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Clonal Rett Syndrome cell lines to test compounds for activation of wild-type MeCP2 expression

Dongbo Yu^a, Fuminori Sakurai^b, David R. Corey^{a,*}^a Department of Pharmacology, UT Southwestern Medical Center at Dallas, Dallas, 6001 Forest Park Road, TX 75390, USA^b Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

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ABSTRACT

Rett Syndrome is an X-linked progressive neurological disorder caused by inactivation of one allele of the *MECP2* gene. There are no curative treatments, and activation of wild-type *MECP2* expression is one strategy for stabilizing or reversing the disease. We isolated fibroblast clones that express exclusively either the wild-type or a 32-bp-deletion mutant form of *MECP2*. We developed a sensitive assay for measuring wild-type *MECP2* mRNA levels and tested small molecule epigenetic activators for their ability to activate gene expression. Although our pilot screen did not identify activators of *MECP2* expression, it established the value of using clonal cells and defined challenges that must be overcome.

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Rett Syndrome (RTT) is a severe neurological disorder that affects 1 in 10,000 girls.¹ After a brief period of seemingly normal post-natal development, symptoms appear that include deceleration in growth, loss of acquired motor and language skills, characteristic hand-writhing movements, muscle hypotonia, and cognitive impairment.^{2,3} Symptoms grow progressively more severe but long-term survival can be achieved with supportive care. There are no curative treatments for RTT, and strategies for slowing, stabilizing, or reversing the disease constitute an urgent yet unmet need.

RTT is predominantly caused by mutations within a single identified gene, methyl CpG binding protein 2 (*MECP2*)⁴, providing us with an obvious therapeutic target. *MECP2* is an X-linked gene that modulates expression levels of large number of genes^{5,6}, with the neuropeptide *BDNF* being a well-studied example.^{7–9}

Since almost all patients are female and the majority are heterozygous for the *MECP2* locus, one intact copy of the wild-type *MECP2* gene exists in all cells of the body. Only a subset of these cells express wild-type *MECP2* due to the process of random X-inactivation during early development, resulting in a mosaic pattern of MeCP2 protein expression throughout the body. Some cells express wild type *MECP2*, while others express the inactive mutant variant and have insufficient MeCP2 function. MeCP2 is expressed across a large number of tissue types¹⁰, and earlier work pointed to the exclusive role of MeCP2's dysfunction in neurons as the cause

of RTT.¹¹ More recent studies have implicated non-neuronal cells, in particular the astrocytes, to be significant players in the pathogenesis of RTT.^{12,13}

Agents that activate expression of the wild-type allele on the silenced X-chromosome might overcome a lack of MeCP2 protein and benefit patients. Support for this hypothesis comes from studies in mouse models with an engineered *MECP2* gene deletion that mimics human RTT.¹⁴ Related studies demonstrated that conditional genetic reactivation of wild-type MeCP2 protein expression in mutant mice can reverse the disease phenotype even in late-stage adult animals.¹⁵ The work has encouraged the belief that RTT can be treated by re-expression of wild-type MeCP2.

Gene therapy provides one strategy for increasing *MECP2* expression. Alternatively, pharmacological reactivation of the wild-type MeCP2 protein expression in cells where it is silenced due to X-inactivation would also be therapeutically beneficial in treating RTT. In vitro cell-based models are valuable tools in studying genetic diseases including RTT.¹⁶ Screening for small molecule gene activators, however, requires development of sensitive assays to detect enhanced expression of *MECP2*. Examining activation in a heterogeneous population of patient cells will be difficult because any upregulation will need to be evaluated against a background of 50% or more expression of wild-type *MECP2*.

For example, an agent that promotes production of MeCP2 at a fraction of the wild-type level would be an excellent starting point for development. This relatively low level, however, would be difficult or impossible to discern against background MeCP2 expression in a heterogeneous cell population. Successful identification

* Corresponding author.

E-mail address: david.corey@utsouthwestern.edu (D.R. Corey).

of lead compounds for gene activation would, therefore, benefit from possession of a clonal cell line with a silent wild type copy of the gene.

To specifically probe for the reactivation of wild-type *MECP2* in cells, we first isolated single cells and established fibroblast lines that express only the mutant copy of *MECP2* gene. We started this process with RTT patient-derived fibroblast cell-line, GM11272 (Coriell Institute). Many RTT cell lines containing different mutations in *MECP2* are available¹⁷ and might have been used, but we chose GM11272 because it contains a 32-bp deletion (1155–1186) in exon 4 of *MECP2* (Fig. 1) that simplifies discrimination of the wild-type and mutant alleles.

We derived single cell clones by diluting a suspension of GM11272 cells to 10 cells/mL and seeding them into 96-well plates at 100 μ L/well. Cell clones with morphology and doubling time similar to the parent strain were then expanded with Minimum Essential Media Eagle (Sigma) supplemented with 15% FBS and 5% MEM non-essential amino acids (Sigma). Their RNA was then harvested and reverse-transcribed into cDNA for PCR analysis to classify each clone as 'wild-type' or 'mutant' with regard to the *MeCP2* expression. We used primers flanking the 32-bp deletion region that yield amplification products of different sizes when run on agarose gel.

Most of the isolated clones expressed wild-type *MeCP2*. The few clones that expressed mutant *MeCP2* and were silent for wild-type *MeCP2* grew relatively slowly. These data suggest that the absence of *MeCP2* is detrimental to cells, but this deficit was not sufficient to prevent isolation of several cell lines with the silent wild-type allele.

Identifying a pharmacological agent capable of reactivating a gene on a silent X-chromosome is challenging and would benefit from sensitive assays that can detect even small amounts of expression. Such low level expression might then serve as a basis for subsequent strategies to increase expression to therapeutically useful levels. For example, we have shown that duplex RNAs complementary to gene promoters can activate gene expression.¹⁸ This activation, however, requires detectable expression of target transcripts at the gene promoter.^{19,20} One approach for achieving useful levels of *MeCP2* would be to use pharmacological agents to prime low level expression, and then use duplex RNA to selectively activate *MeCP2* transcription to a higher level. This approach

might also reduce off-target effects by allowing minimal concentrations of synthetic epigenetic modifiers to be effective.

We developed a 'nested' PCR approach to amplify the cDNA region around the 32-bp deletion in two separate rounds, with each round involving 25–40 amplification cycles (Table 1, Fig. 2). In the first round, an 'outer' primer pair amplifies a 285-bp long fragment with the reverse primer overlapping with the deletion region, thereby making the mutant transcript unable to serve as the template and thus affording allele selectivity. A second round of PCR was added using an 'inner' primer pair with the reverse primer lying entirely within the deletion region, which provides a second layer of allele selectivity and increases the sensitivity of detection for the wild-type transcript.

Even without nested PCR, a population containing just 10% wild-type cells could be unambiguously detected (Fig. 2b). Using nested PCR, however, we could readily detect a wild-type cells at 1:100 dilution. These data suggest that even a 1% upregulation of *MECP2* expression should be detectable.

The 'nested' PCR products were run out on 2% agarose gels, and the presence or absence of a bright band around 250-bp indicates whether detectable levels of wild-type *MECP2* transcript existed in the starting RNA sample. Due to the high sensitivity of the assay, experiments lacking reverse transcriptase were run in parallel as negative controls. The PCR band was excised and cloned into PCR4-TOPO vector (Invitrogen) for sequencing, which confirmed the identity of the amplified inner *MECP2* fragment.

Table 1
Sequences of the primers used in 'nested' PCR

Name	Sequence
<i>1st round (outer pair)</i>	
Forward	5'-AGCGCAAGACCCGGGAGACGG-3'
Reverse	5'-TGGGGTCTCTCGGAGCTCTCGGGCT-3'
<i>2nd round (inner pair)</i>	
Forward	5'-GACCCGGGAGACGGTCAGCA-3'
Reverse	5'-AGTCTCGGGCTCAGGTGGAGGT-3'

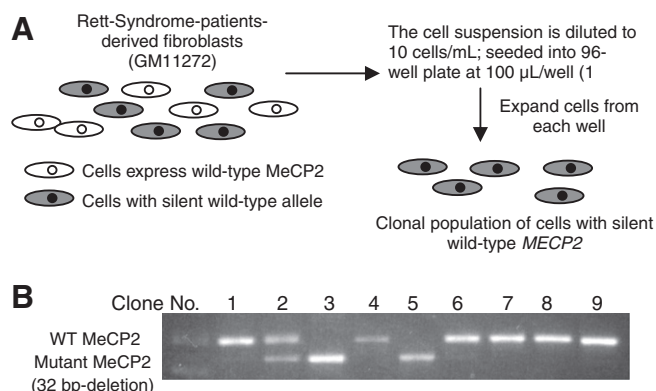


Figure 1. (A) Experimental procedure employed to isolate clones that express only wild-type or mutant *MeCP2*; (B) Determining the expressed *MeCP2* isoform of each expanded fibroblast clone by PCR. RNA is harvested from each clone, reverse-transcribed and amplified using primers flanking the 32-bp deletion. WT-*MeCP2*-expressing clones yielded PCR products with higher molecular weight than the shorter ones produced by the mutant-*MeCP2*-expressing clones. Each lane represents a different clone. 'Clone' 2 likely was the result of two parent cells, one mutant and one wild-type.

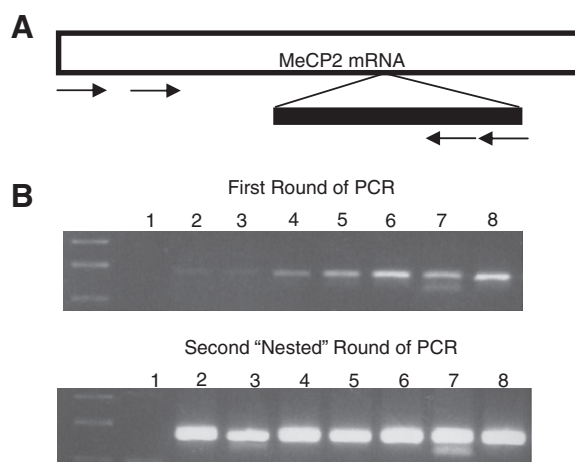


Figure 2. Nested PCR as a selective and sensitive assay for detecting wild-type *MECP2* transcript. (A) Top: schematic showing the relative positions of the nested PCR primers relative to the 32-bp deletion in *MECP2* gene locus; (B) (top) A single round of PCR with deletion-targeting primer pair achieved allele-selectivity but not sensitivity of wild-type *MECP2* transcript detection; (bottom) A second 'nested' round of PCR detects wild-type transcript at as low as 1% of the total RNA mixture while maintaining selectivity in a background of mutant *MECP2* transcript. cDNA template used in each lane (from left to right starting with lane 1) is produced from mixtures of wild-type and mutant clonal fibroblasts with the following ratios: 0:100, 1:99, 5:95, 10:90, 25:75, 50:50, 75:25, 100:0.

Table 2

List of compounds and their maximally tested concentrations

Drug name	Published effective concentration (μM)	Maximum tested concentration (μM)
SAHA	2.5–7.5 ²⁴	1000
RG-108	10 ²⁵	1000
LBH-589	0.1 ²⁶	100
Valproic acid	2000 ²⁷	100,000
Zebularine	100 ²⁸	1000
Trichostatin A	0.2 ²⁹	100
5-aza-dC	0.6 ²⁹	100

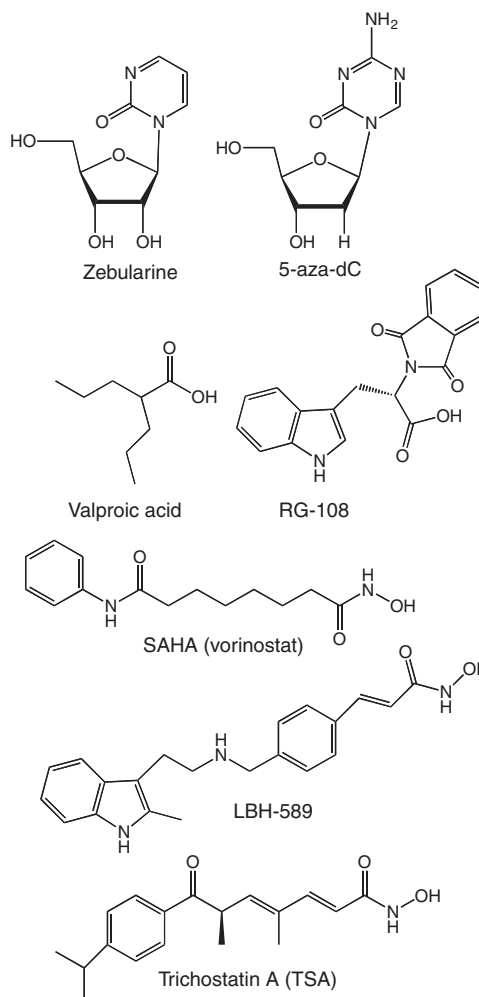
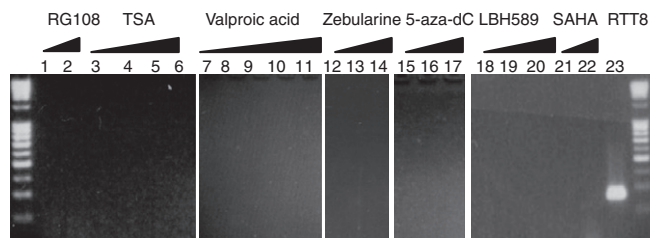
We performed a pilot screening experiment using several compounds previously demonstrated to be epigenetic modifiers capable of reactivating expression of silenced genes in various cell-lines. The compounds were from two major categories: inhibitors of DNA methyl transferases (DNMT) and inhibitors of histone deacetylases (HDAC), the two major classes of enzymes frequently implicated and targeted in epigenetic studies.^{21–29} Trichostatin A (TSA) and 5-aza-dC were dissolved in DMSO at 5 mM stocks; SAHA was dissolved in DMSO at 100 mM stock; LBH-589 and RG108 were dissolved in DMSO at 10 mM stocks; zebularine was dissolved in water at 100 mM stock; valproic acid was dissolved fresh in water to make 1 M stock. We verified the identity of all compounds by mass spectrometry and used them at concentrations above those previously reported to be necessary for gene activation.

Mutant clonal fibroblasts were seeded at 60,000 cells per well in 6-well plates on Day -2. Drug treatment occurred 48 h after seeding on Day 0, with the highest concentrations tested at levels 1000-fold greater than previously reported for gene activation (Table 2). The compounds were mixed in 250 μL media, old growth media was removed from the wells, 1 mL of warm fresh media was added, and 250 μL drug-containing media was added drop-wise in a circular motion over each well. Gentle rocking movements were used to evenly spread the drug over the entire well. Media were changed at 24 h.

Total cellular RNA was harvested on Day 3 (72 h after drug-treatment) by treating with Tri-Reagent (Sigma). Chloroform extraction of the aqueous layer followed by isopropanol and 70% ethanol washes yielded total cellular RNA, which was DNase-I treated and reverse-transcribed to produce cDNA that served as template for detection by nested PCR.

We treated RTT clonal fibroblasts with the epigenetic modifying compounds (Fig. 3). While the cDNA derived from the control wild-type clonal cells repeatedly yielded bright bands near the predicted 250 bp size, none of the mutant *MECP2* fibroblast-derived cDNA consistently produced the expected PCR band regardless of the type of drug treatment received (Fig. 4). Occasionally, bands appeared in mutant cell-cDNA lanes of both RT and no-RT control conditions. These results, however, were not reproducible and can be explained by the high sensitivity of the assay and the potential for artifactual amplification products. It is likely that the bands are due to either genomic DNA contamination of experimental material or trace contamination from the products of previous PCR amplifications. We also tested compounds in combination. TSA/5-azaC, TSA/5-aza-dC/zebularine, RG108/5-aza-dC, RG108/TSA, TSA/5-aza-dC/zebularine/RG108 or TSA/5-aza-dC/zebularine/RG108/LBH589 combinations were inactive. Exposure of cells to 5-aza-dC for up to six days did not activate MeCP expression. Apicidin and MS-275 were also inactive.

Careful handling of pipettes, autoclaving the Eppendorf tubes, using an isolated bench area with constant ethanol wiping, as well as more stringent DNase I treatment conditions (37 °C for 30 min and 75 °C for 15 min) reduced but did not eliminate the occurrence

**Figure 3.** Chemical structures of epigenetic agents used.**Figure 4.** Effect of synthetic agents on reactivation of WT MeCP2. Lanes 1–2: RG-108 at 10 and 100 nM, respectively; lanes 3–6: TSA at 1, 5, 10 and 100 nM; lanes 7–11: valproic acid at 1, 10, 100, 1000 and 10,000 nM; lanes 12–14: zebularine at 1, 10, 100 nM; lanes 15–17: 5-aza-2'-deoxycytidine at 1, 10 and 100 nM; lanes 18–20: LBH-589 at 1, 10, 100 nM; lanes 21–22: SAHA at 10 and 100 nM; lane 23: RTT8 wild-type fibroblast-derived cDNA as template (positive control).

of occasional contaminant bands. To achieve meaningful results, experiments were repeated and results examined together.

Our results highlighted both the strengths and challenges of our approach. Regardless of the agent used, the experiments did not show evidence for reactivation of *MECP2* expression. cDNA from wild-type control fibroblasts, however, consistently produced bright bands showing that *MECP2* can be routinely confirmed by the nested PCR approach. As little as one *MECP2*-expressing cell in 100 can be readily detected. While the sensitivity of the assay made these initial screening experiments more labor-intensive, the sensitivity also ensures that even a small amount of gene

activation can be detected and used as a starting point for future experiments aimed at improving the potency of *MECP2* activation.

Why was activation not observed? Answering this question will require a better understanding of how the *MECP2* gene is silenced during X-chromosome inactivation and how silencing is maintained. Such understanding will facilitate rational design of therapeutic development strategies that can make use of the clonal cell lines and assays described above.

Rett Syndrome is a debilitating neurological disease with a single genetic cause yet without effective treatment. In this study, we explored a small-molecule-based approach in an effort to reverse the disease at its root cause, i.e., the absence of wild-type MeCP2 protein in affected cells. We successfully isolated a number of single cell-derived clones that selectively express only the mutant or wild-type *MECP2* transcript, and developed a nested-PCR-based method that detects low levels of wild-type *MECP2* mRNA. A pilot screen involving 7 commercially available epigenetic compounds did not find an agent that consistently reactivated wild-type MeCP2 in a mutant background. Nevertheless, the sensitivity and robustness of this in vitro system provides us with an important tool for future screening of effective agents against RTT.

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