 1998, Xue et al., 1998, Zhang et al., 1998). The complex is comprised of two enzymatic subunits, histone deacetylase (HDAC1/2) and a chromodomain helicase DNA binding protein (CHD3/4/5), as well as some non-enzymatic subunits, metastasis tumour associated (MTA1/2/3), retinoblastoma binding protein (RBBP4/7), cyclin-dependent kinase 2

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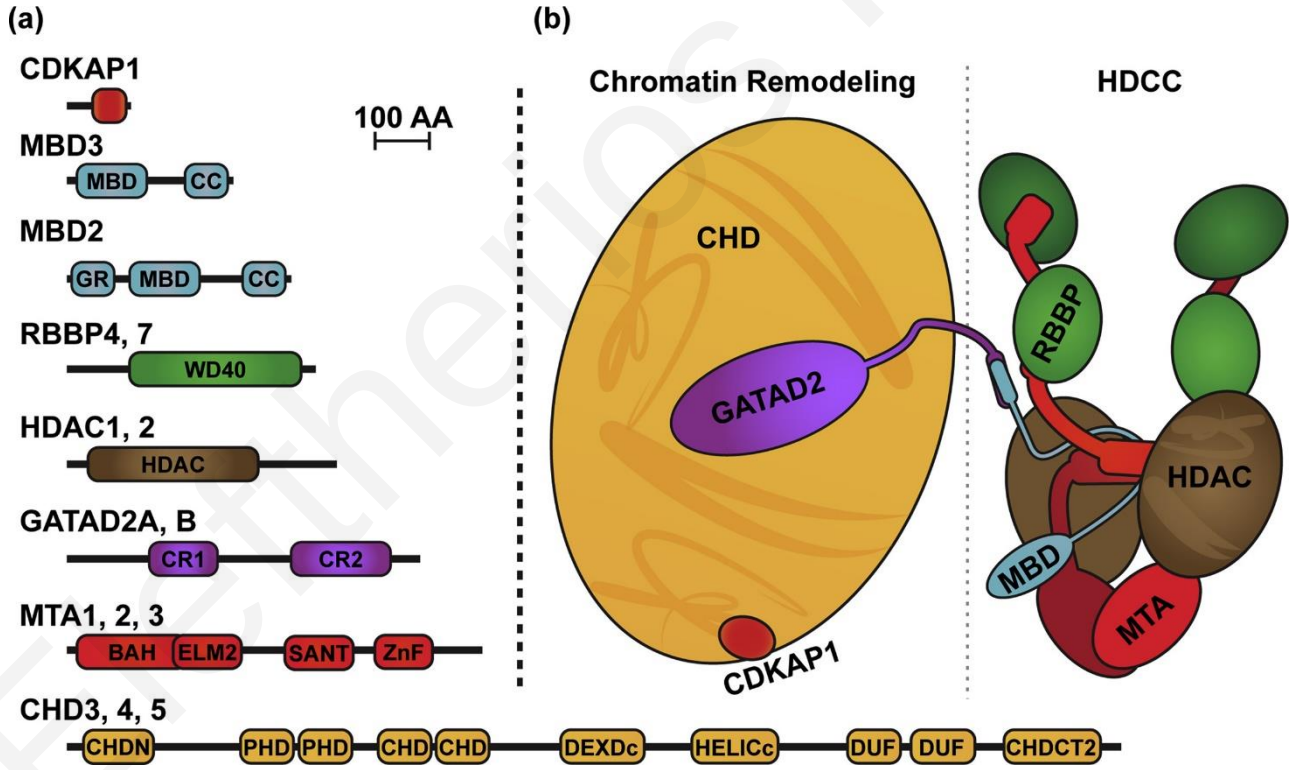
associated protein 1 (CDK2AP1), GATAD2A/2B, and methyl CpG binding domain proteins (MBD2/3) (Figure 4) (Zhang et al., 1999, Xue et al., 1998). Although at first NuRD was associated with transcriptional repression, its target genes and function vary regarding to its different composition of subunits. In particular, MBD2 and MBD3 are mutually exclusive and tether NuRD to different genomic sites (Le Guezennec et al., 2006). Additionally, studies show that NuRD can deviate from its transcriptional repression role and be also used by the cell to fine tune transcription of active genes in embryonic stem cells (Bornelöv et al., 2018). NuRD subunit, MTA1, has been associated with poor prognosis and reported to be overexpressed in various cancer types, including but not limited to breast, colorectal and prostate cancer (Nicolson et al., 2003). Moreover, hypermethylation, through NuRD, can lead to transcriptional silencing of tumour suppressors such as INK4A, RB1 and BRCA1, thus promoting carcinogenesis (McCabe et al., 2009). Consequently, comprehensive understanding of Nurd functions and targets in carcinogenesis is of great importance.



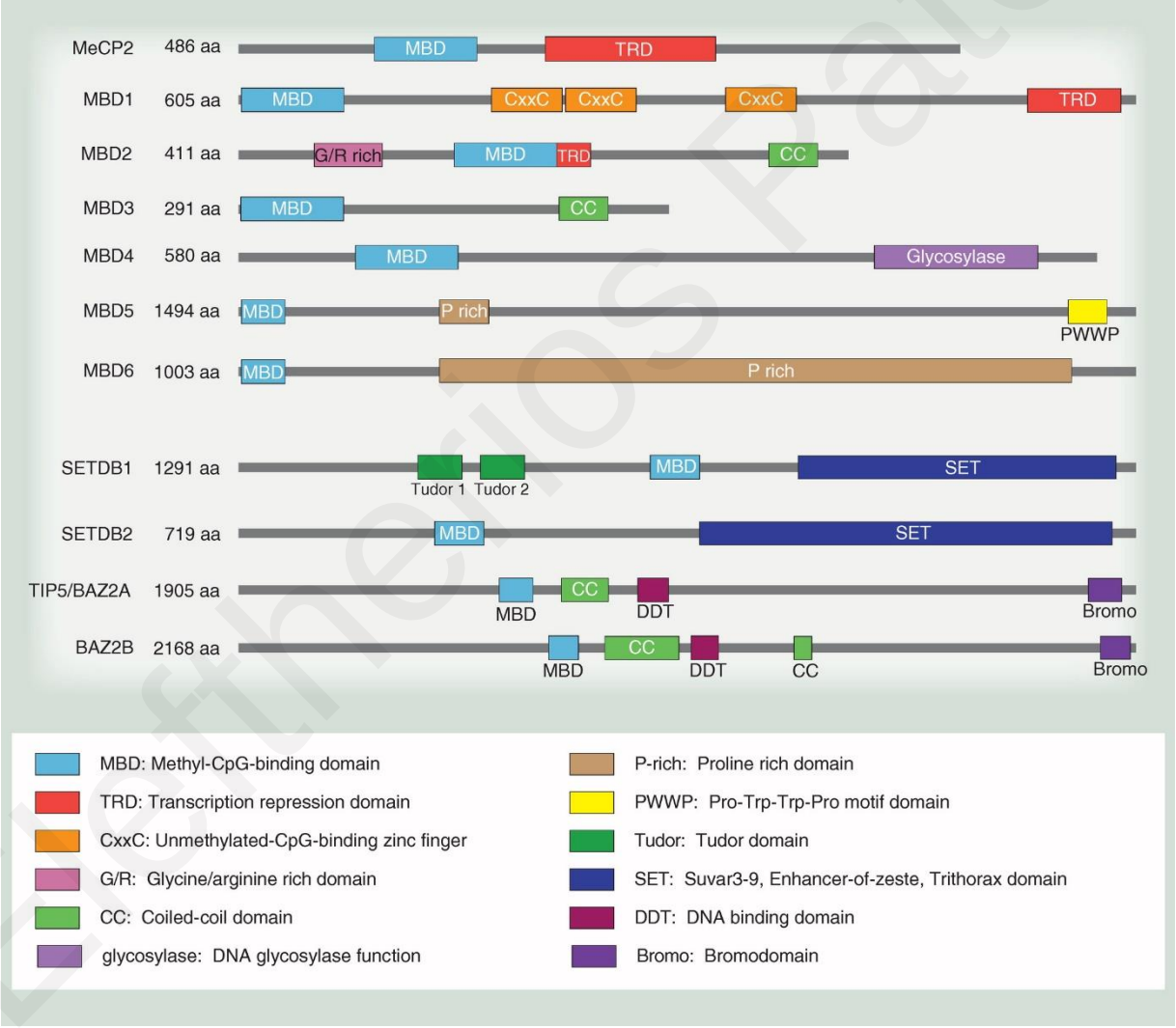
(Leighton & Williams, 2020)

Figure 4: The Nucleosome Remodelling and Deacetylase (NuRD) complex.

A list of the core Nucleosome Remodelling and Deacetylase (NuRD) complex subunits and their individual domains is depicted on the left (A). On the right NuRD is represented in a simplified schematic showing how its subunits interact with each other to form the complex (B).

Methyl CpG Binding Proteins 2&3:

Methylated CpGs are two symmetrical cytosines (5-mCs), methylated by a DNA methyltransferase (DNMT), and can exist in the genome either isolated or highly dense in small genomic regions called CpG islands (Bestor et al., 1988, Gardiner-Garden & Frommer, 1987). MBD2 and MBD3 belong to a broader family of methyl CpG binding proteins that were first characterized in the 90s (Figure 5) (Hendrich & Bird, 1998). MBD proteins are characterised by their ability to bind methylated CpGs through their 85 amino acid MBD domain (Nan et al., 1993, Ohki et al., 2001). MBD2 also consists of a transcriptional repression domain (TRD), a C-terminal coiled coil domain (CC) and an N-terminal glycine-



(Du et al., 2015)

Figure 5: Methyl CpG Binding (MBD) protein family.

A list of proteins that include the Methyl CpG Binding domain (MBD-domain). Additional domains of interest are included, and colour coded according to the legend below.

Eleftherios Patera

arginine (GR) repeat domain (Figure 5) (Boeke et al., 2000, Gnanapragasam et al., 2011). MBD3 is 71% homologous to MBD2, consisting of the same domains except the N-terminal GR-repeat (Figure 5) (Hendrich & Bird, 1998). MBD3 can also bind hydroxy-methylated or non-methylated CpGs because of two amino acid variation in its MBD domain, replacing LYS30 with HIS30 and TYR34 to PHE34 (Saito & Ishikawa, 2002, Yildirim et al., 2011). The binding affinity of methylated CpGs is also different between MBD2 and MBD3. MBD2 exhibits more tighter binding to methylated CpGs whereas MBD3 exerts lower affinity and is rather dynamically diffused in methylates CpGs (Leighton et al., 2022). Both MBD2 and MBD3 are expressed in somatic tissues, and in embryonic cells although MBD2 expression is significantly lower indicating that MBD3 is more important in early development (Hendrich & Bird, 1998, Ohki et al., 2001). This becomes more apparent in the case of MBD knock-outs (KO), MBD2 KO in mice are not lethal whereas MBD3 KO in mice are embryonically lethal (Hendrich et al., 2001). A role they have in common is the regulation of pluripotency through regulating the expression of key reprogramming transcription factors (Kaji et al., 2006, Lu et al., 2014, Markov et al., 2021). MBD2 and MBD3 have been previously associated with carcinogenesis, for example colon, breast and glioblastoma cancer, but their role in cervical cancer remains unknown (Cai et al., 2013, Billard et al., 2002, Zhu et al., 2011).

Significance:

Although screening programs are established, and HPV vaccines are available, cervical cancer remains a burden in developing countries (Arbyn et al., 2020. Previous work from our lab has demonstrated that MBD2 subunit of NuRD complex is replaced by MBD3 in the presence of the oncoprotein E7 (Theofano et al., submitted). To this end, in the current study, we aimed to generate MBD2 and MBD3 knock down C33A cell lines and investigate the role of MBD2/3 in cervical cancer. Our data reveal broad chromatin modification alternations accompanied with decreased expression of core stem cell related genes in our MBD2/3 knock down cell lines. Notably knock down of MBD3 and to a lesser extent MBD2 resulted in cellular growth defect in cervical cancer cells. Current cervical cancer treatments include radical surgery and chemotherapy, both very invasive methods that yet yield a not satisfying, 3 to 5 year, survival rate (Landoni et al., 1997, Morris et al., 1999, Sankaranarayanan et al., 2010). Considering that MBD proteins are affecting gene expression through epigenetic

Eleftherios Patera

regulation, which is reversible, our findings can potentially reveal novel therapeutic targets in cervical cancer and thus pave the way for a better and more optimistic treatment regime.

Materials and Methods

Materials:

Plasmids

Table 1: Transduction Plasmids used.

Name	Usage	Catalogue Number
pSMP-Luc	Knock Down Control	Addgene #36394
pSMP-MBD2-1	MBD2 Knock Down	Addgene #36368
pSMP-MBD2-2	MBD2 Knock Down	Addgene #36369
pSMP-MBD3_1	MBD3 Knock Down	Addgene #36371
pSMP-MBD3_2	MBD3 Knock Down	Addgene #36372

Antibodies

Table 2: Western Blot and Dot Blot Antibodies.

Antibody	Dilution	Catalogue Number
GAPDH	1:1000	Abcam (ab9484)
OCT4	1:800	CST (2750S)
MBD2	1:1000	Abcam (ab188474)
MBD3	1:7500	CST (14540)
5hmC	5:10000	Active Motif (39769)

Eleftherios Patera

Primers

Table 3: RT-PCR Primers used.

Primer's Target	Forward Primer Sequence	Reverse Primer Sequence
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
MBD2	TTCAAGGAGTTGGTCCAGGTAG	GCAGGGTTCTTTTCCACAGC
MBD3	CGTATGGCTCAACACCACG	TCCTGCTTCCTGATGTCCTC
Oct4	GGCATGGACTGTGGTCATGAG	GTGAAGTGAGGGCTCCCATA
Nanog	CCTGTGATTTGTGGGCCTGA	CTCTGCAGAAGTGGGTTGTTTG
Sox2	CGCCCCCAGGGGCAGCAGACTTCACA	CTCCTCTTTTGCACCCCTCCCATTT
Klf4	GAAATTCGCCCGCTCAGATGATGAACT	TCTTCATGTGTAGTAAC GAGGCGAGGTGGT

Methods:

Cell Culture

Cells were always handled under sterile conditions and were cultured in 100mm tissue culture plates in a binder incubator at 37°C, 5% CO_2. 293T cells were grown in DMEM media (Invitrogen #41965062) supplemented with 10% FBS (Invitrogen #10500064) and 1% Penicillin/Streptomycin (Invitrogen #15070063), C33A cells were grown in MEM media (Invitrogen #21090055) supplemented with 10% FBS, 1% L-Glutamine (Invitrogen #25030024) and 1% Penicillin/Streptomycin. Knock down cell lines were grown similar to C33A cells with the addition of 2µg/mL Puromycin (Invitrogen #A1113803). Cells were daily observed under microscope to identify potential contaminations as well as to monitor their growth and were routinely examined for Mycoplasma every two months. When reached approximately 90% confluency, cells were washed once with PBS, detached from their plates using 0.05% Trypsin-EDTA (Invitrogen #25300062) diluted with the appropriate media to be split into multiple plates. When cells were not needed to be in culture they were detached similarly as the splitting procedure, were then centrifuged at 1200 x g for 10 minutes at 4°C, the pellet was resuspended in cryovials with 1mL of the appropriate media supplemented

Eleftherios Patera

with 5% Dimethyl Sulfoxide (DMSO) and were stored short term in -80°C freezer or long term in liquid nitrogen.

Bacterial Culture & plasmid isolation

Near a flame 100ul of bacterial glycerol stock was added to 200mL LB broth and was incubated at 37°C while shaking for 18hours. Liquid bacterial colonies were loaded into 50mL tubes and were centrifuged at 6000 x g for 15 min at 4°C. Supernatant was removed and the bacterial pellets were used for plasmid isolation using Qiagen Midi Kit (Qiagen #12143). DNA concentration was measured using nanodrop 2000c.

DNA digestion

Isolated plasmids (pSMP-MBD2-1, pSMP-MBD2-2, pSMP-MBD3_1, pSMP-MBD3_2) were used for DNA digestion for verification before used in experiments. DNA digestion reactions were set up containing 1µg of DNA, 1ul of restriction enzyme (ClaI, HindIII, or both), and 2ul of the 10x M buffer (Takara # 1034A, # 1060A) and H2O to max volume of 20ul. The mixture was incubated at 37°C for 1 hour to allow the enzymatic activity of the restriction enzymes and then was incubated at 65°C to inactivate the enzymes. 2ul of loading dye was added and the digested DNA, 1µg of non-digested DNA and 5ul of ladder (Nippon # MWD1) was loaded to 1% agarose gel for electrophoresis. Electrophoresis was run at 120V until the DNA bands were separated enough and then it was imaged at G-Box (SYNGENE, model Chemi xR 5).

Transduction

Approximately $1x10^6$ 293T cells were seeded in 100mm tissue culture plates. After 24 hours using Fugene transfection reagent (Promega #E2691) 293T cells were co-transfected with the retroviral vectors (1µg VSV.G, 3µg pUMVC) and 4µg of the appropriate shRNA delivery plasmids (pSMP-Luc, pSMP-MBD2-1, pSMP-MBD2-2, pSMP-MBD3_1, pSMP-MBD3_2). 48 hours post transfection the retrovirus containing media from the 293T cells was collected and filtered through 0.45µm filters, the media was replenished and collected again after 48 hours before safely discarding the cells (all consumables used were bleached before throwing

Eleftherios Patera

away to neutralize any viral particles). Approximately 1x10^5 C33A cells were seeded in six wells and after 48 hours their media was removed and exchanged with the retrovirus containing media that was previously collected supplemented with 1:1000 Polybrene (SIGMA # TR-1003-G). The cells that received the transduction were selected 48 hours post transduction using a 2µg/mL Puromycin supplemented media in which they were kept for future experiments to restrict the growing of cells without the desirable knock down.

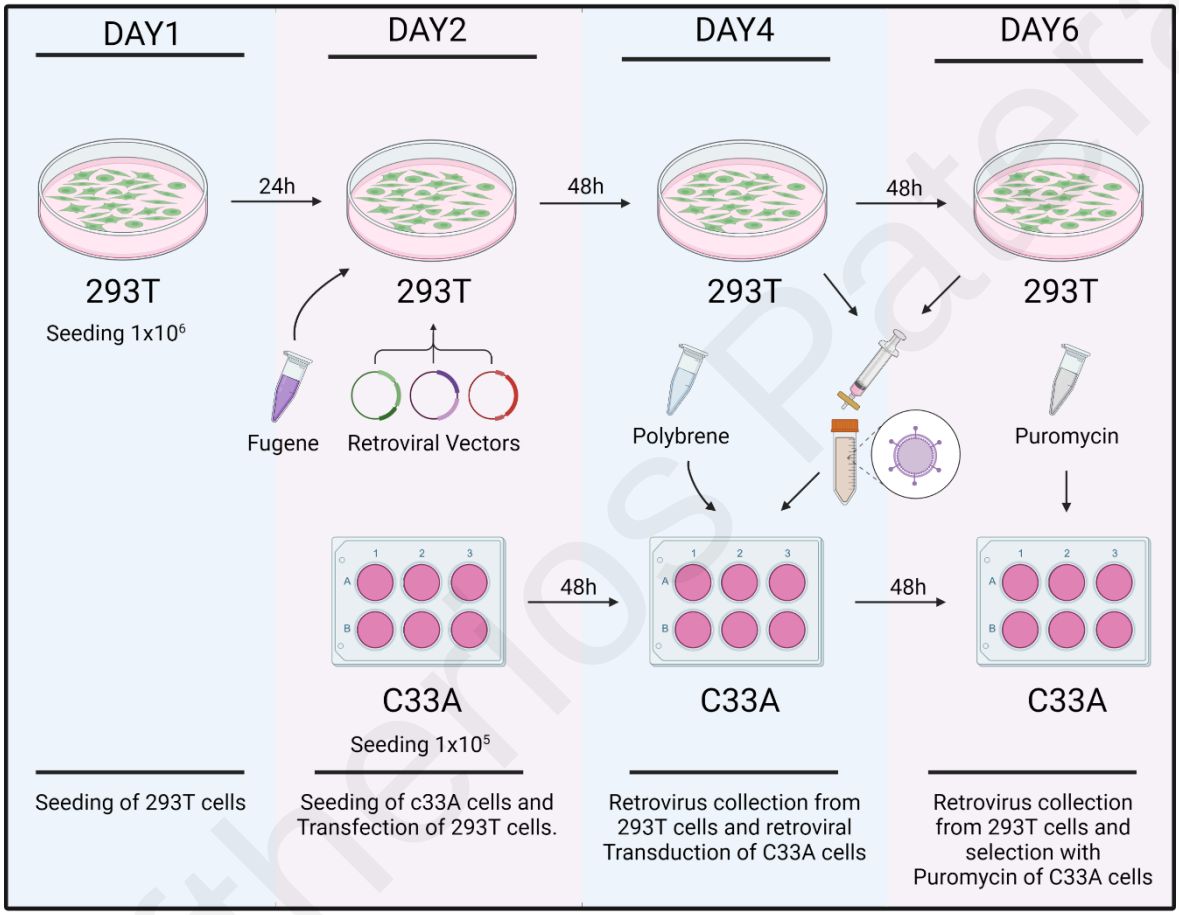


Figure 6: Schematic representation of the Transduction protocol.

Western blot

Cell pellets from a 100mm tissue culture plate were resuspended in 500ul RIPA buffer (150mM NaCl, 5mM EDTA, 50mM Tris-HCL, 1% TritonX-100, 0.1% SDS and 0.5% Sodium Deoxycholate) supplemented with one tablet per 10ml of proteinase inhibitors (ThermoFisher #A32955). The lysates were centrifuges at low speed for 40 minutes at 4°C and then at 13000rpm for 20 minutes at 4°C. Supernatant was placed on new 1.5ml tubes and the protein concentration was identified using Nanodrop. Protein samples and 5ul of protein ladder (BioRad #1610375) were loaded in 10% SDS-PAGE gel for electrophoresis.

Eleftherios Patera

Electrophoresis was performed at 180V until ladder was indicated a sufficient separation of proteins by their molecular weight. Proteins were transferred from the SDS-PAGE to a nitrocellulose membrane (SIGMA #10600007) using a semi-dry transfer apparatus at 20V for 20 minutes. Nitrocellulose membrane was washed thrice with 10mL of TBST for 5 minutes and then blocked with 10mL of 5% BSA (SIGMA #A9647) for 1 hour at room temperature. Following blocking, nitrocellulose membranes were incubated with 10mL of primary antibodies diluted in 5% BSA (SIGMA # A9647-100G) overnight at 4°C. Nitrocellulose membrane was washed thrice with 10mL of TBST for 5 minutes and then incubated in 10mL of secondary HRP-conjugated antibodies diluted in 5% BSA for 1 hour at room temperature. Nitrocellulose membrane was washed thrice with 10mL of TBST for 5 minutes and then imaged in G-Box using ECL (ThermoFisher #WP20005).

Dot Blot

Cell pellets from two 100mm tissue culture plates were used for DNA extraction using the Qiagen Blood and Tissue kit (Qiagen # 69504). DNA concentration was then measured using Nano drop and different dilutions of DNA were prepared for the Dot blot. DNA was denatured at 95°C for 10 minutes and 1.5uL was loaded onto a nitrocellulose membrane. Membranes were blocked using 10mL 5% BSA for 1 hour at room temperature, and then were incubated with the primary antibodies for 1 hour at room temperature. The membranes were then washed thrice with 10mL TBST and then the HRP-conjugated secondary antibodies were added and incubated for 1 hour at room temperature. After washing thrice with 10mL TBS the membranes were imaged at G-box using ECL reagents.

RNA extraction

Cell pellets collected from a 100mm cell culture plates were first resuspended in 1ml Trizol (Invitrogen #15596026) before adding 200ul Chloroform (SIGMA #1024452500). The samples were shaken vigorously and then centrifuged at 12,000 x g for 10 minutes at 4°C. Aqueous phase was separated, 500ul Isopropanol was added and the mixture was vortexed and then incubated for 5 minutes on ice to allow RNA precipitation. After centrifugation at 12,000 x g for 10 minutes at 4°C the supernatant was removed, and the RNA pellet was washed with 500ul of 70% ethanol. RNA was then centrifuged at 12,000 x g for 1 minute at

Eleftherios Patera

4°C, supernatant was removed, and after a brief air dry, the RNA pellet was resuspended in 80ul of DEPC treated H2O. Samples were incubated at 60°C for 10 minutes to dissolve the RNA. Extracted RNA sample concentrations were measured on NanoDrop and stored in -80°C freezer.

cDNA Synthesis and qPCR

Reverse transcription PCR (RT-PCR) was set up using 400ng of RNA, 1ul of iScript Reverse Transcriptase (BioRad #1708891), 4ul of 5x iScript Reaction Mix (BioRad #1708891), adjusted to final volume of 20ul with DEPC treated H2O (Invitrogen #AM9916). Thermocycler incubation was followed as stated in the iScript cDNA synthesis kit protocol. Quantitative PCR (q-PCR) was performed using 1ul of cDNA sample, 5ul KAPA SYBR FAST qPCR Master Mix (2X) (SIGMA #KK4618), 1ul primer mix and 3ul DEPC treaded H2O. Each sample in q-PCR was repeated in triplicates and the relative gene expression was normalised on GAPDH.

Statistical Analysis

All data was analysed using the GaphPad Prism. Paired t-test was performed to acquire p value and statistical significance was achieved at p value less than 0.05.

Eleftherios Patera

Results:

Generation of MBD2 and MBD3 Knock Down C33A cell lines.

To investigate the role of MBD2 and MBD3 in cervical cancer first we aimed to generate MBD2 and MBD3 knockdown C33A cell lines. We initially sought to verify the small hairpin RNA knockdown plasmids (pSMP-MBD2-1, pSMP-MBD2-2, pSMP-MBD3_1, pSMP-MBD3_2) before using them to generate knockdown (KD) cell lines. All four plasmids were generated from George Q Daley's laboratory (Onder et al., 2012) using the same vectors which resulted in plasmids of the length of 6699bp with identical restriction sites. Restriction using both ClaI and HindIII resulted in two fragments of sizes 6036bp and 663bp (**Figure 7A & 7B**) as expected and single digestion with either ClaI or HindIII resulted in a single linear fragment of 6699bp (Figure 1A & 1B), thus confirming the identity of the plasmids. To deliver the shRNA plasmids in C33As, we utilized a retrovirus transduction protocol (summarized in **Figure 7C**). As a negative control we utilized a plasmid expressing a luciferase shRNA which has no target in a normal cell. The resulting MBD2 and MBD3 KD C33A cell lines were kept under puromycin selection indefinitely whilst in culture. Knock down efficiency was assessed both on mRNA and protein level (**Figure 8**). MBD2 mRNA levels were significantly reduced with sh1, with an approximate 53% knock down (**Figure 8A**). Surprisingly, sh2 resulted in an almost two-fold overexpression of MBD2 (**Figure 8A**). MBD3 exhibited an approximate 25% reduction of mRNA with sh2, which is statistically significant, but the biological significance is in doubt (**Figure 8B**). MBD3 mRNA expression had no significant change with sh2. The only significant change in protein expression was observed in the significant reduction of MBD2 levels in sh1 knock down C33As. MBD2 sh2 and both sh RNAs of MBD3 did not achieve any significant change in protein expression. Consequently, further experiments utilized only the MBD2 sh1 and MBD3 sh1 KD C33A cell lines.

Eleftherios Patera

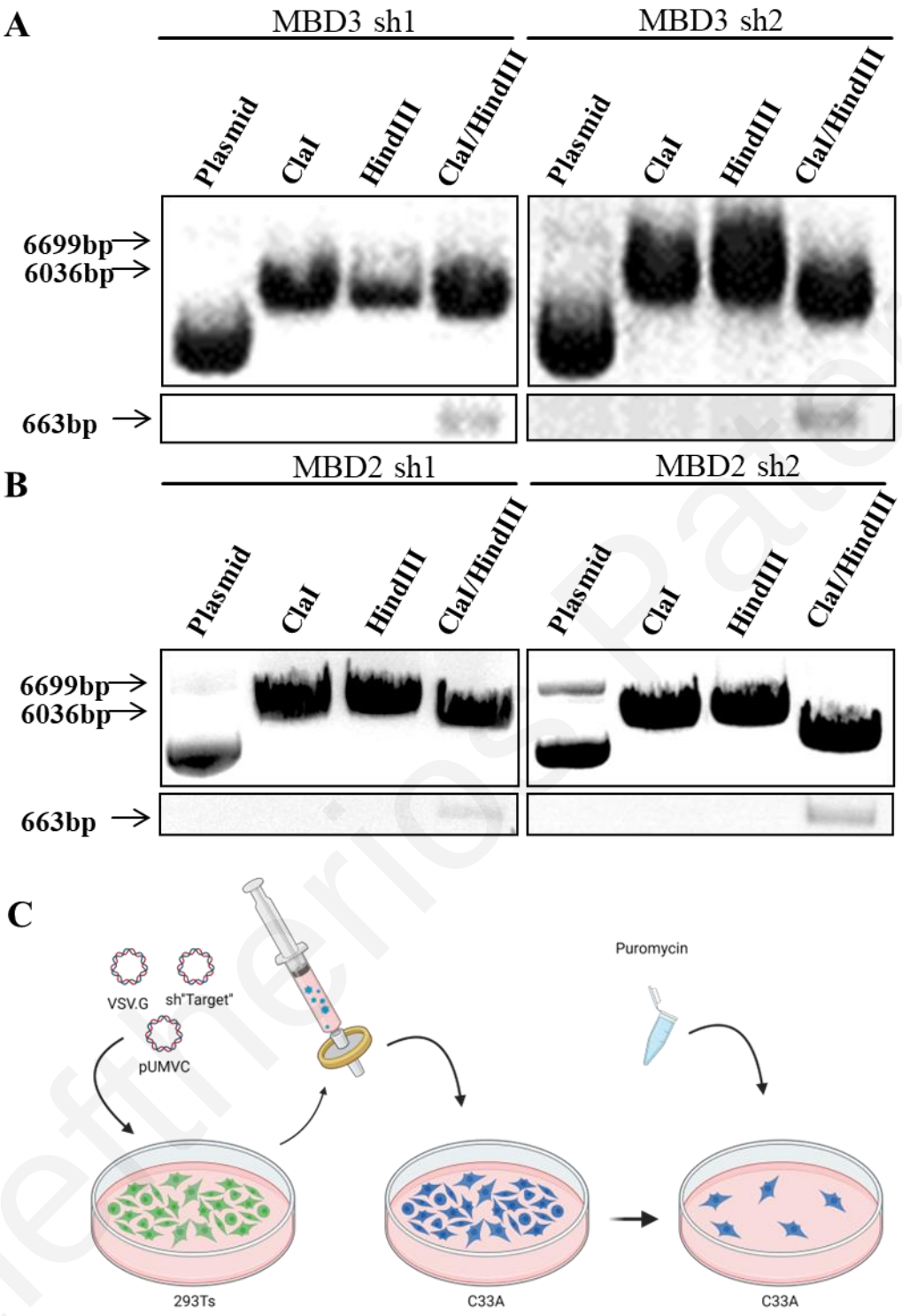


Figure 7: Verification of Knock down plasmids and Transduction set up. The knock down plasmids pSMP-MBD2-1, pSMP-MBD2-2, pSMP-MBD3_1, pSMP-MBD3_2, have been tested with DNA digestion before further usage. Knock down plasmids were at the predicted 6699bp molecular weight when single digested with ClaI or HindIII and produced

two fragments (6036bp and 663bp) when double digested with both ClaI and HindIII **(A, B)**. Schematic representation of the experimental procedure followed during lentiviral transduction **(C)**.

Investigation of cell proliferation changes upon MBD2/MBD3 knock down.

After generating the MBD2 and MBD3 knockdown cell lines, first we wanted to address any changes in cell proliferation and survival. We seeded $1x10^5$ cells in 6 wells and monitored their growth in the span of 5 days (**Figure 9**). We observed that both MBD2 and MBD3 knock down C33A cell lines showed significantly reduced cell numbers on the fifth day (**Figure 9A, 9B**). Additionally, normalising the cell numbers to the control cells show that the relative cell numbers of MBD2 knock down C33As have no statistically significant change (**Figure 9C**), but strikingly, the relative cell numbers of MBD3 knock down C33As showed statistically significant reduction throughout the experiment starting as soon as day one post seeding (**Figure 9D**). Data is not enough to distinguish whether the lower relative cell numbers are a result of decreased proliferation, increased cell death, or both.

Expression changes of Reprogramming and Stem Cell transcription factors.

Since both MBD2 and MBD3 have been previously shown to be important during cell reprogramming by regulating core reprogramming transcription factors (Markov et al., 2021/, Lu et al., 2014) we wanted to see the effect of MBD2 and MBD3 knockdown in C33A in the expression of such transcription factors. To this end, we tested the mRNA levels of Nanog, Sox2, Klf4, and Oct4 using qPCR (**Figure 10**). To our surprise, MBD2 knock down in C33As resulted in statistically significant reduction of Oct4 mRNA levels (**Figure 10A**). Additionally, Sox2 mRNA expression was also significantly reduced in MBD3 knock down C33A cells (**Figure 10B**).

Eleftherios Patera

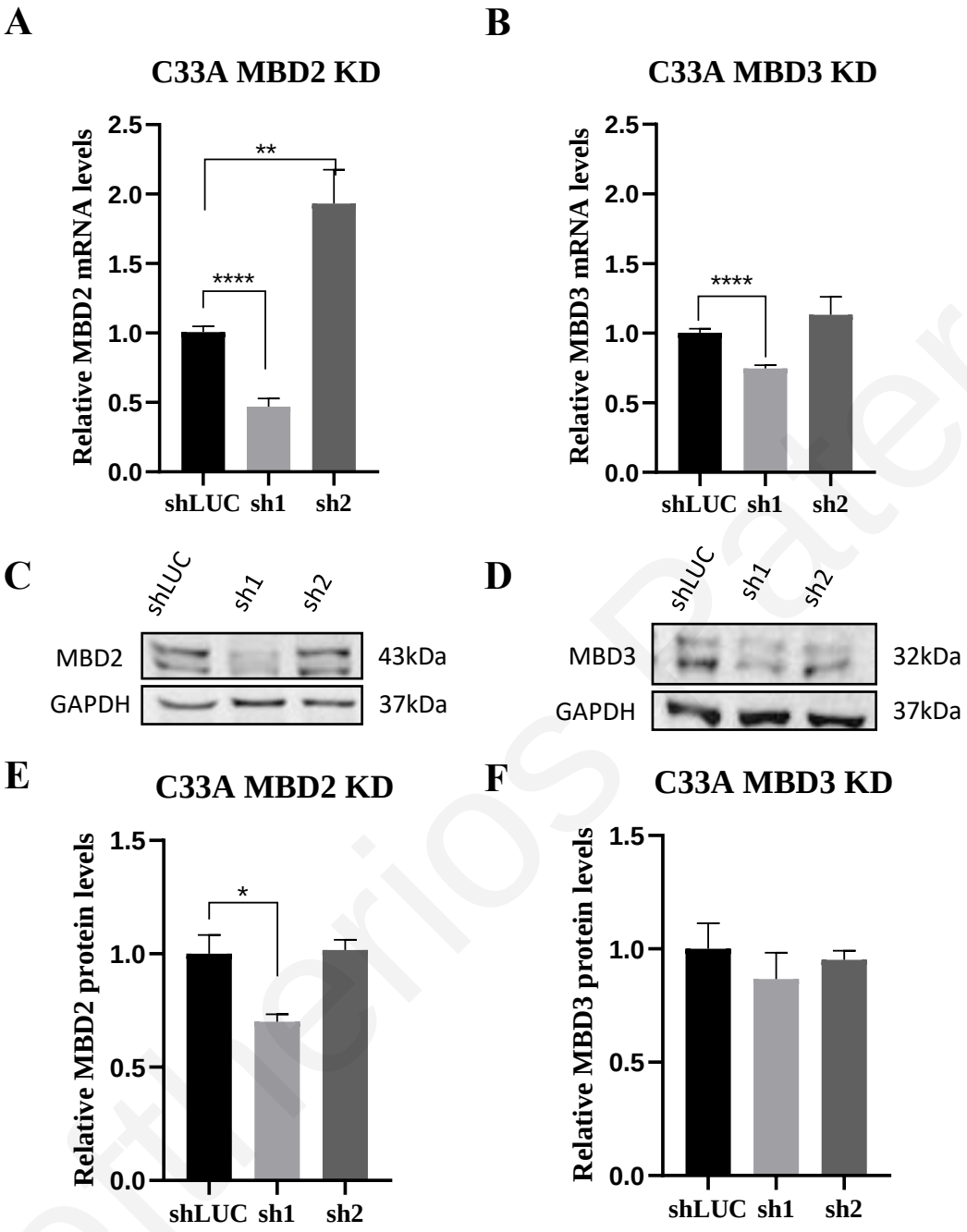


Figure 8: Validation of MBD2 & MBD3 Knockdown efficiency in C33A. Technical triplicates of three different biological replicates were used to determine the relative mRNA of MBD2 & MBD3 using qPCR **(A, B)**. MBD2 and MBD3 sh1 were able to achieve significant knock down (MBD2 sh1 53.04% knock down, and MBD3 sh1 25.35% knock down), MBD2 sh2 resulted in MBD2 overexpression (193% mean), and MBD3 sh2 had no significant change in MBD3 expression **(A, B)**. Representative western blots of MBD2 KD C33A and MBD3 KD C33A lysates **(C, D)**. Quantification of western blots of three

Eleftherios Patera

biological replicates of C33A MBD2 and C33A MBD3 KD **(E, F)**. MBD2 sh1 resulted in significant reduction of MBD2 protein expression (29.95% knock down), MBD2 sh2, MBD3 sh2 and MBD3 sh2 do not show significant changes. Statistical significance was calculated at $p < 0.05$.

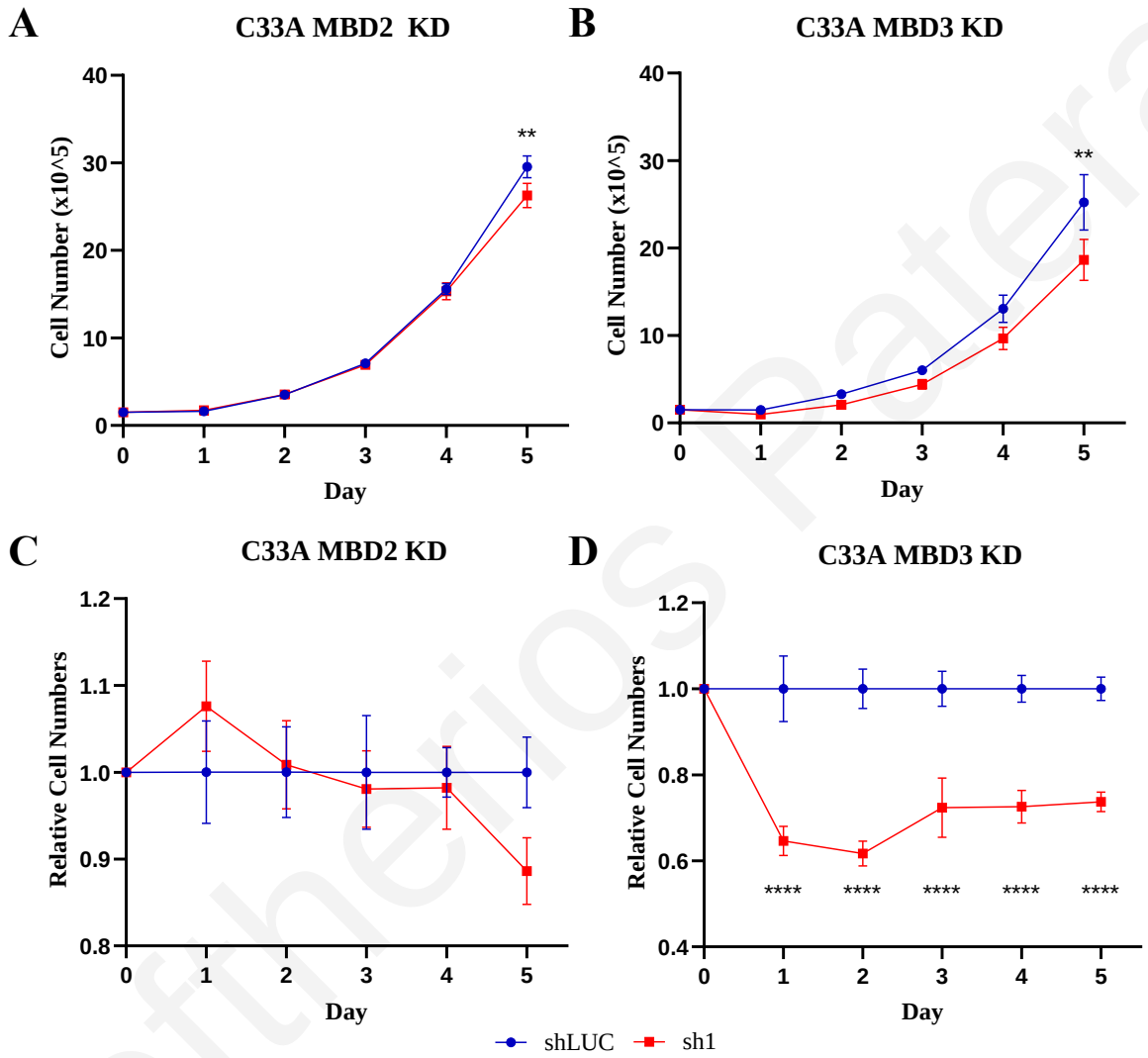


Figure 9: MBD2 and MBD3 KD reduces cell growth of C33A cells. C33A cells with MBD2 and MBD3 KD (**A & C**, and **B & D** respectively) were seeded on six wells with initial cell number of $1x10^5$. Cell numbers of both MBD2 and MBD3 KD C33A were significantly lower than control on the fifth day post seeding (**A, B**). Relative cells numbers of C33A MBD2 and MBD3 KD cells were calculated as the percentage of the average cell number of the control cells (**C, D**). MBD3 KD but not MBD2 KD C33A exhibited significantly lower relative cells numbers compared to control throughout days one to five

Eleftherios Patera

post seeding (**D**). Data was analysed using three biological and three technical replicates. Statistical significance was calculated at $p < 0.05$.

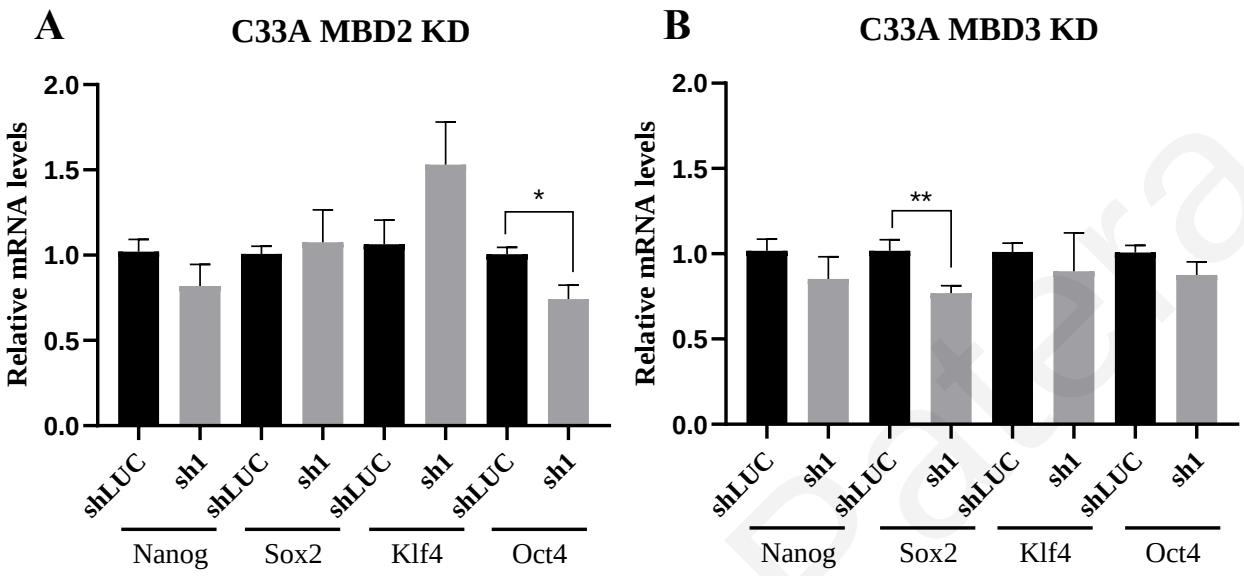


Figure 10: Stem Cell & Reprogramming transcription factors mRNA levels post MBD2 or MBD3 KD in C33As. Results represent the sum of three technical and three biological replicates. Oct4 is significantly reduced in MBD2 but not MBD3 KD C33As and Sox2 is significantly reduced in only the MBD3 KD C33As. Statistical significance was calculated at $p < 0.05$.

MBD proteins can influence genomic modifications in cervical cancer.

Considering MBD3 KD resulted in significantly decreased relative cell numbers as soon as day 1 whereas MBD2 loss was not as severe in C33As (**Figure 9C, 9D**), we turned our focus on fundamental differences between MBD2 and MBD3. Although MBD proteins are known to bind methylated CpG dinucleotides MBD3 was reported to recognize and bind hydroxy methylated (5hmC) CpGs (Yildirim et al., 2011). Consequently, we were interested in investigating the 5hmC levels in our produced cell lines. Using DNA extracts from MBD2 and MBD3 KD C33As we were able to image the 5hmC levels in dot blots (**Figure 11A, 11C**). Quantification of the dot blots revealed that loss of MBD3 has no significant effect on the 5hmC levels, but interestingly, MBD2 loss results in a significant increase of 5hmC in C33As.

Eleftherios Patera

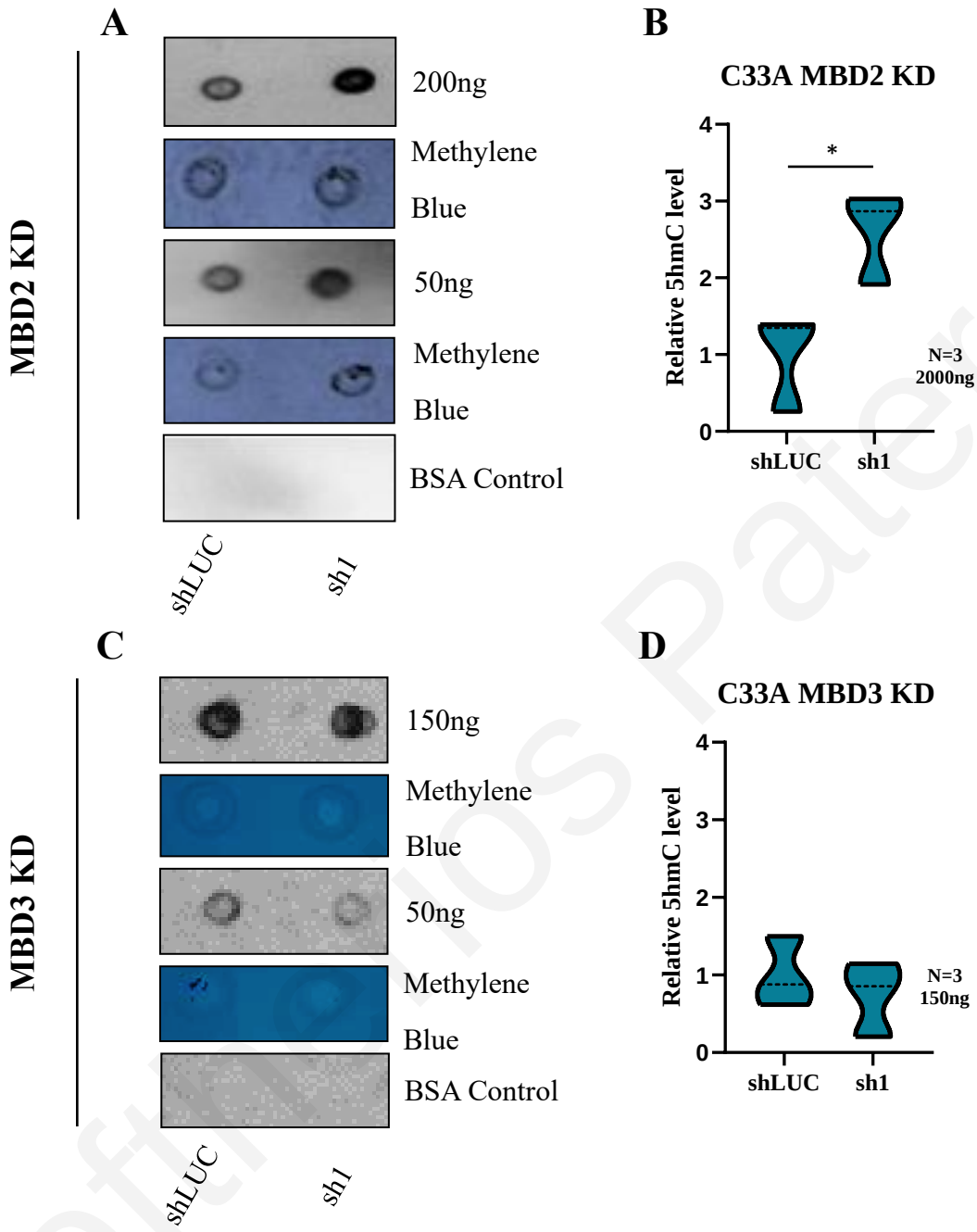


Figure 11: MBD2 but not MBD3 KD induces increased DNA hydroxy methylation (5hmC). DNA extracts (50, 150 or 200 ng) from MBD2 and MBD3 KD C33A cells were used for Dot Blots against hydroxy methylation (5hmC). Methylene Blue was as a loading control and BSA was used as a negative control. Dot blots were repeated in biological replicates and representative images are presented (**A, C**). Dot blots of three biological replicates were quantified using Image J and plotted in violin plots (**B, D**). Statistical significance was calculated at $p < 0.05$.

Eleftherios Patera

Discussion

Despite cervical cancer screening methods have been established and practiced for decades, the lack of contraceptives and vaccine immunization in developing countries leads to cervical cancer mortality rates manifesting withing a concerning range (Arbyn et al., 2020, Shingleton et al., 1995). Taking into account that HPV is the main causative agent of cervical carcinogenesis, a comprehensive understanding of the underlying oncogenic mechanisms of HPV is needed. Previous work from our lab has demonstrated that HPV E7 oncoprotein increases Oct4 expression and causes Oct4 to form interactions with MBD3-NuRN instead of the MBD2-NuRD complex, thereby unveiling a potential role of MBD2/MBD3 dynamics during cervical carcinogenesis (Panayiotou et al., 2020, Panayiotou et al., submitted). In the current study, we opted to investigate the role of MBD2 and MBD3 by generating MBD2 and MBD3 KD C33A cell lines. Our results first indicate the importance of MBD3 in cervical cancer cells, reflected by the observation of significantly lower relative cell numbers, compared to control as soon as 24 hours post seeding (**Figure 9**). Although the acquired data do not distinguish whether the difference is due to alterations in the proliferation rate of MBD3 KD cells or whether it can be rather attributed in elevated apoptosis rates, it is evident that MBD3 KD promotes a growth defect in C33A cells. MBD2 KD does not display a comparable significant effect, and this could be explained by MBD3 compensating for the loss of MBD2, since they both can bind methylated (5mC) CpGs. This consequently could be the reason why MBD3 loss cannot be tolerated by cervical cancer cells, as the hydroxy methylation (5hmC) binding of MBD3 cannot be compensated by MBD2, whose binding is more restricted to 5mC (Leighton et al., 2022). Further characterization of the genomic regions rich in 5hmC in cervical cancer cells and how MBDs interact with those is needed before we could draw further conclusions on the ability of an MBD compensating for the loss of another.

A plethora of studies have already revealed that pluripotency transcription factors, such as Oct4 and Sox2, are associated with cervical cancer (Liu et al., 2014, Yang et al., 2019, Panayiotou et al., 2020). These combined with further studies have already led to the hypothesis that oncogenic viruses can infect stem cells or promote reprogramming of cells

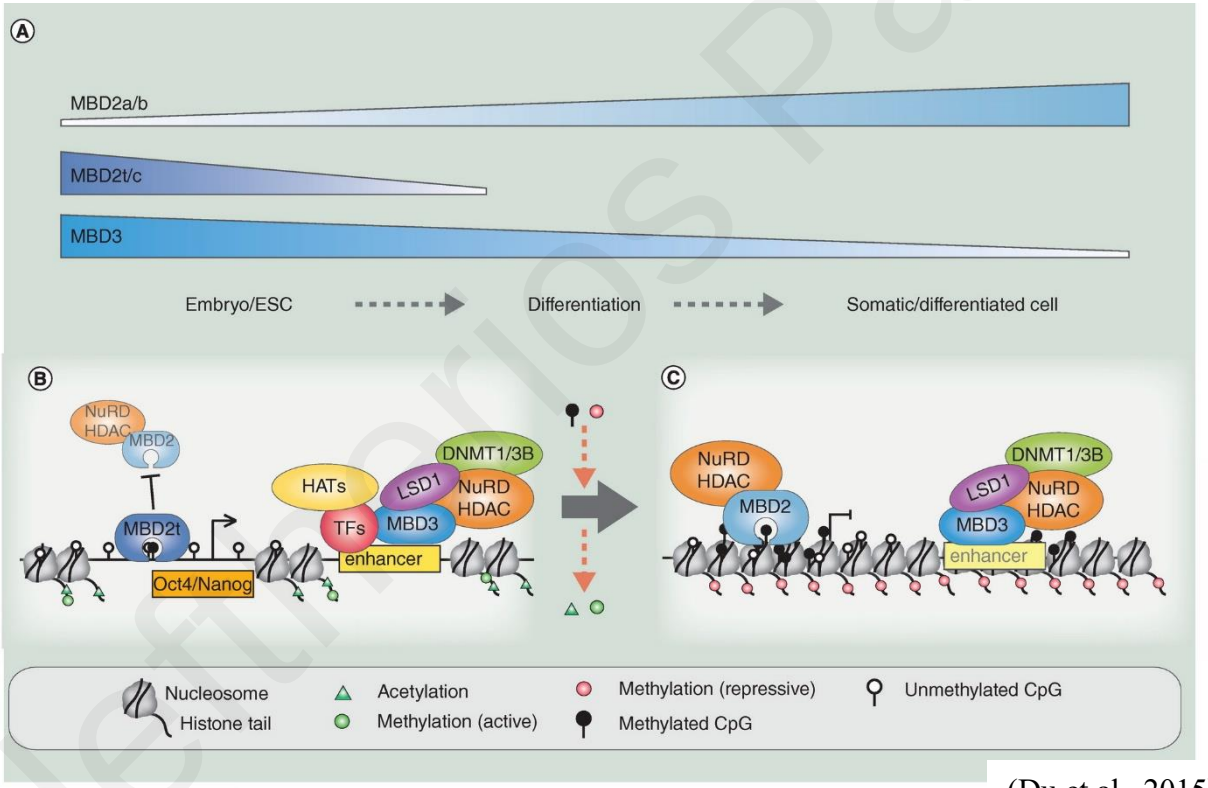
Eleftherios Patera

into pluripotency (Takahashi & Yamanaka, 2006, Yilmaz & Strati, 2019). Furthermore, both MBD2 and MBD3 have been reported to play key role in regulating pluripotency and differentiation of stem cells affecting among others the expression of core reprogramming transcription factors (Kaji et al., 2006, Lu et al., 2014, Markov et al., 2021). Therefore, we were interested in characterising potential changes of pluripotency genes in MBD2 and MBD3 KD cervical cancer cells. Given the association of MBDs with transcriptional repression, we were surprised to notice that Oct4 expression was significantly reduced in MBD2 KD C33As, and Sox2 was significantly reduced in MBD3 KD C33As. Nevertheless, it is important to note that the pluripotency gene expression changes have not yet been verified at the protein level with Western blots, and this is anticipated to be accomplished as part of our future work. A mechanism that could potentially explain the decrease of Oct4 in MBD2 KD C33As is hidden through alternative spliced variants of MBD2. There are three different MBD2 spliced variants, MBD2a, MBD2b and MBD2c (also called MBD2t) that have been identified in the scientific literature thus far (Hendrich & Bird, 1998). MBD2a and MBD2b are almost identical, however, MBD3c lacks both the transcriptional repression domain (TRD) and the C-terminal coiled coil domain (CC), hence rendering it incapable of interacting with the NuRD complex. It has been demonstrated in human embryonic stem cells (hESCs) that Oct4 induces the spliced of the spliced variant MBD2c which can then bind to Oct4 promoter and practically safeguard it from NuRD targeted silencing (Lu et al., 2014). Hence, MBD2c could be manifesting in high enough levels in C33A cells, to act as a safeguard against Oct4-induced silencing, and thereby our generated MBD2 KD (which impacts all known isoforms) expose the Oct4 promoter to be consequently targeted for silencing. Supplementary follow-up experiments are needed to characterise this mechanism. For example, chromatin immunoprecipitation assays (ChIP) could be used to identify changes in NuRD tethering in Oct4 promoter in MBD2 KD, compared to control cells. Alternatively, re-introduction of specific MBD2 variants in MBD2 KD C33As could reveal whether MBD2c alone is capable of rescuing the MBD2 KD cells and promote re-expression of Oc4.

Hydroxy methylation (5hmC) is a DNA modification and a mid-product of the demethylation procedure catalysed by the ten-eleven translocation (TET) enzymes (Zhang et al., 2010, Koh et al., 2011). Accumulating data seem to propose that 5hmC is decreased in cervical carcinogenesis and declined levels of 5hmC correlate with poor survival and cervical cancer progression (Zhang et al., 2016, Wang et al., 2019, Kato et al., 2020). By knowing that and

Eleftherios Petera

also by taking into account that MBD3, a known reader of 5hmC, is necessary for the growth of cervical cancer cells, we were interested in looking into the 5hmC levels in our MBD2 and MBD3 KD cell lines. MBD3 KD C33A exhibited no significant change, but interestingly our MBD2 KD C33A cells have shown significant increase in 5hmC levels. The origin of that is not clear on whether it could be attributed to elevated TET1 levels, or solely to the fact that with the absence of MBD2 binding the 5mC are more exposed to be targeted by existing TET1. As part of our future work, it would be critical to further scrutinize TET1 expression and *in vitro* work with TET1 enzymatic assays could also demonstrate the TET1-protective effect from MBD2 binding. Furthermore, a limitation of the undertaken dot blot assays is that they can only provide an estimate of global levels of 5hmC, although it is of uttermost importance to shed a light on the precise genomic loci that are mostly affected, as this can



(Du et al., 2015)

Figure 1: Regulation of Stem cell related gene expression through MBD variants.

Depiction of MBD3, MBD2a/b and MBD2c gene expression throughout development. MBD2c is more abundant in embryonic stem cells, whereas MBD2a/b are increased in expression throughout differentiation. MBD3 is expressed in both somatic and embryonic cells but exhibits elevated expression in embryonic stem cells (**A**). MBD2c binds methylated sites in the promoter of Oct4 and Nanog and since it cannot interact with NuRD, it protects the silencing of those genes (**B**). MBD2a/b binds exposed methylated sited and promotes gene silencing through NuRD (**C**).

Eleftherios Patera

give a probable answer to how 5hmC changes affect transcriptional programming in cervical cancer. To this end, oxidative bisulfite sequencing can be utilized to map both 5mC and 5hmC profiles (Booth et al., 2013). Understanding the changes of 5mC and 5hmC distribution in the genome of cervical cancer could lead to opportunities for generation of novel epigenetic treatments for cervical cancer.

In addition to the aforementioned future perspectives, an inducible knockdown strategy can be employed instead of stable knockdown. This could shed light into the immediate response of the cells when depleted of MBD2 or MBD3, while also providing better knock down efficiency. In addition, overexpression of MBD2 and MBD3 in C33As could complement our findings by characterizing how the cell responds to elevated levels of MBD2/MBD3. More than that, CRISPR-induced mutagenesis of MBD2 could help restrict the different spliced variants that the MBD2 gene can produce, therefore providing us with a tool to inspect one isoform in isolation of the rest. It is important to also note that the initial findings that led to this project do not imply that MBD2 is reduced from cell, but rather the NuRD associated with Oct4 is bound to MBD3 instead of MBD2 in the presence of E7. Having that in mind, other strategies that do not involve depletion of MBDs could be used to further characterise the MBD dynamics during carcinogenesis.

To conclude, our findings demonstrate that the growth of cervical cancer cells seems to be MBD3 dependent, and disruption in the MBD2/MBD3 expression results in broad changes in genomic modifications, as well as deregulation of expression of core stem cell related genes. More comprehensive understanding of the role of MBD proteins in carcinogenesis is needed for the development of potential novel treatments for cervical cancer.

Eleftherios Patera

Abbreviations:

5hmC: 5 Hydroxyl Methyl Cytosine

5mC: 5 Methyl Cytosine

CC: Cervical Cancer

CDK2AP1: Cyclin-Dependent Kinase 2 Associated Protein 1

CHD: Chromodomain Helicase DNA binding protein

ChIP: Chromatin Immunoprecipitation

CIN: Cervical Intraepithelial Neoplasia

G/R: Glycine/Arginine

HDAC: Histone Deacetylase

HPV: Human Papilloma Virus

KD: Knock Down

KO: Knock Out

MBD: Methyl CpG Binding Domain

MTA: Metastasis Tumour Associated protein

NuRD: Nucleosome Remodelling and Deacetylase complex

q-PCR: Quantitative Polymerase Chain Reaction

RBBP: Retinoblastoma Binding Protein

RT-PCR: Reverse Transcription Polymerase Chain Reaction

TRD: Transcription Repression Domain

Eleftherios Patera

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Eleftherios Patera

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