

Research Update July 2006: Where do we Stand Now?

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RSRF held its annual Research Symposium from June 25th-28th at the Eaglewood Resort just outside Chicago, Illinois. Over a hundred scientists gathered to discuss their latest research findings and gain a broad perspective on the field of MeCP2 research as a whole. By all counts, this was RSRF's most stimulating symposium yet — in large part because interest in the biology of Rett syndrome has acquired a momentum that is stimulating dialogue across disciplines in an unprecedented fashion.

The meeting began on Sunday afternoon with a “State of the Research” talk by Huda Zoghbi, M.D. Dr. Zoghbi's lab published their discovery of the genetic basis of Rett syndrome in October 1999. Since that time, the Zoghbi lab and numerous others have striven to understand how mutations in MeCP2 (the acronym for “methyl CpG-binding protein 2”) lead to the complex and devastating symptoms of Rett syndrome. Approximately fifty parents and family members attended the talk and engaged in lively discussions afterward. From my perspective as a newcomer to the Rett community, I was impressed with the level of enthusiasm for research evidenced by the audience; it's very clear Rett parents are committed to doing whatever they can to support scientists as they work toward a treatment.

It was also very clear, from both Dr. Zoghbi's talk and dozens of presentations throughout symposium, that the field has made significant gains in understanding 1) the range of symptoms that are caused by MeCP2 mutations, 2) how MeCP2 is involved in regulating the expression of other genes, and 3) what MeCP2 enables neurons to accomplish. The rest of this article will attempt to describe the current consensus in these areas in terms that will be accessible to the novice yet interesting to those more experienced in all matters concerning MeCP2.

A primer in the genetics of Rett syndrome: X, Y, and XCI

Most cases of Rett syndrome are sporadic, meaning that they are not inherited from earlier generations. This, coupled with the rarity of classic Rett, made it difficult to track the genetic basis of the disorder, because there were few clues as to where in the genome to look for a mutation. The identification of a few families with more than one affected member, however, helped researchers to map the gene to a region on the X chromosome, one of the sex-determining chromosomes. (Females have two X chromosomes whereas males have an XY pair.) After narrowing the candidate region on the X chromosome to a few hundred genes, researchers began the painstaking process of analyzing one gene after another to find the Rett syndrome mutation. To do this, they had to compare DNA samples from affected girls and their families: a disease-causing mutation should occur in the daughter but not in either parent. It took over a decade of intensive searching, but a gene called *MECP2* was eventually found to be the cause of 95% of Rett syndrome cases.

This discovery shed light on why classic Rett syndrome develops primarily in girls. Since females have two X chromosomes in every cell, one copy is randomly inactivated to shut down gene expression and keep the gene dosage equivalent to that of a male, who has only one X. This process in females is known as X chromosome inactivation (XCI). Because the inactivation is

(usually) arbitrary, the X chromosome with a mutated copy of *MECP2* will be inactivated in some cells of Rett syndrome girls, thereby mitigating the effect of the mutation to a certain extent and allowing survival and mostly normal infant development before the full-blown disease appears. (If X chromosome inactivation is skewed away from the usual 50:50 on/off ratio, the disease can be either much more mild or much worse, depending on whether most cells express the healthy or the mutant *MECP2*.) A male doesn't have the option of shutting down expression of his X chromosome, so any mutation on the X is going to exert its full effects. Some families with recurrent Rett syndrome have given birth to sons with a severe neonatal encephalopathy that is fatal within a few months; the mutations that cause this very severe disease in males are the same as those that will cause classic Rett syndrome in females. More severe mutations in *MECP2* could cause male fetuses to die before term or shortly after birth (and never be diagnosed). Conversely, when male children bear a mild mutation in *MECP2* that would cause little or no effect if it were in a female child, they tend to develop mental retardation with seizures and motor disorders or even early onset schizophrenia or manic-depressive illness.

As is often the case with biology, there are exceptions to these general rules about random X inactivation and males with mutations on the X chromosome. For some reason, a number of girls with *MECP2* mutations have skewed X inactivation favoring cells expressing the healthy version of the MeCP2 protein. (This favorable XCI occurs in female mice, too.) If the skewing is particularly favorable—say, 90% of cells express the normal *MECP2* and only 10% express the mutant form—girls with mild mutations might not show any symptoms, while girls with very severe mutations could exhibit a much milder disease than would otherwise be expected. And there are two ways in which boys may develop a syndrome that looks just like classic Rett: aneuploidy (an abnormal number of chromosomes) and somatic mosaicism. If a boy has Klinefelter syndrome, for example, he possesses an extra X chromosome, so his karyotype is XXY instead of XY; if he bears a *MECP2* mutation on one of the X chromosomes, he could develop the classic form of Rett syndrome. These kinds of abnormalities occur at fertilization, and all the cells produced by the many subsequent stages of cell division will carry the same mutation. Somatic mosaicism arises when the mutation is not inherited from the parental sperm or egg but occurs some time after conception, so that only some cells carry the mutant gene. In this case, if a male fetus somehow acquires a mutation in *MECP2* in one of his cells, he would express the mutant protein only in cells descended from that “mother cell.” In this sense, somatic mosaicism has much the same effect as random X inactivation in females.

While we're on the topic of genetic abnormalities, we should point out that *losing* MeCP2 function due to mutation is not the only way to wreak havoc in the brain. Last year, a Belgian group identified four male patients who suffered from *too much* MeCP2 through duplications of the *MECP2* region. In other words, the gene itself was perfectly fine, but it was expressed at twice the normal levels. Surprisingly, these patients had some symptoms very reminiscent of Rett: head growth deceleration, hypoactivity, seizures, little or no speech, apparently severe cognitive impairment. (One patient with a triplication has also been found, and his symptoms were even worse.) MeCP2 levels are just as important in mice: transgenic mice that overexpress MeCP2 also develop a neurological syndrome very reminiscent of that seen in the Rett mouse models. This is one significant clue that *MECP2* gene dosage is very finely regulated in the brain. Too much and too little are equally detrimental.

MeCP2 and the developing brain

In normal development, genes are turned on at different, but precisely regulated, times and places in the body. This program of gene expression is roughly analogous to a long and complex musical score being brought to life by an orchestra: not all the instruments play all the time, or even at the same time, nor do they play the same notes or at the same dynamic strength. The *MECP2* gene encodes a protein (also called MeCP2, but written without italics and with a small “e”) that helps orchestrate the “rests” that certain genes must take during this developmental symphony.

MeCP2 expression in the developing brain is itself finely tuned: it appears first during embryogenesis in those brain regions that govern essential functions such as breathing, but its expression slowly crescendos and spreads as our neurons mature in childhood. MeCP2 appears to be crucial to the ability of neurons to respond to stimuli as we learn and form memories—technically speaking, this is known as “activity-dependent synaptic plasticity”—which would explain why Rett children do relatively well until several months after birth, when the ability to acquire language and other fine skills seems to evaporate. Mice in which *Mecp2* was deleted only in mature neurons in the brain (after the pups had been born and weaned) provide support for this notion: they develop a neurological syndrome that resembles Rett. What is puzzling is why too much MeCP2 is deleterious to neurons. The mice that overexpress MeCP2 actually show a *greater* ability to learn in early life, up until about 12 weeks of age (which is young adulthood for a mouse), but then they develop seizures and other features reminiscent of Rett in mice and die prematurely. Once again, too much MeCP2 is as bad as too little. This may be problematic for gene therapy approaches: some neurons in Rett girls express the normal protein (remember random X inactivation), and expressing more MeCP2 in these neurons would harm the cells.

MeCP2 quiets gene expression at multiple levels

MeCP2 was initially discovered as a transcriptional repressor, a protein that keeps genes from being expressed. Genes that are meant to be silenced will acquire a methyl group consisting of carbon and hydrogen atoms that attaches to a DNA sequence in the gene’s control center (called a promoter region). The head of the MeCP2 protein anchors itself to the methylated DNA, while the tail recruits other molecules to the target gene that change its electrical charge and collapse it into a very compact form. In its new, dense geometry the gene becomes inaccessible to transcription factors and cannot be expressed (or, to put it another way, the gene is silenced). If a defective MeCP2 cannot bind properly to the methylated DNA, or if it can bind the DNA but cannot recruit the other molecules that help in the silencing, the gene will be expressed—it will blare its notes at the wrong time. Since MeCP2 likely has a number of target genes it normally silences, the result of MeCP2 dysfunction is a neural cacaphony.

More recent work has shown that MeCP2 silences genes at the chromatin as well as the DNA level. DNA would take up an enormous amount of space if it were simply laying around the nucleus as a double helix millions of base-pairs long. It is much more efficient for the cell to package DNA by winding it tightly around nucleosomes that are in turn linked together — imagine a series of spools wrapped in and connected with the same DNA thread. The collection

of spools is called chromatin. (Turn the coiling up a couple of notches and you get complex structures called solenoids, minibands, and ultimately chromosomes.) Last year, scientists discovered that MeCP2 can help assemble chromatin into “silent loops” that are inaccessible to transcription factors. Such loops are absent in the brains of MeCP2 null mice, leaving genes such as *Dlx5* and *Dlx6* in active chromatin regions where they can be (inappropriately) expressed.

Other factors involved in regulating gene expression might produce syndromes similar to Rett

MeCP2 is not the only protein involved in silencing other genes, but it was the first such protein found to cause a human disease. There are other methyl-binding proteins, all of which may help regulate gene expression. Mutations in any of these proteins (or proteins that interact with them) could be responsible for atypical Rett syndrome or other disorders such as autism, severe neonatal encephalopathy, and non-syndromic mental retardation. In fact, although MeCP2 mutations account for over 95% of all classic Rett syndrome cases, they have been identified in only about half the cases diagnosed as atypical Rett. Could there be factors that help regulate MeCP2 activity that could cause a Rett-like syndrome? Apparently yes: in 2004, two different groups found that mutations in another X-linked gene, *CDKL5* (cyclin-dependent kinase-like 5), are responsible for a number of cases of atypical Rett. MeCP2 and *CDKL5* are expressed in a very similar spatio-temporal pattern during brain development (at least in mice) and there is evidence that *CDKL5* indirectly regulates MeCP2 activity. Unfortunately, the function of *CDKL5* is not yet understood. Additional research to define the pathway of this interaction could identify new proteins that cause other Rett syndrome-like conditions in children who do not carry mutations in either MeCP2 or *CDKL5*.

Closer scrutiny of mouse models reveals new abnormalities

The Rett field has benefited tremendously from being able to study and compare various mouse models that bear different sorts of mutations in *MECP2*. The first animal model is usually a knock-out, i.e., one in which the gene of choice is deleted from the animal’s genome, in the hopes that what goes wrong will then tell us a lot about that gene’s normal function. Two different MeCP2 null mouse lines have been studied, and they do develop symptoms that are reminiscent of Rett syndrome in human children (see Table 1): a period of generally typical development that is interrupted at about 4-6 weeks with tremors and spasticity, and progresses to breathing dysrhythmias and further deterioration. A mouse model that more closely resembles human Rett syndrome was made by inserting one of the milder mutations in MeCP2 that causes Rett in girls. The mutation causes MeCP2 to be truncated at amino acid 308, making it shorter than normal, and the mice track the human disease with surprising fidelity. Each model has its advantages, and closer examination of the mice in the coming years will continue to produce new insight into both normal and abnormal development. Recently, for example, a group of scientists revisited the null mice in the first days of life, and they found that they *do* show early signs of neurological impairment — as early as 5 days after birth — and act quite differently from normal mice in their response to social isolation. Clinicians are realizing the same is true of some children in that the “period of normal early development” may not be so normal. As researchers learn more about the nuances of mouse behavior and neurological function, parents and

clinicians will be better equipped to screen for very early signs of development going awry in children.

Table 1 presents several of the most-studied mouse models and the main conclusions that can be drawn from the published studies.

Mouse model	symptoms	reference
MeCP2 null	By 6 weeks: tremors, hypoactivity, spasticity. Irregular breathing by 10-12 weeks. Death at 12 weeks MeCP2 is more important for postnatal development than embryogenesis.	Guy et al. Nat Gen 2001 Chen et al. Nat Gen 2001
MeCP2 null	By 5 days: Dramatic increases in ultrasonic vocalization in response to social isolation; sensory reflex and behavioral response abnormalities MeCP2 deficiency <i>does</i> cause early abnormalities that are not apparent on gross inspection.	Picker et al., Neuroreport 2006
Deletion of MeCP2 in the brain at 12 days gestation	Identical to null mice; selective deletion of MeCP2 in <i>postnatal</i> neurons produced same phenotype, but delayed. Again, early development is not much hindered by loss of MeCP2, but postnatal maturation requires it.	Chen et al. Nat Gen 2001
Selective deletion of MeCP2 in postnatal forebrain	Hindlimb claspings, impaired motor coordination, increased anxiety, and abnormal social behavior. Normal locomotor activity and unimpaired context-dependent fear conditioning, MeCP2 loss in even <i>one</i> brain region, after birth, is sufficient to cause severe dysfunction.	Gemelli et al. Biol. Psych. 2006
MeCP2 ³⁰⁸ (creates a truncated protein)	Onset by 6 weeks: tremors, hypoactivity, spasticity, seizures, ataxia, irregular breathing, paw stereotypies, social behavior abnormalities, scoliosis/kyphosis. Premature death. Spatial memory, contextual fear memory,	Shahbazian et al. Neuron 2002

	<p>and social memory impaired.</p> <p>This model very closely tracks the human disease and will be very useful for studies of possible treatments as well as providing insight into what happens when MeCP2 is merely dysfunctional as opposed to totally absent.</p>	Moretti et al. J Neurosci 2006
MeCP2 over-expressed at twice the normal levels	<p>Enhanced learning early on (10 weeks), but after 20 weeks, develop spasticity, stereotypies, seizures, progressive motor deterioration, ataxia, altered synaptic plasticity, premature death.</p> <p>Too much MeCP2 is dangerous.</p>	Collins et al. Hum Mol Genet 2004
Normal MeCP2 expressed in post-mitotic neurons under the <i>tau</i> promoter in MeCP2 null mice	<p>Equivalent to wild-type mice. In other words, expression of MeCP2 just in maturing neurons is sufficient to prevent mice from developing Rett-like symptoms.</p> <p>MeCP2 dysfunction outside of the central nervous system does not contribute significantly to Rett, and even delayed expression of the normal protein is beneficial to neurons. This raises hopes that there is a window of opportunity for therapeutic intervention.</p>	Luikenhuis et al. PNAS 2004

Progress in identifying targets of MeCP2 silencing

If MeCP2 normally silences genes, the next question is, naturally, “which genes?” A number of labs are tackling this question. Using DNA microarrays, which involve a “gene chip” the size of a saltine cracker that contains thousands of genes, one group compared gene expression in wild-type and null mice at two different ages to capture the profiles both before and after symptom onset. Eleven genes are misexpressed in the null mice, 5 of which are regulated by hormones that are secreted in response to stress (Nuber et al., Hum Mol Gen 2005). This interesting result needs to be followed up to establish whether these genes actually contribute to the symptoms of Rett syndrome, but it is intuitively appealing to think that we might be closer to understanding why Rett children are so vulnerable to anxiety.

Other groups are studying gene expression changes that may be specific to very particular cell types, and still others are taking a candidate-gene approach in which an educated guess is followed up by analyses to determine whether the gene in question is affected by MeCP2 dysfunction. It is not always easy to determine how this effect is mediated, however: take the case of brain-derived neurotrophic factor (BDNF). This protein supports the growth and differentiation of neurons and synapses; BDNF is stimulated when neurons are actively encoding

information. Chronic exposure to the stress hormone corticosterone decreases BDNF expression in rat brains and leads to atrophy of areas that are responsible for learning and memory. Similar atrophy has been found to take place in humans suffering from depression, and BDNF levels are reduced in people suffering from Alzheimer's disease and Huntington's disease as well. Clearly, BDNF is very important to healthy minds, and the possible role of BDNF in Rett is intriguing. Yet it remains unclear whether BDNF is up- or down-regulated in Rett. Some evidence suggests that it is expressed more in MeCP2 null mice (which one might expect, if MeCP2 normally silences this gene), whereas other data indicate that its levels are much lower. What is certain is that BDNF levels can exacerbate or improve the phenotype of MeCP2 null mice: loss of BDNF makes MeCP2 null mice worse, whereas increasing BDNF levels improves both the activity levels and lifespan of MeCP2 null mice. Because BDNF is expressed in response to neural activity, the apparent reduction in BDNF in MeCP2 null mice could be a secondary one, i.e., loss of MeCP2 reduces overall neuronal activity and thus indirectly reduces BDNF expression.

One challenge in assessing gene expression in the brain is the complexity of the organ. So many different regions, so many specialized cell types: each is likely to have their own unique gene expression profile, which in turn might change over time in unpredictable ways. Rett syndrome, and other disorders caused by various mutations in MeCP2, might be caused by changes in a small number of genes or hundreds.

Opening up a whole new set questions

As if transcriptional silencing and chromatin looping weren't enough tasks for one protein, there seems to be yet another way for MeCP2 to influence gene expression that doesn't have to do with silencing them. It has, rather, to do with modifying the kind of protein that is produced by a process called alternative splicing. If you ever wondered how billions of unique creatures could be created out of a mere 25,000 genes, herein lies part of the answer: by snipping out various pieces of the DNA sequence of a gene, or selecting different promoters or cleavage sites, messenger RNA might be able to produce thousands of different proteins or "splice variants" from just one gene. One particular fruit fly gene has *over 38,000* splice variants. (So much for the central dogma of molecular biology: "one gene, one protein.") These variant proteins can have different activities and different levels of expression.

Because alternative splicing occurs in response to changing conditions or varying stages of development, it is not surprising that the brain would be fairly humming with proteins that are splice variants of genes expressed elsewhere in the body. So when researchers discovered that MeCP2 interacts with YB-1, a protein well-known for its role in RNA splicing, the next logical step was to check out splicing patterns in MeCP2 mice. It turns out that MeCP2³⁰⁸ mice show a different splicing pattern in a number of genes. Strangely enough, one of those genes is *Dlx5*, which is also subject to transcriptional silencing by MeCP2-mediated chromatin looping. Could this indicate that the activities of MeCP2 in silencing and splicing are somehow coordinated? One might imagine that when the normal MeCP2 silencing complex is released from a target gene about to be expressed, MeCP2 becomes free to interact with the splicing machinery of the cell. The fact that *Dlx5* is subject to two types of MeCP2 regulation might also suggest that MeCP2 is not a global transcriptional repressor but instead acts on a handful of genes.

In sum

Progress in research more often than not raises more questions than it answers. Rett research seems particularly apt to throw us a curve, starting with the discovery that the gene that is mutated is not the direct cause of symptoms. The involvement of MeCP2 in chromatin looping, alternative splicing, and helping neurons to encode new information in response to stimuli are salient discoveries of the past year. We still have no answers to the questions of how to treat Rett syndrome, how to prevent it, or how to cure it, but the field is gaining momentum. It will be fascinating to see where we stand in June 2007.

Question and Answer session following Huda Zoghbi's talk

Dr. Zoghbi's talk was followed by a lively question-and-answer session. Here are some of the questions raised and highlights of the ensuing discussion:

What about the 4% of Rett girls who don't appear to have a MeCP2 mutation?

There are parts of genes that regulate the expression of the gene – that could be happening in these cases. Such a mutation can still cause loss of function, without altering the gene itself. You can also inactivate a protein by adding a methyl group or a phosphate. There is a precedent for this: some cases of muscular dystrophy are caused by protein modification (glycosylation in this instance). There are basically four ways you can cause trouble with a protein:

Don't make the protein

Change the protein by altering an amino acid (mutation)

Regulate the protein's expression

Alter the protein's activity by adding a methyl group or a phosphate or glycosylation

Large deletions occur in 10% of cases. Is this a worse mutation to have?

Not necessarily. Some deletions produce classic RTT, some milder forms of the disease. Because the cell is not happy with such a severely defective MeCP2, the cell doesn't survive, leading to favorable X chromosome inactivation.

Why has Rett research moved so fast?

Three main reasons. First, foundations like RSRF provide funding for projects that might not be fundable through other mechanisms, and RSRF has also allowed researchers to leverage greater funding from other sources. Second, the problem is frankly very interesting: it gets at the fundamentals of epigenetics as well as how the brain works. Third, Rett allows you to study several diseases at one time – not just because MeCP2 causes a variety of symptoms but also because of overlap with other diseases such as autism and Parkinson's disease.

Could you induce patch repair of the mutation?

RSRF is funding a study to address this question, but the problem is that every cell has the mutation. It also would not be helpful in cases where the gene is truncated.

What do we know about the emotional aspects of the phenotype?

Unfortunately, not much. We need to find the neurons responsible for anxiety, and we might be getting closer as we work to identify targets of MeCP2 activity.

Would you recommend more intense somatosensory stimulation, since somatosensory input seems to be dampened in Rett?

I think that the girls definitely need stimulation, but these girls tend to be anxious, so I would be gradual and gentle... we need to pay careful attention to how each girl responds to different stimuli and what pleases her vs. stresses her out.

What can we do to accelerate research?

Funding is critical, as it allows us to take risks. Keep engaging the scientific and medical communities in defining what we know and don't know. Open-ended research: cannot tell scientists what to study, because one never knows where an interesting line of inquiry will begin or end up. We also need to better describe and quantify clinical features so we can measure improvements when we start clinical trials.

My daughter performs better (follows commands) when she is in the swimming pool with her therapist. Could it be that fear of drowning enhances her performance?

Anxiety could upregulate epinephrine, but we have to consider the possibility that being surrounded by water provides proprioceptive stimulation that enables her to know where her body is or comforts her in some way.

[Author note: Parkinson's disease patients have difficulty initiating movements, even when they know what they want or need to do; autistic children also have a problem with volition. Time Magazine had an excellent cover story on autism ("Inside the Autistic Mind") in their May 15, 2006 issue that touches on this issue. There is evidence that this problem of the will is manifested in mice as well: older Rett mice that are quite sick won't move unless they are placed on a rotating rod. It may well be that girls understand more than they can show us, because of a problem with volition.]

Should we do newborn screening?

When we have a treatment, absolutely. What I'd like to see is whole population screening to find out if there is any correlation between slight mutations in MeCP2 and susceptibility to neurodegenerative diseases such as Parkinson's.