Functional assessment of MeCP2 in Rett syndrome and cancers of breast, colon and prostate

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Functional assessment of MeCP2 in Rett syndrome and cancers of breast, colon and prostate

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Abstract

Ever since the first report that mutations in methyl-CpG-binding protein 2 (MeCP2) causes Rett syndrome (RTT), a severe neurological disorder in females world-wide, there has been a keen interest to gain a comprehensive understanding of this protein. While the classical model associated with MeCP2 function suggests its role in gene suppression via recruitment of co-repressor complexes and histone deacetylases to methylated CpG-sites, recent discoveries have brought to light its role in transcription activation, modulation of RNA splicing and chromatin compaction. Various post-translational modifications (PTMs) of MeCP2 further increase its functional versatility. Involvement of MeCP2 in pathologies other than RTT, such as tumorigenesis however, remains poorly explored and understood. This review provides a survey of the literature implicating MeCP2 in breast, colon and prostate cancer.

Introduction

MeCP2 is an X-chromosome associated gene, identified as the founding member of methyl-CpG binding domain (MBD) containing protein (Lewis et al. 1992). Our current knowledge regarding MeCP2 remains incomplete, despite its discovery some 24 years ago. The requirement of MeCP2 function is exemplified most notably in pathological conditions linked with RTT and other disorders namely classical autism, neonatal encephalopathy and X-linked mental retardation (Amir et al. 1999; Couvert et al. 2001; Lam et al. 2000; Beyer et al. 2002; Schanen et al. 1998). RTT involves an apparent normal development of female(s) upto 7-18 months of age during which they achieve the expected motor, social and language milestones, followed by regression of acquired skills (Hagberg et al. 1983; Hagberg and Witt-Engerstrom 1986). While RTT is most

frequently seen in females than males (1 in every 15,000 females) (Hanefeld 1985), the latter have a shorter lifespan and often develop fatal neonatal or congenital encephalopathy (Kankirawatana et al. 2006; Villard et al. 2000). MeCP2 is an epigenetic reader that recognizes and binds to not only 5-methyl cytosine but also, 5-hydroxymethyl-cytosine on DNA (Mellen et al. 2012). Recent works have implicated MeCP2 in various cancers (Song et al. 2016; Muller et al. 2010; Ballestar et al. 2003). Works from our own lab suggests the potential role MeCP2 plays in the progression of colon cancer (Pandey et al. 2015).

Herein we begin with an introduction of MeCP2 followed by the multi-faceted functions it is involved in, by interacting with various co-regulators or by undergoing multiple PTMs. This review article examines the evidence for involvement of MeCP2 in the cancer of the breast, colon and prostate. We stress the notion that MeCP2 is a key epigenetic protein that is aberrantly regulated in cancer.

MeCP2: Structure and function

The pioneering works from Bird lab identified a nuclear protein, MeCP which preferentially bound methylated DNA without any sequence specificity and was thought to play a role in the inaccessibility of methylated CpG (Meehan et al. 1989; Meehan et al. 1990). Later experiments from the same lab demonstrated that MeCP was actually two distinguishable proteins – MeCP1 and MeCP2, with MeCP1 needing at least twelve symmetrically methylated CpGs while MeCP2 was able to bind a single methylated CpG base pair (Meehan, Lewis, and Bird 1992). Corroboration of the methyl-binding activity of MeCP2 came from studies by Lewis et al. in 1992. They showed that MeCP2-LacZ fusion protein was incapable of localizing to centromeric heterochromatin in methyl-

transferase-deficient mouse cells. By constructing deletions of full length MeCP2, Nan et al. discovered the minimal region (MBD, ~85 amino acids long) required to bind DNA containing one or more symmetrically methylated CpGs. Additionally they also demonstrated that MBD binds to DNA as a monomer (Nan, Meehan, and Bird 1993).

Wakefield et al. in 1999, proposed the solution structure of recombinant MBD of MeCP2 via nuclear magnetic resonance (NMR) spectroscopy. They reported the structured core (amino acids 95-162) adopted a novel fold and formed a wedge shaped structure composed of a four-stranded anti-parallel β -sheet on one side and an α -helix on the C-terminus end. Two anti-parallel β -strands are connected by an unstructured loop of five amino acid residues comprised of one positively charged and two polar residues (Wakefield et al. 1999). They also report that the DNA binding surface of MeCP2, the MBD, is rich in basic amino acids, lysine and arginine. The Bird lab recently published a high-resolution X-ray co-crystal structure of MBD bound to symmetrically methylated DNA (20 bp fragment of mouse brain-derived neurotrophic factor, BDNF) (Ho et al. 2008). Their model contradicted the previous proposed hypothesis that the stability of MBD-DNA interaction is brought about by hydrophobic association. The study revealed that the methyl-group predominately makes contact with tightly bound water molecules and that MeCP2's MBD, instead of recognizing the methylated CpG per se, interacts with the water molecules at the major groove of the methylated DNA (Ho et al. 2008).

Transcription repression domain (TRD) of MeCP2 was next characterized using an *in vitro* β -actin transcription assay, wherein the effects of fusion proteins made using Gal4 DNA binding domain and varying fragments of MeCP2 fragments were tested for

their ability to repress a reporter gene construct containing Gal4 binding sites near the promoter (Nan, Campoy, and Bird 1997). Rigorous experimentation led to the identification of a minimal MeCP2 region encompassing amino acids 205-310, sufficient to cause transcriptional silencing of a reporter gene construct. Both native and recombinant rat MeCP2 led to transcriptional repression *in vitro* from methylated promoters but not from non-methylated promoters (Nan, Campoy, and Bird 1997). Recent findings from Huda lab have allowed for the characterization of two clusters of AT-Hook domains within MeCP2 (Baker et al. 2013). Such domains are found to be highly conserved among species and play a key role in DNA binding of chromatin-associated, non-histone proteins of the high-mobility group (HMG), where they were described for the first time (Reeves and Nissen 1990). Figure 1B shows the location of AT-Hook domains in the two MeCP2 isoforms.

Recent investigations have identified intrinsic disorder in MeCP2 e2 isoform using FoldIndex algorithm (Adams et al. 2007). Over a concentration range of ~1000 fold, MeCP2 is found to be monomeric in both low and high salt solutions. Circular dichroism spectroscopy has predicted MeCP2 monomer to be ~60% unstructured in conditions where it preferentially recognizes CpG dinucleotides and can form compact chromatin (Adams et al. 2007). The same study carried out experiments involving protease digestion of MeCP2 and identified structurally distinct domains which are collectively organized into tertiary structure possessing coil-like hydrodynamic properties, exhibiting the intrinsic disorder in MeCP2 sequence. Both MBD and TRD when expressed as individual fragments have the potential to function as non-specific DNA binding

segments. The various structural attributes of MeCP2 provide a basis for its multi-functional role *in vitro* and *in vivo* (Adams et al. 2007).

MBD-dependent functions

A schematic representation of the MeCP2 domains is shown in Figure 1B. In an effort to discover protein factors mediating methyl-dependent repression, the same group used co-immunoprecipitation (Co-IP) assays and showed that TRD interacts with a co-repressor complex comprised of mSIN3A and histone deacetylases to result in the alteration of chromatin structure via post-translational modifications of histone tails (Nan et al. 1998; Jones et al. 1998). These two studies were instrumental in demonstrating that firstly, an indirect method exists by which MeCP2 can introduce alterations in chromatin architecture by recruiting histone deacetylases, and secondly that DNA methylation and histone deacetylation, the two global gene regulation mechanisms, can be linked by MeCP2 (Figure 2), together with the finding that pharmacological inhibition of deacetylase activity can partially relieve the methylation-dependent silencing. Such partial alleviation of transcription repression was indicative of involvement of other potential repressive modes.

MeCP2 interacts with various proteins to execute its functions in regulating cellular physiology. In an *in vitro* myogenesis system, MeCP2 N-terminus has been shown to associate with heterochromatin protein 1 (HP1) via Co-IP assays (Agarwal et al. 2007). Using immunocytochemistry, the study reported that HP1 localization to heterochromatin correlated with MeCP2 presence. HP1 is known to bind with H3K9me3 and interact with Suv39H1 methyltransferase (Yamamoto and Sonoda 2003), further linking the presence of MeCP2 at heterochromatic regions.

137 Maintenance DNA methyltransferase I (DNMT1) is known to interact with the TRD of
138 MeCP2 (Kimura and Shiota 2003), indicating that MeCP2 may potentially be involved in
139 faithful copying of methylation marks during DNA replication. Contrary to this, works
140 from (Nan, Meehan, and Bird 1993), underscored that binding to hemi-methylated DNA
141 of MeCP2 MBD, is negligible and so does not support the finding of hemi-methylated
142 DNA being bound by MeCP2.

143 Using Co-IP assays, Bird group found that methyl-binding domain of MeCP2 interacts
144 with α -thalassemia/mental retardation, X-linked, ATRX protein (a SWI2/SNF2 DNA
145 helicase/ATPase that is mutated in ATRX syndrome) (Nan et al. 2007). They reported
146 that point mutations within the MBD of MeCP2 that caused RTT syndrome inhibited its
147 interaction with ATRX. In a DNA methylation-dependent manner, MeCP2, can recruit
148 the helicase domain of ATRX to heterochromatic foci in living mouse cells, allowing for a
149 tempting speculation that ATRX may be involved in the epigenetic regulation of genes
150 bound by MeCP2 (Nan et al. 2007).

151 A recent study revealed that MeCP2 is required for ATRX and CTCF binding to the *H19*
152 ICR and further suggested that MeCP2 and ATRX are involved in maintenance of
153 nucleosome configuration conducive for CTCF binding to bring about regulation of gene
154 expression at a subset of imprinted domains (Kernohan et al. 2014).

155 MeCP2 TRD binds c-Ski, encoded by the proto-oncogene c-Ski (Kokura et al. 2001).
156 Immunostaining analysis has demonstrated co-localization of MeCP2 and c-Ski in the
157 nuclear heterochromatic region. The magnitude of repression brought about by Gal4-
158 MeCP2 fusion protein was abolished following over expression of dominant negative
159 form of c-Ski. Also, antibody-mediated neutralization of Ski and Ski-related novel

proteins, c-Ski and sno, led to the abrogation of transcription repression by Gal4-MeCP2 which suggest a role of the ski gene family in methylation-dependent transcription repression.

MeCP2 TRD and the amino-terminal domain were demonstrated to interact directly with the ETS domain of PU.1 (ETS family of factors) by various pull-down and immunoprecipitation assays (Suzuki et al. 2003). PU.1 transcriptional activity was found to be repressed by MeCP2 on a reporter construct containing trimerized PU.1 binding sites and that this transcription suppression can be rescued by TSA, a histone deacetylase inhibitor. By performing pull-down assays, it was discovered that MeCP2 preferentially associates with the complex containing PU.1-mSIN3A-HDAC1 and not with PU.1-CBP. ChIP-PCR assays using undifferentiated murine erythroleukemia cells (MEL) demonstrated that MeCP2 and PU.1 are enriched at PU.1-binding site present on the reporter construct and that present in the intron (intervening sequence 2 region) of β -globin gene, known to regulate its gene expression. It was also shown that during the course of erythroid differentiation of MEL cells, PU.1-mSIN3A-HDAC1 complex disappeared from this region (Suzuki et al. 2003). Thus by forming a complex with mSIN3A and HDAC1, MeCP2 can repress PU.1. While underscoring the multifunctional nature of this protein, a recent review describes the various protein factors MeCP2 associates with (Guy et al. 2011). Figure 3 illustrates the minimal region required for the interaction of different candidate binding partners with MeCP2 e2 isoform.

MeCP2 Isoforms

Our understanding of MeCP2 location, expression patterns, and functionality increased with the discovery of a previously unknown isoform MeCP2B (Mnatzakanian et al. 2004). The new isoform e1, or MeCP2B, is a splice variant whose translation start site begins at exon 1 of MeCP2, foregoes exon 2 and encompasses full-length exons 3 and 4 to give rise to a 498 amino acid long protein, while the known isoform e2 or MeCP2A begins at exon 2, followed by full-length exons 3 and 4 to yield a 486 amino acid protein (Figure 1). Hence the two isoforms differ at their N-terminus. MeCP2 e2 is expressed in skeletal muscle, liver, spleen and prostate while MeCP2 e1 is expressed primarily in fetal and adult brain and brain sub-regions. In the adult human brain, MeCP2 e1 expression is ten times the expression of MeCP2 e2 (Mnatzakanian et al. 2004). These findings indicate that important differences between these two isoforms remain to be identified.

MeCP2 post-translational modifications (PTMs)

MeCP2 PTMs make possible the intricacies of MeCP2 transcriptional modulation. While many PTMs of MeCP2 have been found to date, phosphorylation of MeCP2 remains the most well studied. MeCP2 phosphorylation was first noted in 2003 when Chen et al (Chen et al. 2003) found that MeCP2 selectively binds to the promoter III of BDNF gene and functions to negatively regulate its expression. They discovered that membrane depolarization of rat cortical neurons in response to increased KCl triggers calcium-dependent phosphorylation and exiting of MeCP2 from BDNF promoter and facilitates chromatin remodeling to allow for BDNF transcription. Hence, MeCP2 is pertinent in regulating genes involved in neuronal activity and the dysregulation of that role might be the causative factor for RTT.

205 *De novo* phosphorylation of MeCP2 at S421 (serine residue at 421 position) in cultured
 206 rat neurons, triggered by neuronal activity followed by an influx of calcium was reported
 207 in 2006 (Zhou et al. 2006). In this study, it was demonstrated that phosphorylation of
 208 MeCP2 at serine 421 residue was selectively induced *in vivo* in response to synaptic
 209 activity driven via either light exposure or via pharmacologically induced seizures in
 210 rodents. Phosphorylation at this site results in de-repression of *BDNF* transcription and
 211 regulates MeCP2-dependent dendritic growth and spine maturation. Western blot
 212 analysis from whole cell extracts prepared from a 4-week old male mouse revealed that
 213 ser421 phosphorylation occurred specifically in the brain and not in any other tissue
 214 analyzed. These observations indicate that by inducing phosphorylation of MeCP2,
 215 neuronal activity orchestrates a gene expression program to bring about nervous
 216 system maturation and the disruption of which may underlie the RTT phenotype in
 217 individuals with MeCP2 mutation.

218 Mass spectrometry analysis has shown serine 80 residue of MeCP2 to be
 219 phosphorylated in rodents either basally or when subjected to seizures (Tao et al.
 220 2009). Neuronal activity triggers dephosphorylation of ser80 while S80A mutant
 221 contributes to reduced association of MeCP2 to several gene promoters and
 222 concomitantly altering their expression (Tao et al. 2009).

223 Using a dephosphorylation mimetic MeCP2 S421A mouse, it was shown *in vivo*, that
 224 loss of ser421 phosphorylation leads to an abnormal development of dendrites and
 225 synapse, and a defective behavioral response to new experience (Cohen et al. 2011),
 226 suggesting that RTT is a disorder of experience-dependent neuronal development.

227 Studies using tandem mass spectrometry (MS/MS) have reported the presence of
228 multiple MeCP2 PTMs, viz., phosphorylation, acetylation and ubiquitination (Gonzales
229 et al. 2012) in SH-SY5Y cells ectopically expressing MeCP2. Gonzales et al. discovered
230 that phosphorylation at S80 or S229 residue of MeCP2 selectively alters interactions
231 with chromatin factors such as SMC3 and HP1, and co-factors such as SIN3A and YB-
232 1, *in vivo*. MeCP2 phosphorylation was found to be indispensable to bring about
233 differentiation-induced activation and/ or repression of receptor tyrosine kinase (*RET*),
234 and early growth response factor 2 (*EGR2*), MeCP2 target genes (Gonzales et al.
235 2012). It is worth mentioning that MeCP2 was found to be acetylated at lysine residues
236 305, 307 and 321.

237 Phosphotryptic mapping was utilized in 2013 to identify three novel MeCP2
238 phosphorylation sites (S86, S274, and T308) following neuronal stimulation (Ebert et al.
239 2013). Various agents such as those that increase intracellular cAMP level, neuronal
240 activity, or BDNF, can differentially induce phosphorylation of these sites, suggesting
241 the epigenetic regulatory role of MeCP2 of gene expression, by integrating various
242 environmental signals. T308 phosphorylation was shown to prevent the interaction of
243 MeCP2 TRD and the nuclear receptor-corepressor (NCoR) complex and hence
244 decreasing MeCP2's ability to cause transcription repression (Ebert et al. 2013).
245 Neuronal stimulation in knock-in mice harboring a typical human RTT missense
246 mutation R306C, failed to elicit T308 phosphorylation, indicating that loss of MeCP2
247 T308 phosphorylation may potentially contribute to RTT. Ebert et al., further showed
248 that mice bearing MeCP2 T308A mutation exhibited reduced induction of a subset of
249 neuronal activity-regulated genes and to RTT-like phenotype. Hence, neuronal

stimulation-dependent T308 phosphorylation regulates MeCP2's interaction with the NCoR complex and that RTT phenotype in humans could be attributed, in part, to the failure of activity-dependent phosphorylation of MeCP2 at T308 residue and the subsequent loss of phosphorylation-controlled MeCP2-NCoR complex interaction. Figure 3 illustrates the distribution of different phosphorylation sites over MeCP2 domain map. Excellent reviews have highlighted the role of multiple other MeCP2 phosphorylation sites (Bellini et al. 2014; Ausio, Martinez de Paz, and Esteller 2014). The role of protein acetylation in regulating cell physiology is becoming increasingly appreciated (Yang and Seto 2008). Recent studies have reported MeCP2 to be acetylated (Choudhary et al. 2009; Zocchi and Sassone-Corsi 2012; Gonzales et al. 2012; Pandey et al. 2015), and because little is known about how acetylation influences MeCP2 function, it remains an active area of research. Over expression of SIRT1 deacetylase was shown to result in reduced acetylation of mouse MeCP2 e1 isoform at lysine 464 (K464) or human lysine 461, when MeCP2 was ectopically expressed in HEK293 cells (Zocchi and Sassone-Corsi 2012). ChIP-qPCR analysis of hippocampus tissue revealed that MeCP2 enrichment at exon IV of *BDNF* was significantly increased in SIRT1^{Δex4} mice relative to wild-type control mice and was linked to decreased BDNF expression in SIRT1^{Δex4} mice (Zocchi and Sassone-Corsi 2012). In an attempt to identify endogenously acetylated lysine(s) in MeCP2 in multiple cancer cell line models, we pharmacologically inhibited SIRT1 and using tandem MS/MS, identified various acetylated lysines (K22, K135, K171, K200, K256, K271 and K289) (Pandey et al. 2015). Our acetylation mimetic mutant, MeCP2 K171Q, elicited decreased binding to chromatin remodeling proteins, ATRX and HDAC1, as compared

273 MeCP2-WT and was associated with retarded growth and proliferation of RKO colon
274 cancer cells in which it was over expressed (Pandey et al. 2015). Figure 3 illustrates the
275 distribution of different acetylation sites over MeCP2 e2 isoform domain map.

276 Sumoylation of MeCP2 has been found to occur on K223, that brings about the
277 recruitment of deacetylase complex HDAC1/2 and the deacetylation mimetic mutant,
278 K223R, inhibits transcriptional repression function of MeCP2 in primary cortical neurons
279 (Cheng et al. 2014). Furthermore, sumoylation at K223 plays a pertinent role in
280 regulating the formation of excitatory synapse.

281 Many other MeCP2 PTMs such as the methylation and ubiquitination of lysine residues
282 have been reported elsewhere (Bellini et al. 2014). The above mentioned PTMs of
283 MeCP2 elevate its ability to function dynamically within various cells of the body,
284 thereby necessitating the need to characterize other modifications of MeCP2.

285 **TRANSCRIPTIONAL ACTIVATOR**

286 While attempting to delineate the molecular mechanism(s) fundamental to
287 neuropsychiatric disorders resulting from either MeCP2 depletion or over-expression
288 (clone including regions responsible for both e1 and e2 isoforms) in mice while
289 analyzing patterns of gene expression in mouse hypothalamus, it was observed that,
290 although in both cases, the expression levels of thousands of genes was altered,
291 majority of the genes (~85 %) were surprisingly upregulated by MeCP2 (Chahrour et al.
292 2008). MeCP2 was found to interact with the transcriptional activator, cyclic-AMP
293 response element binding protein 1 (CREB1) while occupying the promoter of an
294 activated target gene did not interact with the same while occupying a repressed target
295 gene promoter. Interestingly, findings from the same lab later extended the observation

in the cerebellum, wherein a multitude of genes were found to be upregulated in MeCP2-Tg mice (over-expressing MeCP2) and downregulated in MeCP2-null mice consistence with MeCP2's role as a transcriptional modulator capable of both increasing and decreasing the expression of genes (Ben-Shachar et al. 2009). Such investigations are indicative that gene expression alterations in various regions of the brain occur following MeCP2 loss or gain of function. The role of MeCP2 as a modulator has been recently review by others (Delcuve, Rastegar, and Davie 2009). Figure 4 illustrates how MeCP2 can function as a transcriptional activator.

REGULATION OF RNA SPLICING

Examples mentioned above provide insights to the various roles MeCP2 plays by interacting with multiple proteins. MeCP2 e2 isoform can associate with DNA/RNA binding protein, Y-box binding protein (YB-1) and regulate reporter minigene splicing (Young et al. 2005). The same group further demonstrated that RNA mediates interaction between YB-1 and MeCP2 and that this interaction was not disrupted when R106W-MeCP2 (RTT mutant form of MeCP2) was used or a functional methyl-binding domain of MeCP2 is not absolute for its interaction with YB-1. Such results together with the findings that a mouse model of RTT (MeCP2-308 mutant) shows abnormal alternative splicing pattern as compared to MeCP2-WT (Young et al. 2005), is indicative of MeCP2's role in regulating splicing.

It was later reported that MeCP2 is increasingly present at the heavily methylated alternatively spliced exons (ASEs) and that inhibition of DNA methylation results in firstly, abnormal splicing of ASEs and secondly, disrupts the methylation specific targeting of MeCP2 (Maunakea et al. 2013). Genetic ablation of endogenous MeCP2

was found to cause increased acetylation of histone H4 and abnormal skipping events associated with ASEs which over-lapped to a great degree with the skipping of exons observed when HDACs were pharmacologically inhibited. These findings thus suggest that intragenic CpG-methylation enhances recognition of exons by serving as a docking site for MeCP2 which in turn brings about the recruitment of HDACs to maintain the local hypoacetylation of histones to modulate alternative RNA splicing.

MBD-INDEPENDENT FUNCTIONS

A rapid increase in MeCP2 research has occurred ever since the discovery that MeCP2 is an etiological factor for Rett syndrome (RTT) (Amir et al. 1999). As opposed to the earlier methyl-binding activity of MeCP2, recent findings uncover its ability to bind to chromatin and DNA independent of CpG-methylation status (Georgel et al. 2003; Nikitina et al. 2007; Yasui et al. 2007; Chahrour et al. 2008; Ben-Shachar et al. 2009). Studies discussed below involve the usage of MeCP2 e2 isoform.

In vitro studies demonstrating histone deacetylase-independent compaction via formation of secondary and tertiary chromatin structures assembled by MeCP2 led to its understanding as a complex nuclear protein, with a key role in globally regulating chromatin architecture (Georgel et al. 2003). This study employed sophisticated electron cryo-microscopy to characterize complexes formed between non-methylated 12-mer nucleosome arrays and recombinant human MeCP2, or specific missense (R133C) or non-sense (R168X) MeCP2 mutants and revealed the novel finding that chromatin condensing region(s) of MeCP2 reside in domains other than the methyl-binding domain.

Such findings served as a foundation for a later study that utilized various truncated forms of human MeCP2 to demonstrate that the methylation independent interaction with nucleosome positioning-sequence was mediated by the C-terminal domain, and also indicated a partial methyl-specific interaction being mediated via the MBD (Nikitina et al. 2007). Results from electron microscopy analysis revealed the capability of MeCP2 to modulate nucleosome array structure by trans-interaction with multiple arrays and in-cis compaction of an array.

In an attempt to identify novel genes targeted by MeCP2, Horike et al., sequenced MeCP2-binding sites in mouse brain and mapped an imprinted gene cluster on chromosome 6 that contained two genes, *Dlx5* and *Dlx6* (Horike et al. 2005). These genes showed two fold higher expression in MeCP2-null mice than those of MeCP2-WT mice. Analyzing ChIP-coupled loop assays showed that MeCP2 mediated the formation of silent chromatin-derived 11-kb chromatin loop at the *Dlx5-Dlx6* locus and that this loop was absent in MeCP2-null mice. While this indicates a new mechanism of MeCP2 driven gene regulation, recent works from a study has refuted against these findings (Schule et al. 2007).

Involvement in Breast Cancer

One of the most common malignancies observed in women is breast cancer and it remains one of the leading causes of mortality worldwide, despite the progress made in the past 15-20 years (Yang et al. 2015; Murphy and Morris 2012).

An early report linking MeCP2 with breast cancer via in vitro studies showed that partially methylating the promoter region of breast cancer predisposition gene *BRCA1*, results in a MeCP2-dependent inhibition of gene expression (Magdinier et al. 2000). It

364 was reported that dysregulation of MBD domain containing proteins MeCP2 and MBD2,
365 occurs in human breast cancer, following the quantification of their transcripts in normal
366 breast and in benign and neoplastic breast tumors via RT-PCR analysis (Billard et al.
367 2002).

368 A study in 2003 for the first time established a link between MeCP2 mRNA and
369 estrogen receptor status. The authors not only showed high MeCP2 mRNA expression
370 levels in neoplastic tissues than in non-neoplastic tissues, but also discovered higher
371 MeCP2 transcript levels in estrogen receptor positive (ER⁺) breast cancer samples than
372 those present in estrogen negative breast cancer specimen (Muller et al. 2003).

373 In an effort to demonstrate existence of gene specific binding or association pattern of
374 MBD proteins in cancer cells, *Ballestar et al*, showed that MeCP2 was one of the select
375 MBD proteins that was uniquely in association with Ras association domain family 1A
376 gene (*RASSF1A*), ectonucleotide pyrophosphatase/ phosphodiesterase 4 (*ENPP4*),
377 phosphatidylinositol 4-phosphate 5-kinase (*PIP5K*) and retinoic acid receptor B2 gene
378 (*RARB2*) promoters (Ballestar et al. 2003). Such studies reveal that while certain
379 methylated CpG sequences interact with only one MBD protein, others can associate
380 either multiple or all of them. Thus, breast cancer involves MeCP2-dependent inhibition
381 of *BRCA1* gene expression, together with elevated MeCP2 gene expression in
382 neoplastic tissues and increased localization of MeCP2 at the promoters of various
383 tumor suppressor genes (TSGs).

384 An investigation aimed at characterizing MeCP2 target sequences in MCF-7 breast
385 cancer cells, employed a preparative-ChIP assay to create a library containing MeCP2
386 bound sequences, such as the retrotransposons (mostly with Alu repeats), matrix-

387 associated regions (MARs), CpG islands, and others (Koch and Stratling 2004). The
 388 study used multiple techniques including indirect immunofluorescence microscopy and
 389 concludes that the preference of MeCP2 at these regions is due to its sequence
 390 recognizing information potential (guanine nucleotide bases adjacent to CpG or TpG) as
 391 demonstrated in earlier foot-printing assays (Weitzel, Buhrmester, and Stratling 1997).
 392 Epigenetic silencing of estrogen receptor- α (ER- α) gene as a result of CpG
 393 hypermethylation correlates with the interaction and binding of MBD proteins including
 394 MeCP2, histone deacetylase 1 (HDAC1), and DNA methyl-transferases (DNMTs)
 395 proteins to the *ER* promoter in MDA-MB-231 breast cancer cells, and whose treatment
 396 with 5-aza-2'-deoxycytidine (inhibitor of DNMTs) and trichostatin A (inhibitor of HDAC
 397 activity) results in the vacating of the promoter by repressor complex comprising of
 398 MeCP2 and other MBD proteins, together with DNMTs and HDAC1 (Sharma et al.
 399 2005). This treatment further correlates to increased transcription activation marks
 400 (H3Ac, H4Ac and H3K4me2) and decreased repressive marks (H3K9me2), leading to
 401 the re-expression of *ER- α* gene. Such studies involving the use of epigenetic drugs
 402 allows for a deeper understanding of the fundamental molecular mechanisms of
 403 chromatin remodeling resulting in ER reactivation. Findings from such studies aid in
 404 developing therapeutic strategies against breast cancer via epi-drugs. It was later
 405 shown that the repressive complex comprising of MeCP2, HDAC1 and DNMT1, while
 406 present at *ER- α* promoter in ER-negative MDA-MB-468 cells, remained absent at the
 407 same region in ER-positive MCF-7 cells (Rasti et al. 2012). MDA-MB-468 cells express
 408 a mutant p53 whose binding to HDAC1, DNMT1 and MeCP2 is not altered at *ER- α*
 409 promoter. Investigating *ER- α* gene silencing in these cells showed that down regulating

410 DNMT1 or HDAC1 led to the disassembly of mutant p53 and the repressive complex
411 from *ER-α* gene promoter and this was consistent with the ER re-expression following
412 partial demethylation of *ER-α* promoter (Arabsolghar, Azimi, and Rasti 2013). HDAC1
413 plays a key role in *ER-α* gene silencing due to its deacetylase activity and for the
414 assembly of DNMT1 in the repressive complex. Also, mutant p53 binds to *ER-α* gene
415 promoter via direct association with HDAC1 and indirect association with DNMT1 and
416 MeCP2 in 468 cells (Arabsolghar, Azimi, and Rasti 2013).

417 An inhibitor of zinc-dependent deacetylases, valproic acid (VPA), brings about the
418 shutoff of *ER-α* promoter in a reversible manner via invoking recruitment of MeCP2
419 transcription repressor resulting in decreased levels of mRNA encoding *ER-α* and
420 subsequent loss of the protein in ER-positive breast cancer cells (Reid et al. 2005).

421 While characterizing epigenetic deregulation status in three breast cancer cells
422 representing different stages of human breast cancer, *Tryndyak et al.*, showed
423 significant epigenetic alterations in breast cancer cells as compared to normal
424 mammary epithelial cells MCF-10-2A and demonstrated that the more malignant MDA-
425 MB-231 cells have a greater loss of CpG methylation followed by aberrant expression of
426 DNMT1, MBD proteins: MeCP2 and MBD2, reduced Suv4-20h2 leading to loss of
427 H4K20me3, and H4-hyperacetylation as compared to MCF-7 cells (Tryndyak,
428 Kovalchuk, and Pogribny 2006). Such results suggest that malignant breast cancer cells
429 are associated with greater epigenetic deregulation, including aberrant expression of
430 MeCP2, which may potentially be an indicative of aggressive tumor phenotype during
431 tumorigenesis.

Epigenetic changes in mammary gland following exposure to ionizing radiation (IR) and the induced carcinogenesis in a rat model of breast cancer has been studied. IR exposure results in global DNA hypomethylation together with reduced levels of *de novo* DNA methyltransferase (DNMT3a and 3b), maintenance methyltransferase (DNMT1) and MeCP2 and linked to cell cycle control mechanisms and was in part associated to activation of DNA repair pathway (Loree et al. 2006). Following irradiation, apoptosis was significantly increased and was paralleled by a noticeable increase in cellular proliferation.

Mechanistic studies involving malignant breast cancer have shown that kallikrein-related peptidase 6 (KLK6), TSG silencing is due to formation of repressive chromatin mediated by histone deacetylation and CpG methylation-dependent binding of MeCP2 at its proximal promoter (Pampalakis et al. 2009). Stable overexpression of KLK6 in MDA-MB-231 cells exhibits reversal of malignant phenotype i.e., decreased proliferation rates, reduced cellular motility, inhibition of anchorage-independent growth, and reduced *in vivo* orthotopic tumor formation in severe combined immunodeficiency (SCID) mice. This suggests that via potential inhibition of epithelial-to-mesenchymal transition (EMT), KLK6 might exert a protective role against metastatic breast cancer.

Utilizing a luciferase assay, it has been shown that in MCF-7 cells, MeCP2 can repress select target gene promoters namely, *CD44*, phosphatase and tensin homologue (*PTEN*), *Cyclin D2* and glioma-pathogenesis related protein 1 (*GLIPR1*) (Muller et al. 2010).

Bisulfite sequencing analysis revealed that leukemia inhibitory factor (LIF) regulating progression of various types of cancer, is hypermethylated within the promoter region,

455 in normal breast epithelial cells but highly demethylated with cancer progression (Shin,
456 Park, and Jang 2011). In line with this observation, enrichment of MeCP2 and
457 H3K9me2 decreased with breast cancer progression while H3K4me2 activation mark
458 increased inversely together with increased binding of Sp1 transcription factor at such
459 hypomethylated sites. Genetic ablation of LIF lead to substantial reduction of cellular
460 growth and colony formation in breast cancer cells analyzed (Shin, Park, and Jang
461 2011).

462 Immunohistochemistry analysis reveals MeCP2 protein to be significantly higher in
463 invasive ductal carcinoma (IDC) specimen than in non-neoplastic lesions (Xu et al.
464 2012).

465 Upregulated in breast cancer, a disintegrin and metalloprotease domain-containing
466 protein 12 (*ADAM-12*) is subjected to transcriptional suppression by Z-DNA-forming
467 negative regulatory element (NRE) present at its 5'-UTR, facilitates binding to
468 epigenetic regulator, MeCP2 and nuclear family of transcription factors, NF1C and
469 NF1X, contributing to its low expression in normal cells and tissues (Ray et al. 2013).

470 Natural compounds including curcumin, epigallocatechin gallate (EGCG), resveratrol,
471 and others have been shown to cause reversal of epigenetic modification by reducing
472 the protein expression of DNMT1, MeCP2 and HDAC1 (Mirza et al. 2013).

473 Anti-cancer peptide SA12 binds to MeCP2 *in vitro* and results in upregulation of p53 and
474 PTEN expression and further inducing apoptosis by targeting Bcl-2 family and related
475 caspases in SKBr-3 cells (Yang et al. 2015).

476 Analysis of ~9221 human tumor samples from TCGA has allowed for the finding that
477 MeCP2 is a frequently amplified oncogene in a number of tumors including triple

negative breast cancer (TNBC) (Neupane et al. 2016). Over-expressing MeCP2 e2 isoform in human mammary epithelial cells (HMECs) enables anchorage independent growth to a level almost similar to that resulting from an infection with activated HRAS while the longer e1 isoform fails to enable such growth (Neupane et al. 2016). The same study further showed that while both the isoforms could activate PI3K pathway, only MeCP2 e2 isoform is capable of activating MAPK pathway in HMECs during growth factor deprivation. Such investigations have revealed MeCP2 functionality in different cellular contexts.

Above mentioned studies underscore the alteration in epigenetic process in ER-positive and TNBC cells and the role MeCP2 plays in silencing TSGs or promoting cancer survival.

Involvement in Colon cancer

Strong E-cadherin (*ECAD*) TSG expression and a lack of promoter CpG methylation in normal colonic mucosa and other well-differentiated adenocarcinoma has been observed, while both mucinous adenocarcinoma and signet-ring cell carcinoma display complete CpG methylation and strong MeCP2 expression patterns when 29 cases of colorectal carcinoma were analyzed in Indonesia (Darwanto et al. 2003). Loss of MeCP2 expression was found to be correlated to ECAD re-expression.

Patients with RTT are known to have gastrointestinal pathologies, owing to loss of parasympathetic control. A recent study reported the risk of colon cancer in a RTT patient (Yilmaz et al. 2014). Genome-wide studies in YB5 cells (a clonal derivative of the SW48 colon cancer cell line), demonstrated that re-expression of basally silenced and hypermethylated genes by the combinatorial action of carboplatin and decitabine

501 was significantly better than when induced by the two drugs alone. The combination
502 also brought about the inhibition of HP1 α expression accompanied by increase in
503 H3K4me3 and H3K9ac at the reactivated gene promoters together with decreased
504 binding of MeCP2 and MBD2 (Qin et al. 2015). Hence the epigenetic synergy shown by
505 the combination of a platinum analog, carboplatin, and decitabine can be used treat
506 various cancers.

507 As mentioned earlier, specifically targeting SIRT1 deacetylase by pharmacological
508 drugs, we discovered MeCP2 to be endogenously acetylated in RKO colon cancer cells
509 (Pandey et al. 2015). K171 residue identified via mass spectrometry analysis as an
510 acetylated site was particularly interesting as it was conserved from *Xenopus* species to
511 *Homosapiens*. Chromosomal associated proteins such as HDAC1, MeCP2 and ATRX
512 are known to bring about chromatin remodeling including repression of TSG expression.
513 We observed reduced binding of ATRX and HDAC1 to our acetylation mimetic mutant,
514 MeCP2 K171Q (Pandey et al. 2015). A different approach involving the pharmacological
515 inhibition of SIRT1 too showed consistent result. Transient and stable over expression
516 of K171Q mutant in colon cancer cells showed retarded growth and proliferation as
517 compared to those over expressing MeCP2-WT. This shows the role of specific MeCP2
518 lysine residue in regulating cell physiology and growth characteristics.

519 A recent study demonstrated that genetic ablation of MeCP2 in DLD-1 colorectal cancer
520 cells results in cell cycle arrest at G0/G1 phase, decrease in cell viability, and also leads
521 to inhibition of cell migration (Song et al. 2016). Results from this study that inhibition of
522 MeCP2 function in DLD1 cells leading to decreased cell growth are in consensus with
523 our study of MeCP2 K171Q mutant showing retarded growth in RKO cells. This

suggests that MeCP2 may serve as a potential target towards treating colorectal cancer.

In an unbiased screen for identification of drugs which reactivated basally suppressed genes in colon cancer cells, led to the identification of classical epigenetic drugs (inhibitors of DNA methylation and histone deacetylation) and 11 other pharmacological drugs which led to induction of methylated-CpG island promoters of not only endogenous TSGs but also the basally silenced promoters driving a GFP based reporter gene in multiple colon cancer cells (Raynal et al. 2016). Most of these pharmacological drugs were cardiac glycosides that did not alter DNA CpG-methylation locally or histone modifications (acetylation and methylation) globally, but instead affected calcium signaling by altering calcium-calmodulin kinase II (CamKII) activity thereby causing nuclear exclusion of MeCP2. Further inhibition of CamKII activity prevented gene reactivation and rescued drug induced cancer cell toxicity and killing (Raynal et al. 2016). These findings indicate that calcium signaling pathway can be targeted to prevent MeCP2-mediated TSG silencing in colon cancer.

Involvement in Prostate cancer

Prostate cancer remains to be the first and most common cancer affecting men in the United States and is a disease of older men (Mehta and Armstrong 2016). In a majority of prostate cancers, glutathione S-transferase 1 (*GSTP1*) gene promoter is subjected to hypermethylation and inactivation. While studying epigenetic regulation of *GSTP1* gene, Clark group has discovered that (Stirzaker et al. 2004); firstly, histone H3 acetylation is independent of gene expression. Secondly, seeds of DNA methylation promotes histone deacetylation. Thirdly, spreading of DNA hypermethylation across CpG islands is not

547 associated to MeCP2 but MBD2 binding and finally, H3K9me2 histone methylation
548 ensues only after histone deacetylation and is linked to hypermethylation of CpG
549 islands. Such findings provide insight into the molecular and mechanistic events
550 responsible for aberrant hypermethylation of CpG-islands and hence suppression of
551 such TSGs in prostate cancer cells.

552 Inhibition of MeCP2 expression has been shown to prevent growth in prostate cancer
553 cells, while its ectopic expression confers growth advantage (Bernard et al. 2006). Such
554 over-expression of MeCP2 allows androgen dependent LNCaP cells to grow and
555 maintain the tumorigenic phenotype in absence of androgen stimulation by preserving a
556 constant level of c-Myc during an androgen depleted condition. Furthermore, MeCP2
557 over-expressing LNCaP cells were demonstrated to have a functional p53 pathway and
558 retain their sensitivity to chemotherapeutic drugs (Bernard et al. 2006).

559 Tissue inhibitor of matrix metalloproteinase-2 (*TIMP2*) involved in cell growth and
560 invasion, is a TSG mostly expressed in normal tissues but silenced in glioblastomas and
561 lung tumors (Mohanam et al. 1995). *TIMP2* has a CpG methylated gene promoter and
562 remains suppressed in prostate cancer cells while is expressed in normal prostate
563 epithelial cells (Pulukuri et al. 2007). ChIP assay revealed presence of MeCP2 at
564 methylated *TIMP-2* gene promoter in prostate cancer cells, while absent in normal cells
565 and combinatorial treatment of decitabine and TSA restored its expression and as a
566 consequence resulted in reduced tumor cell invasion. Prostate tumors expressing low
567 *TIMP-2* levels showed CpG methylation of its gene promoter (Pulukuri et al. 2007).
568 These findings indicate that reduced *TIMP-2* gene expression is linked to its promoter

CpG methylation and MeCP2 localization, and that this may play a key role in progression of prostate cancer during invasion and metastasis.

MeCP2-knockdown in PC3 prostate cancer cells results in low proliferating cells compared to parental cells or those that are deficient in MBD1. MeCP2 deficient cells are also associated with elevated apoptosis while MBD1-deficient and parental cell show almost on par apoptosis (Yaqinuddin et al. 2008). Assays such as the Boyden chamber invasion and wound healing migration assays reveal that MeCP2-knockdown cells were both less invasive and migratory than MBD1-deficient cells. mRNA expression profiles of MeCP2- and MBD1-depleted cells show significant differences when compared to each other, and also when compared to the control (Yaqinuddin et al. 2008). Thus, MeCP2 and MBD1 have non-redundant functions in prostate cancer cells, or that their gene suppression affects independent cellular processes, and each protein could regulate distinct sets of genes involved in proliferation, invasion, migration and apoptosis.

Growth arrest and DNA damage inducible, alpha (GADD45alpha) plays a key role in DNA repair, cell cycle control and apoptosis (Zhan 2005). GADD45alpha expression in PC3 cells is higher than that in LNCaP and DU145 cells by 12- and 17-fold respectively (Ramachandran et al. 2009). An inverse correlation exists between GADD45alpha expression and methylation at 5' four CpG region in the proximal promoter of the gene. Reactivation of GADD45alpha expression has been seen in DU145 and LNCaP cells following pharmacological inhibition of DNA methylation. MeCP2 localizes to the 4 CpG region in DU145 cells and its knockdown leads to increased GADD45alpha expression. Increased GADD45alpha expression via either 5-azacytidine pre-treatment or by

recombinant GADD45alpha expression, leads to increased sensitivity to docetaxel (Ramachandran et al. 2009). Hence GADD45alpha is epigenetically regulated and serves as a potential target for treatment against prostate cancer.

A recent study aimed at investigating the importance of green tea polyphenols (GTPs) showed that human prostate cancer LNCaP cells when exposed to GTPs (1-10 µg/ml) for 1-7 days results in GSTP1 re-expression in a concentration- and time-dependent manner which was associated with decreased DNMT1 activity (Pandey, Shukla, and Gupta 2010). Time-dependent GTP treatment of LNCaP cells results in decreased transcript and protein levels of HDAC 1-3; MBD1, MBD4 and MeCP2, and increased levels of acetylated histones H3 and H4. Exposing LNCaP cells to GTP does not lead to global hypomethylation as indicated by methylation-specific PCR results for LINE-1 promoter, infact maintains genomic integrity as prometastatic gene *S100P* expression is decreased in contrast to treatment with decitabine that results in global hypomethylation and increased *S100P* expression (Pandey, Shukla, and Gupta 2010). GTP thus acts as a chemotherapeutic agent due to lack of its toxicity and ability to alter DNA methylation and histone modifications.

In DU145 cells it has been demonstrate that MeCP2 binds to the methylated target gene promoters, *Cyclin D2* and *GLIPR1*, and that this binding is lost following treatment with decitabine (Muller et al. 2010). Above studies thus accentuate that epigenetic deregulation in prostate cancer involves aberrant enrichment of MeCP2 at hypermethylated gene promoters and that either removal of CpG methylation or targeting MeCP2 itself is key to regain TSG re-expression and decreasing cancer growth.

Exposing LNCaP cells to curcumin (CUR) has been demonstrated to result in demethylation of the proximal 14 CpG sites of *Neurog1* gene and result in the restoration of its expression (Shu et al. 2011). While having limited effects on the expression of MBD proteins; MBD2, MeCP2, and DNMT proteins; DNMT1 and DNMT3a, CUR exposure led to significantly reduced binding of MeCP2 at Neurog1 gene promoter and had varying effects on the expression of HDACs, but decreased the total HDAC activity. Cur treatment resulted in decreased H3K27me3 binding at the Neurog1 gene promoter as well as a decrease at the global level in LNCaP cells (Shu et al. 2011).

MeCP2 knockdown in PC3, LNCaP, and normal murine NIH3T3 cells results in a defective cell cycle progression and a significant decrease in cellular proliferation, together with cellular aggregation in S/G2M phase of cell cycle without leading to either a severe apoptosis- and/ or senescence-like outcome (Babbio et al. 2012). MeCP2-depleted PC3 cells show substantial decrease in lamin A, lamin B1, lamin C and lamin B receptor (LBR), and immunofluorescence microscopy on such cells demonstrate the appearance of an altered nuclear lamina and nuclear rim (Babbio et al. 2012). Such results indicate that cells deficient in MeCP2 lose their ability to correctly assemble their nuclear envelope (NE) as they have decreased levels of NE components, like lamins and LBR, and hence are associated with decreased cellular proliferation and viability. MeCP2 may thus potentially act as a “bridge” between chromatin and NE, facilitating stability of the chromatin at nuclear periphery.

Exposure of LNCaP and DU145 cells with sulforaphane (SFN), an organosulfur compound rich in cruciferous vegetables results in decreased activity and expression of

human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, that is associated with alterations at the hTERT promoter region in chromatin dynamics and compaction involving histone PTMs, H3K4me2 and H3K18Ac (Abbas et al. 2016), linked with prostate cancer recurrence (Seligson et al. 2005). ChIP-qPCR indicated that MeCP2 occupancy is elevated at hTERT promoter region with increased nucleosomal density following SFN treatment (Abbas et al. 2016). These results underscore the role SFN mediates in suppression of hTERT in prostate cancer cells and the inherent capacity of such organosulfur compounds to change the chromatin dynamics and provide a novel dietary approach by which phytochemicals provide chemoprevention.

Summary

This review highlights various intricate roles MeCP2 plays in different cellular contexts. Owing to its unusual structural features and continually increasing list of PTMs and candidate binding partners, MeCP2 can differentially regulate gene expression. There is a slow-yet steady increase in appreciation that such features of MeCP2 are exploited by cancer cells. The dire consequence of elevating or suppressing MeCP2's role in pathogenic condition like cancer mentioned herein, make them attractive targets for therapeutic intervention. Nonetheless, MeCP2's involvement in the basic regulatory aspects in different tissues such as the brain, becomes a limitation. There is thus an increasing need for gaining a deeper comprehension of MeCP2 biology.

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Figure legends

Figure 1. Approximate representation of MeCP2 isoforms and their domain maps.

A. MeCP2 e1 isoform is encoded by exons 1, 3 and 4 and has a translational start site (TSS) in exon 1 while MeCP2 isoform 2 is encoded by exons 2, 3 and 4 with its TSS located in exon 2. **B.** Domain map of MeCP2 isoforms is shown. N, N-terminal; MBD, Methyl-binding-domain; A-T Hook domain; TRD, Transcriptional repression domain; His-rich, Histidine-rich domain; Pro-rich, Proline-rich domain, C, C-terminal.

Figure 2. MeCP2 is transcriptional suppressor. MeCP2 recognizes CpG-methyl groups on DNA via its MBD domain and recruits co-repressor mSIN3A and HDAC1 to cause histone deacetylation, chromatin compaction and hence target gene suppression.

Figure 3. Distribution of different post-translational modifications (PTMs) on MeCP2 domain map. Approximate distribution of different PTMs over MeCP2 (e2 isoform) domain map is shown. Amino acids marked in black, red and blue color respectively represent, sites for acetylation, phosphorylation and sumoylation. An approximate amino acid scale is represented at the top of the domain map. Below the domain map are candidate binding partners of MeCP2 showing minimal region required for interaction (represented by horizontal lines).

Figure 4. MeCP2 is a transcription activator. MeCP2 interacts with CREB1 at target gene promoters and provides accessibility to RNA Polymerase II to result in gene expression.

Figure 1

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Figure 2

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Figure 3

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Figure 4

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