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**Isolation of *MECP2*-null Rett Syndrome patient hiPS cells and isogenic controls through X-chromosome inactivation**

Objectives:

Rett Syndrom (RTT) is a neurodevelopmental autism spectrum disorder that affects 1 in every 10,000 live female births. 95% of RTT patients have a loss-of-function mutation in an X-linked gene encoding methyl-CpG binding protein 2 (MECP2). MECP2 functions as a transcriptional activator and repressor by binding to methyl-CpG binding domain (MBD) or recruiting chromatin remodeling proteins via the transcriptional repression domain (TRD). The most common forms of RTT are caused by missense or nonsense mutations leading to a homomorphic MECP2, while null mutations leading to complete absence of protein are very rare. Most of our knowledge of RTT has resulted from the study of *Mecp2* mutant mouse models because access to patient neurons, such as postmortem tissues, is limited and may not reflect the early onset symptoms of RTT since the disease onset in RTT mice is during adulthood. Previous studies have shown that mouse models do not fully represent the human neurological condition. For these reasons, a method allowing for affected neurons directly from RTT patients would be advantageous to make further progress in the understanding the role of MECP2 of RTT in humans. In this publication, Cheung et al. aim to isolate *MECP2*-null human induced pluripotent stem (hiPS) cells and create an isogenic control through a nonrandom pattern of X-chromosome inactivation to be used in future research of MECP2.

Experimental Procedure and Results:

Preliminary screening for mutations in a classic RTT patient using multiple ligation-dependent probe amplification showed a deletion of exons 3 and 4 of *MECP2*. The complete RTT phenotype was seen in this patient. A skin punch biopsy was taken, fibroblasts were expanded, and DNA harvested for genotyping to precisely map the Δ3-4 *MECP2* mutation. Quantitative polymerase chain reaction (qPCR) was performed on Δ3-4 fibroblasts with primers spanning the *MECP2* locus to determine copy number variations (Fig. 1A and B). The regions of the breakpoints were identified, allowing Δ3-4 *MECP2* spanning primers to be designed to amplify the mutant *MECP2* allele (Fig. 1C). Sequencing revealed a pair of deletions, g.61340\_67032delinsACTTGTGCCAC and g.67072\_67200del. The larger deletion (g.61340\_67032delinsACTTGTGCCAC) was associated with an insertion of 11bp, removing the entirety of exon 3 and the 5’ end of exon 4, including the MDB and TRD (Fi. 1A). They detected an *AluSx* element spanning the 5’ end of the larger deletion, which could trigger *Alu* recombination-mediated deletions. The origin of the 11bp insertion was not able to be determined due to its relatively small size making it insufficient to perform a genome search. However, the occurrence of an insertion with a deletion has been observed previously by Kidd et al. and is thought to be an inversion of the local genomic sequence (2010). A 3bp microhomolgy was seen flanking the smaller deletion (g.67072\_67200del.) which could trigger a microhomolgy-mediated process such as microhomolgy-mediated end joining or microhomolgy-mediated break-induced replication. Therefore, it seems as if the two deletions may have been caused by two separate mechanisms.

The predicted severity of the Δ3-4 *MECP2* mutation seen in this RTT patient prompted the generation of hiPS cells. Δ3-4 fibroblasts were transduced with *OCT4, SOX2, KLF4*, *c-MYC* retroviral vectors and EOS lentiviral vector that reports pluripotency. Three Δ3-4 hiPS cell lines propagated extremely successfully under puromycin selection driven by the EOS-pluripotent reported and were subjected to further characterization. Δ3-4 hiPS cells were pluripotent and fully reprogrammed as indicated by the hiPS cell markers *REX1, ANCG2, DNMT3B,* and TRA1-60 (Fig. 2A and B). Their ability to differentiate into three germ layers was shown *in vitro* via embryoid body formation (Fig.2C) and *in vivo* via teratoma formation by injection into immunodeficient mice (Fig. 2D). DNA fingerprinting by short tandem repeat analysis indicated that all RTT-hiPS cells originated from one parental fibroblast and were not from contaminating human embryonic stem (hES) cells within the laboratory (Fig. 2E).

It was recently demonstrated by Tchieu et al. (2010) that female hiPS cells retain an inactive X-chromosome. Also reported in the same study, individual hiPS stem cell lines exhibit a nonrandom pattern of XCI, as they reflect the XCI status of the single fibroblast from which they were derived. This is a useful characteristic in the study of heterozygous X-linked diseases, such as RTT. It allows for the generation of hiPS cell lines that express either the wild-type (WT) or the mutant form of the protein depending on the pattern of XCI. This could ultimately allow the generation of isogenic control and experimental hiPS cell lines. Taking this characteristic of hiPS cells into account, they sought to determine if RTT-hiPS cells retain an inactive X-chromosome. Then, if so, is it a nonrandom pattern of XCI.

To investigate the XCI status of Δ3-4 hiPS cells, they probed for expression of *XIST* RNA by RNA fluorescence *in situ* hybridization (RNA-FISH). They observed a single *XIST* RNA signal in 67-100% of Δ3-4 hiPS cell colonies (Fig. 3A). They noted that of the 73% Δ3-4 hiPS #6 colonies where a single *XIST* RNA signal was detectable, 38% of the colonies, characterized as mixed, were below the threshold for a positive colony (>90% of cells per colony). They further examined the XCI status of the Δ3-4 hiPS cells by immunocytochemistry for histone H3 lysine 27 trimethylation (H3K27me3), a repressive chromatin mark that accumulated on the inactive X-chromosome during the initiation of XCI. Similar results to *XIST* RNA were seen. They observed a single H3K27me3 signal in 55-100% of Δ3-4 hiPS cell colonies (Fig. 3B). The combination of the mentioned data along with a skewed XCI pattern (see below) suggests Δ3-4 hiPS cells retain an inactive X-chromosome. The lack of a single *XIST* RNA and H3K27me3 signal can be interpreted as: i) the loss of an X-chromosome, ii) the reactivation of the inactive X-chromosome, or iii) an inactive X-chromosome that has lost the *XIST* RNA and H3K27me3 but remains transcriptionally suppressed. They ruled out the possibility of the loss of an X-chromosome by observing a normal karyotype (Fig. 2G). They also excluded the possibility that RTT-hiPS cells carry two active X-chromosomes since neuronal derivitives of RTT hiPS cells do not exhibit random XCI (see below). The final possibility is that the female hiPS cells can be subject to the loss of *XIST* RNA and other repressive chromatin marks during *in vitro* cultures.

To investigate the pattern of XCI in Δ3-4 hiPS cells, they used the androgen receptor (AR) assay which detects the heterozygous trinucleotide repeat polymorphism in the first exon of the X-linked *AR* gene by PCR to distinguish maternal from paternal X-chromosomes. Genomic DNA was digested with methylation-sensitive enzymes prior to PCR to allow the detection of the inactive X-chromosome. The assay revealed that Δ3-4 fibroblasts exhibited a nonrandom pattern of XCI (69:31) as shown by the detection of two different sized amplicons of the *AR* gene (Fig. 4A). All of the Δ3-4 hiPS cell lines exhibited an extreme XCI skewing pattern (96:4 to 99:1). Also, Δ3-4 hiPS #6 and #37 skewed towards the same parental X-chromosome being inactivated, while Δ3-4 hiPS #20 skewed towards the alternative parental X-chromosome being inactivated.

The importance of the XCI pattern in RTT is the expression pattern of the WT or mutant MECP2 in neurons. To further asses whether RTT hiPS cells retain an inactive X-chromosome despite the loss of *XIST* RNA and H3K27me3 in some cells and whether the XCI skewing pattern does indeed represent a nonrandom XCI pattern, they examined the expression of MECP2 in Δ3-4 hiPS cells. They did this by allele-specific expression analysis using reverse transcriptase PCR (RT-PCR) and qualitative (q)RT-PCR in Δ3-4 hiPS cells using primers exclusively specific to the WT *MECP2* transcripts. WT *MECP2* transcripts were detected in Δ3-4 hiPS #6 and #37 but not in Δ3-4 hiPS #20, suggesting that Δ3-4 hiPS #20 expresses the mutant Δ3-4 *MECP2* transcript (Fig. 5A and B). This supports the results of the AR assay showing that Δ3-4 hiPS #6 and #37 have the same parental X-chromosome inactivated, while Δ3-4 hiPS #20 has the alternative inactivated (Fig. 4A).

Finally, they sought to determine whether the pattern WT and mutant expression of MECP2 is maintained upon differentiation of the Δ3-4 hiPS cells. Since RTT is primarily a neurodevelopmental disorder, they performed directed differentiation of Δ3-4 hiPS #20 and #37 cells into the neuronal derivatives. They chose to focus on Δ3-4 hiPS #20 and #37 because they exhibit alternative parental XCI. The characteristics of the null mutation allow for direct visualization of the WT MECP2 but not the mutant Δ3-4 hiPS at the protein level via immunocytochemistry. They raised an antibody against the C-terminus of MECP2 and Δ3-4 hiPS cells were seen to differentiate into MAP2-positive neurons (Fig. 5E). Co-labeling for MECP2 detected WT MECP2 protein expressed in the nuclei of Δ3-4 hiPS #37-derived neurons but not in the Δ3-4 hiPS #20-derived neurons (Fig. 5E). qRT-PCR further confirmed the results as WT *MECP2* transcripts were detected exclusively in Δ3-4 hiPS #37-derived neurons. They preformed a final AR assay that confirmed similar skewing pattern seen in the parental hiPS cell lines excluding the possibility that XIST RNA- and H3K27me3-negative Δ3-4 hiPS cells carry two active X-chromosomes. From this data they concluded that the nonrandom monoallelic expression of MECP2 in Δ3-4 hiPS cells is maintained through neuronal differentiation and that Δ3-4 hiPS 37 is an isogenic control of the mutant Δ3-4 hiPS #20.

They then demonstrated the utility of the isogenic control and mutant Δ3-4 hiPS cells by performing phenotyping of soma size in Δ3-4 hiPS cell-derived neurons. They observed that mutant Δ3-4 hiPS #20-derived neurons exhibited a significant reduction in soma size compared with the isogenic control (Fig. 5G). This was consistent with previous findings in Mecp2 -/y mice and postmortem tissues that MECP2 expression from RTT patients.

Conclusion:

Cheung et al. established isogenic RTT-hiPS cell lines that express either the WT or mutant allele of MECP2. They mapped the functionally null mutation in *MECP2* for a classic RTT patient that consisted of a pair of deletions. One of the deletions, also associated with an insertion, removes exon 3 and the 5’ end of exon 4. This deletion ultimately removed the entire MBD and TRD from the *MECP2* gene. The pair of deletions seems to be caused by two different mechanisms. The larger deletion caused by an *Alu* recombination-mediated process, while the smaller deletion may be caused by microhomology-mediated process. They established Δ3-4 hiPS cell lines from patient fibroblasts and were shown to be pluripotent and fully reprogrammed. Female RTT-hiPS cells were seen to retain an inactive X-chromosome as suggested by the expression of a single *XIST* RNA signal and H3K27me3 signal in the RTT-hiPS cell colonies. Extreme XCI skewing suggests that the pattern of XCI is nonrandom as detected by the AR assay. They obtained Δ3-4 hiPS cell lines that have alternative parental X-chromosomes inactivated. This XCI pattern allowed for the isolation of a pair of isogenic control and experimental Δ3-4 hiPS cell lines. Δ3-4 hiPS #37 and #6 but not Δ3-4 hiPS #20 expressed WT *MECP2* transcripts. Furthermore, when directed differentiation was performed towards their neuronal lineage on Δ3-4 hiPS #20 and #37, WT MECP2 transcripts and protein were detected only in the neuronal derivatives of Δ3-4 hiPS #37 but not those of Δ3-4 hiPS #20. This indicated that the Δ3-4 *MECP2* mutation results in the complete absence of functional protein and is hence a functionally null mutation. From these results, they concluded that Δ3-4 hiPS #37 is as isogenic control for the mutant Δ3-4 hiPS #20.

Future Research:

Cheung et al. isolated and isogenic system for Δ3-4 *MECP2* deletion. This system is advantageous for several reasons. For disease phenotyping, appropriate healthy control hiPS cells care essential and isogenic cells from the same patient eliminate the diversity present in different genetic backgrounds that exist when comparing cell lines from different individuals. These slight differences in genetic information cannot be entirely excluded from research. Furthermore, isogenic Δ3-4 hiPS cell lines may respond to directed differentiation cues in a more uniform manner than hiPS and hES cells from different individuals. It is possible that further downstream applications with Δ3-4 hiPS cell lines will be able to identify phenotypes that are specific to the *MECP2* mutation. Also, it would be interesting to mix the mutant and isogenic control Δ3-4 hiPS cells in different relative proportions prior to differentiation and show the mosaic mutant and WT expression pattern that exists in some RTT females.

The null mutation mapped in this study is advantageous for rescue experiments that could result in a pharmaceutical breakthrough. Also, the generation of Δ3-4 hiPS-derived neurons allows the study of MECP2 function in human neurons and they can be available for disease phenotyping that has traditionally been difficult due to lack of human tissues from RTT patients for research purposes. It may be possible to use these hiPS-derived neurons for future drug screens to develop treatments of RTT.

Citation:

Cheung A., Horvath L.M., Grafodaskaya D., Pasceri P., Weksberg R., Hotta A., Carrel L., Ellis J. (2011) Isolation of *MECP2*-null Rett Syndrome patient hiPS cells and isogenic controls through X-chromosome inactivation. *Human Molecular Genetics*, **20**, 2103-2115.

Refrences:

Tchieu, J., Kuoy, E., Chin, M.H., Trinh, H., Patterson, M., Sherman, S.P., Aimiuwu, O., Lindgren, A., Hakimian, S., Zack, J.A. et al. (2010) Female human iPSCs retain an inactiveXchromosome. Cell Stem Cell, **7,** 329–342

Kidd, J.M., Graves, T., Newman, T.L., Fulton, R., Hayden, H.S., Malig, M., Kallicki, J., Kaul, R., Wilson, R.K. and Eichler, E.E. (2010) A human genome structural variation sequencing resource reveals insights intomutational mechanisms. Cell**, 143,** 837–847.