Review Article

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A review of Rett syndrome (RTT) with induced pluripotent stem cells

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Abstract: Human induced pluripotent stem cells (hiPSCs) are pluripotent stem cells generated from somatic cells by the introduction of a combination of pluripotency-associated genes such as OCT4, SOX2, along with either KLF4 and c-MYC or NANOG and LIN28 via retroviral or lentiviral vectors. Most importantly, hiPSCs are similar to human embryonic stem cells (hESCs) functionally as they are pluripotent and can potentially differentiate into any desired cell type when provided with the appropriate cues, but do not have the ethical issues surrounding hESCs. For these reasons, hiPSCs have huge potential in translational medicine such as disease modeling, drug screening, and cellular therapy. Indeed, patient-specific hiPSCs have been generated for a multitude of diseases, including many with a neurological basis, in which disease phenotypes have been recapitulated *in vitro* and proof-of-principle drug screening has been performed. As the techniques for generating hiPSCs are refined and these cells become a more widely used tool for understanding brain development, the insights they produce must be understood in the context of the greater complexity of the human genome and the human brain. Disease models using iPS from Rett syndrome (RTT) patient's fibroblasts have opened up a new avenue of drug discovery for therapeutic treatment of RTT. The analysis of X chromosome inactivation (XCI) upon differentiation of RTT-hiPSCs into neurons will be critical to conclusively demonstrate the isolation of pre-XCI RTT-hiPSCs in comparison to post-XCI RTT-hiPSCs. The current review projects on iPSC studies in RTT as well as XCI in hiPSC were it suggests for screening new potential therapeutic targets for RTT in future for the benefit of RTT patients. In conclusion, patient-specific drug screening might be feasible and would be particularly helpful in disorders where patients frequently have to try multiple drugs before finding a regimen that works.

Keywords: Human induced pluripotent stem cells (hiPSCs); methyl-CpG binding protein 2 (MECP2); Rett syndrome (RTT); therapy; X chromosome inactivation (XCI)

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Introduction

The recent successful generation of induced pluripotent stem cells (iPSCs) from patients' somatic cells by ectopically expressing four transcription factors allows the derivation of pluripotent stem cells from patients with Mendelian genetic or non-genetic diseases (1,2). Human induced pluripotent stem cells (hiPSCs) are pluripotent stem cells generated from somatic cells by the introduction of a combination of pluripotency-associated genes such as OCT4, SOX2, along

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with either KLF4 and c-MYC or NANOG and LIN28 via retroviral or lentiviral vectors (3-5). Most importantly, researchers determined that iPSCs were remarkably similar to embryo-derived stem cells with respect to gene expression profile, epigenetic marks, and fate potential (6). Although it is clear that iPSCs are not identical to embryonic stem cells as they retain an epigenetic memory reflecting the tissue of origin (7,8). Most iPSCs lines pass the most stringent tests of pluripotency, self-renewal, multilineage potential and, for mouse iPSCs, germline transmission (the ability to generate most mouse tissues after injection into an early embryo, including germ cells) (9,10). In addition, hiPSCs have huge potential in translational medicine such as disease modeling, drug screening, and cellular therapy. Indeed, patient-specific hiPSCs have been generated for a multitude of diseases, including many with a neurological basis, in which disease phenotypes have been recapitulated *in vitro* and proof-of-principle drug screening has been performed (11-15).

Rett syndrome (RTT) is an X-linked dominant severe neurodevelopmental disorder, first described in 1966 by German literature Dr. Andreas Rett (16). But it would not be until 17 years later that, RTT became recognized in the medical community when Dr. Bengt Hagberg, a Swedish neurologist, and his colleagues reported 35 cases of RTT in the English language (17). RTT is one of the most common causes of mental retardation, with an incidence of 1 in 10,000–15,000 female births (18). RTT is unique in that it is the only pervasive developmental disorders (PDD) that occur almost exclusively in females and has an identified genetic cause (16,17,19). RTT is now recognized as a pan-ethnic disorder and presents an ever widening clinical phenotype (20). For a long time, RTT was thought to be an X-linked dominant condition occurring almost exclusively in females. The clinical phenotype can be variable, and the distinction between classical RTT and variant RTT has been made by delineation of 5 clinical variants: the infantile onset seizure variant, the congenital variant, the 'formefruste', the late childhood regression variant, and the preserved speech variant (21). Most individuals with classic RTT have methyl-CpG binding protein 2 (MECP2) mutations at Xq28 (22,23). However, practitioners should also be aware that the *MECP2* mutation, believed to be the cause of RTT, has also been seen in other phenotypic groups, including those with diagnoses of X-linked mental retardation and girls with autism. Diagnosis of RTT must be made clinically, as MECP2 mutations result in a wide variety of phenotypes within and outside of RTT (21,23-26). MECP2 is an X-linked gene subject to random X chromosome inactivation (XCI) resulting in mosaic expression of mutant MECP2.

Recently, observed that female hiPSCs retain an inactive X-chromosome in a non-random pattern (27), in contrast to their mouse counterparts which reactivate the inactive X-chromosome thus carrying two active X-chromosomes and exhibit random XCI upon differentiation (28). This pattern of XCI in female hiPSCs provides prospects to isolate isogenic control and experimental hiPS cell lines for heterozygous X-linked diseases, such as RTT. The first neurodegenerative diseases modelled using hiPSCs were monogenetically inherited, rare and fatal disorders: smooth muscle atrophy (SMA) and familial dysautonomia (FD) (29). iPSCs have been used to model human neuronal diseases, including spinal muscular atrophy, FD, and amyotrophic lateral sclerosis (29-31) including RTT (14,32-35).

Clinical overview and behavioural changes of RTT

Clinical features of RTT are postnatal progressive neurodevelopmental disorder that manifests in girls during early childhood; however, not all the symptoms are prominent initially, but rather appear over stages. RTT is characterized by a specific developmental profile, with the diagnosis of RTT being based on a consistent constellation of clinical features and the use of established diagnostic criteria. The diagnostic criteria for classical RTT include a normal prenatal and perinatal period with normal developmental progress for the first months of life. Patients with RTT appear to develop normally up to 6–18 months of age. The child with RTT seemingly achieves appropriate milestones, including the ability to walk, and some patients even say a few words. The majority of RTT girls lose verbal expressive language, although some retain some speech or single word expressions. Alternative forms of communication that may be used include communication boards, technical devices, and switch activated systems. These are used for making choices and facilitate environmental access. Some girls are also able to communicate through eye pointing, gestures, body language, and hand pointing. These abilities need to be recognized and encouraged. As the syndrome progresses, patients lose purposeful use of their hands and instead develop stereotypic hand wringing or washing movements, and in some cases clapping, flapping, and mouthing of the hands. One early indicator of neurological involvement is the deceleration of head growth, leading to microcephaly by the second year of life. Major symptoms of RTT include reduced head growth, social withdrawal,

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weight loss, loss of previously acquired skills including purposeful hand use and expressive language, gait ataxia, stereotypic movement of the hands, and autonomic dysfunctions such as respiratory distress (18). The earliest autonomic perturbation is hyperventilation during wakefulness. Breathing irregularities such as breath holding and hyperventilation, episodes of motor activity such as twitching, jerking, or trembling, or a cardiac arrhythmia associated with a prolonged QT interval are most commonly confused with seizures. One of the most arduous features of RTT is the occurrence of seizures, which range from easily controlled to intractable epilepsy, with the most common types being partial complex and tonic-clonic seizures (36). Other autistic features also manifest, including expressionless face, hypersensitivity to sound, lack of eye-to-eye contact, indifference to the surrounding environment, and unresponsiveness to social cues (37). It is frequently mentioned that 26% of deaths in women with RTT are sudden and unexpected, often implying that these are due to cardiovascular or respiratory arrest (38). The largest RTT mortality reported, 305 deaths, is from the North American RTT Database. Unfortunately, incomplete reporting precluded analysis of the cause of death in this study (39). There is a suggestion that, serotonin may be involved with autonomic cardiovascular dysfunction in RTT. Plasma levels of serotonin are lower in RTT subjects and there is a negative correlation between plasma serotonin and low frequency: high frequency power ratio for pulse interval, an index of sympathetic over activity (40). In addition, levels of the serotonin metabolite 5-hydroindoleacetic acid are low in cerebrospinal of affected individuals (41).

Brain structure and function of RTT patients

The characteristics that distinguish the recently evolved primate and human brain from other mammalian brains include the proportionally larger growth of the cerebral cortex, the diversification of cortical area maps and a much additional extensive degree of connectivity. Neuroimaging studies have revealed structural and functional brain abnormalities in many neuropsychiatric conditions, often preceding the onset of symptoms (42). For example, at the earliest ages assessed, children with the attention deficit hyperactivity disorder have significantly less cerebral cortical volume than typically developing children (43). The earliest stages of brain development are implicated in the trajectories leading to the manifestation of neuropsychiatric disorders. Development of the nervous system proceeds through a set of complex checkpoints which arise from a combination of sequential gene expression and early neural activity sculpted by the environment. Genetic and environmental insults lead to neurodevelopmental disorders which encompass a large group of diseases that result from anatomical and physiological abnormalities during maturation and development of brain circuits. The link between MECP2 and RTT is fascinating because RTT is one of a small group of ASDs that gives us the opportunity to study mutations in a single gene and how they affect sequential phenotypic checkpoints of brain development leading to neuropathological endpoints in psychiatric disorders (44).

Almost two decades of research on RTT has led to the development of an intriguing story of how a single transcription factor can play a crucial role in neuronal development, synaptic maturation, and plasticity. Synaptic scaling is forms of homeostatic plasticity in which average neuronal activity levels are modulated to allow for dynamic adjust of synaptic strength to promote stability of neuronal circuits (45). Recent evidence shows how MeCP2 mediates activity-dependent synaptic scaling in rat hippocampal cultures (46). The increase in neuronal activity upon bicuculline treatment leads to an increased level of MeCP2 expression, which in turn binds to the GluR2 promoter and recruits a repressor complex to inhibit its expression and availability of these molecules at the synapse. Regulating AMPA receptor GluR2 subunit expression is one direct way to mediate an adaptive response that regulates synaptic strength and prevents recurrent circuit excitation. Epilepsy is often seen in RTT patients and often difficult to treat (47). Microglia may also influence the onset and progression of RTT. Elevated levels of glutamate, released from microglia, may cause abnormal stunted dendritic morphology, microtubule disruption, and damage to postsynaptic glutamatergic components making microglial glutamate synthesis or release a potential therapeutic target for RTT (48,49). Since most cases of RTT are caused by mutations in the *MECP2* gene, it is assumed that convulsions are based on genetic mechanisms, however, the balance of excitation and inhibition is also believed to play a critical role in the progression of the disease during early development. Although the primary function of MeCP2 in normal brain development remains unclear, it is becoming increasingly evident that there is a complex interplay of genes and environment which results in the synaptic and circuit-level defects in brain function.

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MeCP2 expression and functions

Neurodevelopmental disorders include a wide range of diseases characterized by impairment of neuronal function during brain development. They have a strong genetic component; though they can result from a single mutation, they are more commonly multigenic (50). Mecp2, a nuclear protein, belongs to a family of transcription factors that bind to DNA. The human MECP2 gene consists of four exons resulting in expression of two protein isoforms due to alternative splicing of exon 2. These splice variants differ only in their N-terminal and include the more abundant MeCP2-e1 isoform as well as the MeCP2-e2 isoform (51-53). It contains three domains: methyl-binding (MBD), transcriptional repression (TRD) and the C-terminus; plus two nuclear localization sequences (NLS) (18,54,55). In addition, MECP2 has a large, highly conserved 30-untranslated region that contains multiple polyadenylation sites, which can be alternatively used to generate four different transcripts. The specific role of MeCP2 in transcription and translational control might vary depending on the different molecules recruited and protein-protein interactions. This complexity, for example, is shown in the regulation of one of the most important targets of MeCP2 in the central nervous system: brain-derived neurotrophic factor (BDNF). MeCP2 regulates BDNF expression by binding to promoter IV and repressing its transcription until MeCP2 is phosphorylated and released via a neuronal activity-dependent mechanism. MECP2 functions as a transcriptional regulator by binding to the genome in a DNA methylation-dependent manner via its methyl-CpG binding domain and recruiting chromatin remodeling proteins via its TRD domain (56-61). The MECP2 gene has some unique characteristics: (I) it is mainly affected by *de novo* mutations, due to recurrent independent mutational events in a defined "hot spot" regions or positions; (II) complex mutational events along a single allele are frequently found in this gene; (III) most mutations arise on paternal X-chromosome. The recurrent point mutations involve mainly CpG dinucleotides, where C>T transitions are explained by methylation-mediated deamination.

MeCP2 expression pattern within different brain regions follows the developmental maturation of the central nervous system, being initially detected in the earliest developing structures such as brainstem and thalamus (62,63) and the elevated levels of MeCP2 expression immature neurons are maintained throughout adulthood, implying its importance in post-mitotic neuronal function. Gene expression studies show that different brain regions are enriched with different splice variants; MeCP2e2 is prevalent in dorsal thalamus and layer of the cortex while MeCP2-e1 is detected in the hypothalamus (51). Recent results suggest that MeCP2-e2 isoform is upregulated in Aβ-treated cortical neurons and promotes neuronal death in postmitotic neurons, a pathway normally inhibited by forkhead protein FOXG1 (64). Since MeCP2 is expressed in mature neurons and its levels increase during postnatal development, MeCP2 may play a role in modulating the activity or plasticity of mature neurons. Consistent with this, MECP2 mutations do not seem to affect the proliferation or differentiation of neuronal precursors. MeCP2 is also an "intrinsically disordered" protein with long stretches of unorganized segments, without standard three-dimensional secondary structure, proposed to participate in the formation of a flexible scaffold required for multiple biological interactions (65). Although the exact mechanisms that regulate the complex MECP2 expression patterns are not yet fully understood, a recent study identified the core promoter and several cis-regulatory elements that drive MeCP2 expression (66). These regulatory sequences may dictate the spatial and temporal patterns of MeCP2 expression. The role of MeCP2 in the development and maturation of the nervous system compared to the maintenance of adult neurons is not yet fully elucidated.

Mecp2 in RTT patients

RTT is a rare monogenetic disorder included in the ASDs and is caused by mutations in the MeCP2 gene. Several mutations introduce premature stop codons throughout the gene and are predicted to result in a null allele. Cheung *et al.* and Schanen *et al.* have reported that phenotypic analysis of same genotype controls and neurons obtained from altered hiPS cells are the source to know the development of RTT and denotes the importance of MECP2 in human neurons (33,67). Consistent with RTT animal models and RTT post-mortem human brain tissue (68), both groups detected a decrease in cell soma size of RTT neurons compared with non-affected controls. MeCP2 null and conditional mutant mouse models with cell-type or area specific loss of MeCP2 in the brain show phenotypic features that resemble some features of RTT patient symptoms. All these models have been generated by mutating the mouse endogenous Mecp2 gene or by the introduction of the human MECP2 gene with a representative RTT mutation. There seems to be an association between higher degree of anxiety and reduced

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social interest so mouse background has to be taken into account when comparing behavior phenotypes (69-71). Interestingly, the recent literature has started to address whether different translation types and levels of MeCP2 lead to variation in the anxiety and social phenotype: the complete lack of protein might produce a stronger phenotype with less anxiety-like behaviour, whereas a truncated protein could generate mouse lines with increased stress (72). BDNF is critical for neuronal development, synaptic maturation, and plasticity through the activation of specific neurotrophic tyrosine kinase receptor type 2 (TrkB), which, in turn, activates signal transduction pathways such as PLCγ, PI3K/Akt, and MAPK/ERK that regulate protein synthesis and neural function by activating PSD95 (73). This pathway is of central importance to the expression and amelioration of the RTT phenotype. The regulation of protein synthesis via the PI3K pathway has been proven to be crucial in synaptic function, dendrite structure, and plasticity (74-76). All of these functions have been shown to be compromised in RTT (77,78). Recently, direct evidence has demonstrated the dysregulation of the entire Akt/mTOR axis in MeCP2 null mice, giving a molecular theoretical framework for the mechanism of action of genes regulated by MeCP2, like BDNF (79). Retrotransposon DNA elements are thought to be preferentially regulated in neurons by MecP2, suggesting the importance of examining MecP2 function in differentiated neural cells rather than in pluripotent cells or non-neural clinically accessible tissues (80).Understanding the correlation between the functional interaction of the different Mecp2 transcripts produced by these models with other anxiety-related genes like corticotrophin releasing hormone will be capital, not only to explain the mice phenotypic variance but for clinical applicability (81-83).

hiPSC in RTT

The generation of hiPSCs from RTT patients represents an inexhaustible source for *in vitro* derived patient-specific neurons, assuming that RTT-hiPSCs can be expanded indefinitely with a normal karyotype and stable genome and the generation of patient-specific hiPSCs from RTT girls has been an area of intense research as several groups have reported the generation of such cells (14,32-35,84,85). RTT-hiPSCs generated by different groups have similar properties as they carry pathogenic mutations in MECP2 or CDKL5 and are pluripotent *in vitro* and *in vivo*. Essentially, RTT-hiPSCs can be differentiated into affected neurons and exhibit RTT-associated phenotypes *in vitro* and can be rescued by transgene expression or drug treatments. The female RTT-iPSCs thus generated exhibited the reactivation of randomly inactivated fibroblast X chromosome and expressed both WT and mutant MeCP2 from two active X chromosomes. When differentiated into the neuronal lineage, RTT-iPSCs recapitulated the *in vivo* phenotypes, including synapse defects, smaller soma size, altered calcium signaling, and electrophysiological defects. In 2009, Hotta *et al.* derived an iPSC line from an 8-year-old Rett patient possessing the heterozygous R306C missense mutation in MECP2, which disrupts with normal neuronal maturation. MECP2 binds to methylated DNA, thus its function is directly related to epigenetic status. During reprogramming, there is large scale (86) erasure of epigenetic marks (87). However, evidences show that selective loss of MECP2 in forebrain GABAergic neurons can phenocopy aspects of autism and Rett's disorder (88), enhances the likelihood that an iPSC-mediated approach will shed useful light on this disorder.

RTT-hiPSC retained the MECP2 mutation, is pluripotent and fully reprogrammed, and retained an inactive X-chromosome in a non-random pattern. Analysis of isogenic control and mutant hiPS cell-derived neurons represents a promising source for understanding the pathogenesis of RTT and the role of MECP2 in human neurons. These neurons are useful for investigating the pathogenesis of RTT and have potential for use in drug screens and identification of novel compounds for therapy (14,33-35,84,85). For this impending to be realized, efficient protocols that direct differentiation into adult stage neurons of defined subtypes may be required (89). However, with the generation of RTT-hiPSCs from multiple groups, the XCI status of RTT-hiPSCs, and more generally, female hiPSCs, has been variable. Some researchers (33,34,84,85) reported the generation of RTT-hiPSCs that retain the Xi (post-XCI) from the founder somatic cell it was derived from, while others (14,90) reported the generation of some RTT-hiPSCs that reactivate the Xi of the founder somatic cell and hence carry two active X-chromosomes (pre-XCI).

XCI or Lyonization

Mutations in MECP2 appear to give a growth disadvantage to both neuronal and lymphoblast cells, often resulting in skewing of X inactivation that may contribute to the large degree of phenotypic variation. During eutherian mammalian development, females randomly inactivate one

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of the two X chromosomes in a process called XCI (91). XCI occurs during female development when one of the two X-chromosomes is randomly inactivated such that approximately half the cells inactivate the maternally derived X-chromosome, while the other half inactivates the paternally derived X-chromosome (92). Most healthy human females consist of mosaic cell populations with respect to XCI pattern that follows a bell-shaped curve with a median value of 50% (93). XCI is the mammalian strategy to equalize X-linked gene dosage between XX females and XY males and involves transcriptionally silencing the majority of genes on one X chromosome in females (94,95). In onset, XCI is random and either the maternally or paternally inherited X-chromosome is silenced in each cell. Consequently, that X-chromosome remains the XCI throughout all future cell divisions (94). Upon Xist upregulation, the Xi is heavily epigenetically remodeled, in many ways similar to other silenced genes throughout the genome. Epigenetic marks associated with the Xi include CpG island promoter DNA methylation (96,97), incorporation of histone variant MacroH2A, and modification of core histones (98-100). An early event that follows XIST accumulation is the recruitment of the polycomb complex PRC2 that induces histone H3 trimethylation at lysine 27 (101-103). Other epigenetic features, such as DNA methylation, accumulate later and are important in the maintenance of XCI. Altogether these many alterations function with XIST to create a silenced nuclear compartment (104) that is spatially sequestered to the periphery of the nucleus and is cytologically recognizable as the darkly staining Barr body (105).

XCI in RTT

A complexity of the RTT story is that the MECP2 gene is located on the X-chromosome and is influenced by XCI. In females, only one of the two X-chromosomes is active in each cell and the choice of which X-chromosome is active is usually random, such that half of the cells have the maternal X-chromosome active and the other half have the paternal X-chromosome active. Therefore, a female with an MECP2 mutation is typically mosaic, whereby half of her cells express the wild-type MECP2 allele and the other half express the mutant MECP2 allele. Occasionally, cells expressing the wild-type MECP2 allele divide faster or survive better than cells expressing the mutant allele, which therefore results in a non-random pattern of XCI and amelioration of the RTT neurological phenotypes. Although XCI is random in most cases, it can occasionally be non-random which could lead to phenotypic variability in RTT patients depending on the extent of favourable XCI skewing (106). The best examples for illustrating the dramatic effects of XCI patterns in RTT are monozygotic twins who manifest very different phenotypes (107). In addition, skewed XCI patterns occur in brain regions of female mice heterozygous for a mutant MECP2 allele, where phenotypic severity correlates with the degree of skewing (108).

Directed differentiation of post-XCI RTT-hiPSCs will yield homogeneous cultures of neurons that express either WT or mutant MECP2 allowing simpler analysis of a population of cells without influence from the opposite allele being expressed as in pre-XCI RTT-hiPSCs (33). Archer stated that the generation of isogenic control and mutant RTT-hiPSCs allow the mixing and matching of WT and mutant expressing cells in different proportions which provide an opportunity to study the effects of XCI skewing as observed in RTT patients (106). Furthermore, it will also allow the mixing and matching of different cell types such as neurons and glia to study the non-cell autonomous effects of non-neuronal cell types in RTT has become apparent in the recent RTT literature (48,109-111). In addition, X-chromosome contains a high density of genes important for brain development and reproduction, and ID is approximately three times more often related to genes on the X versus autosomes (112). To address the role of telomeres in reprogramming, Colman and colleagues introduced an exogenous telomerase reverse transcriptase (TERT) transgene into fibroblasts to prevent skewing (85). Upon differentiation, pre-XCI RTT-hiPSCs underwent random XCI and generated a mosaic culture of fibroblasts that expressed either the WT or mutant MECP2, thereby restoring the lost population expressing Xu. Although not demonstrated, the reprogramming of such fibroblasts expressing Xu should also yield isogenic control and mutant post-XCI RTT-hiPSCs. Together, these data suggest that there is an inherent difference in the two parental X-chromosomes, independent of the MECP2 mutation, which impacts the reprogramming efficiency of any given cell. This will ultimately impact one's ability to generate isogenic control and mutant post-XCI RTT-hiPSCs from fibroblasts that have been extensively passaged. Therefore, the generation of isogenic control and mutant post-XCI RTT-hiPSCs should be more efficient from primary fibroblasts at earlier passages while the mosaic culture is still present (85).

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On the other hand, the pre-XCI RTT-hiPSCs reported by Park and colleagues lacked H3K27me3 marks and expressed MECP2 twofold compared to male hESCs, consistent with a class I assignment (35). However, this approach does not exclude the possibility of class III hiPSCs that have lost XCI marks resulting in reactivation of X-linked genes (113). Likewise, the undifferentiated pre-XCI RTT-hiPSCs reported by Muotri and colleagues lacked XCI marks, such as XIST RNA and H3K27me3, and expressed MECP2 in a biallelic manner (14).

Post-XCI RTT-hiPSCs

Plath and colleagues findings confirmed that the non-random monoallelic post-XCI status of female hiPSCs can be exploited to generate isogenic control (expressing WT MECP2) and mutant (expressing mutant MECP2) hiPSCs from the same individual (27). Similarly, Renieri and colleagues and Chang and colleagues isolated isogenic control and mutant RTT-hiPSCs from some female patients carrying heterozygous mutations in the X-linked CDKL5 gene and MECP2, respectively (34,84). RTT-hiPSCs were post-XCI as they displayed non-random XCI skewing by AR assay and expressed CDKL5 or MECP2 in a non-random monoallelic manner allowing the isolation of isogenic control and mutant RTT-hiPSCs. Altogether, these studies demonstrate that post-XCI RTT-hiPSCs can be isolated, and become a particularly strong model system that allows direct comparison of mutant and isogenic control RTThiPSCs from the same individual (33,34,84).

The XCI status of female hiPSCs has been investigated by several groups and they have generated hiPSCs from RTT patients (14,33-35,84,85,101). Post-XCI RTT-hiPSCs with non-random XCI will yield isogenic WT or mutant MECP2 expressing RTT-hiPSCs. Differentiation of post-XCI RTT-hiPSCs will yield cultures that maintain this non-random monoallelic expression pattern allowing the direct comparison of WT and mutant neurons. Conversely, pre-XCI RTT-hiPSCs will carry two Xa and upon differentiation would yield a mosaic culture of WT or mutant MECP2 expressing neurons similar to RTT patients. When pre-XCI RTT-hiPSCs were differentiated into neurons, there was a mosaic expression of MECP2-positive and -negative neurons. The XCI status of hESCs can be categorized into three classes as proposed by Lee and colleagues (113). Similarly, the XCI status of hESCs has been highly variable (114-123). Most RTT-hiPSCs reported are either Class II or III as they are post XCI, exhibit non-random XCI skewing resulting in non-random monoallelic expression of MECP2 and maintain this expression pattern upon differentiation into neurons (33,34,84,85). XCI marker analyses have identified examples of RTT-hiPSCs that can be classified as class II or III based on XIST RNA-FISH and/or H3K27me3 IF (33,35,85,113,116,118-122), while other studies cannot conclusively place their RTT hiPSCs within class II or III (34,84).

Class II hESCs carry a Xi with a non-random skewing pattern resulting in non-random monoallelic expression of X-linked genes (113,118-120,123). It is thought that class I hESCs represent the most pristine pluripotent stem cells present in the human blastocyst which contains two Xa (124). However, the culturing of class I hESCs can result in a spontaneous transition into class II hESCs in which XCI initiates and up-regulates XCI marks (113). Class II hESCs, upon culture and/or cellular stresses such as freeze/thaw cycles, can also lose XCI marks such as XIST RNA and repressive chromatin marks, and thus transition into class III hESCs (113,118,119,121,122).

First, all post-XCI RTT hiPSCs generated to date were reprogrammed by integrating viral vectors (33,34,84,85). Given that each RTT-hiPSC line is expected to be an independent clone, they will harbor unique vector integration sites. Therefore, methods of reprogramming such as non-integrating viral vectors (125,126), non-replicative episomal vectors (127), and/or mRNA- (128), miRNA- (129,130), or protein-based (90) reprogramming methods will be required to overcome this limitation. Secondly, the process of reprogramming itself leads to the accumulation of diverse abnormalities in the genome of hiPSCs (131-135). Therefore, each hiPSC line will carry unique abnormalities that may affect the subsequent phenotype of each hiPSC line. It is, thus, imperative to study more than one hiPSC line per individual, affected and/or unaffected, to ensure the validity of any phenotypes.

Post-XCI hiPSCs were prone to losing XCI marks such as XIST RNA, EZH2, macroH2A1, and H4K20me1 upon extended passaging. However, these hiPSCs retained a transcriptionally silent Xi. This reiterates that evaluation of XCI marks such as XIST RNA and chromatin marks and their mediators is not sufficient to determine XCI status in hPSCs (113,119,122,136). Finally, these data suggest that the non-random XCI nature of female hiPSCs can be exploited to generate isogenic control and mutant hiPSCs from the same individual carrying heterozygous mutations in X-linked genes. However, there are also potential disadvantages to post-XCI RTT-hiPSCs. From a biological

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standpoint, one could question whether post-XCI RTT-hiPSCs are fully reprogrammed given that the Xi did not reactivate. This is likely in part due to suboptimal culture conditions and reprogramming methods required to capture and stabilize RTT-hiPSCs in a pre-XCI state (122,136,137). Although post-XCI RTT-hiPSCs have been shown to exhibit non-random monoallelic expression of MECP2, the possibility that they have partial-XCI cannot be excluded (33,34,84,85).

Differentiation of post-XCI RTT hiPSCs, regardless of the presence or absence of XCI marks, will show a continued presence or absence of XCI marks, respectively, resulting in non-random XCI skewing and monoallelic expression pattern of X-linked genes (27,33,85,113,118-123,136). Altogether, the analysis of XCI upon differentiation of RTT-hiPSCs into neurons will be critical to conclusively demonstrate the isolation of pre-XCI RTT-hiPSCs in comparison to post-XCI RTT-hiPSCs.

Pre-XCI RTT-hiPSCs

Generation of pre-XCI hPSCs will provide an invaluable *in vitro* system to study XCI in humans, which will be essential as the mouse may not be quite as similar as once thought (124,138-141). Pre-XCI RTT-hiPSCs are advantageous because their differentiation initiates random XCI allowing generation of a mosaic culture of neurons expressing WT or mutant MECP2 (14,35). This provides a clinically relevant system as RTT patients are mosaic with respect to WT and mutant MECP2 expression. However, such a mosaic culture may introduce a new variable as one needs to ensure that all differentiation experiments yield similar XCI skewing ratios.

Milestone for the drug therapies in RTT patients

The generation of patient-specific hiPSC has major implications for translational medicine, such as disease phenotyping, drug screens, and cell therapy. Indeed, hiPSC have been generated from a variety of diseases where specific phenotypes have been observed *in vitro* and proof-of-principle drug screens have been performed (3,29-32,142-145). Disease models using iPS from RTT patients' fibroblasts have opened up a new avenue of drug discovery for therapeutic treatment of RTT (14,35). Excellent RTT mouse models have been created to study the disease mechanisms, leading to many important findings with potential therapeutic implications. These include the identification of many MeCP2 target genes, better understanding of the neurobiological consequences of the loss- or misfunction of MeCP2, and drug testing in RTT mice and clinical trials in human RTT patients. However, because of potential differences in the underlying biology of humans and common research animals, there is a need to establish cell culture-based human models for studying disease mechanisms to validate and expand the knowledge acquired in animal models. Therapy has emphasized risperidone, but some atypical antipsychotic medications have been helpful, as have robotic aids, massage, hyperbaric oxygen, and music. Treatment with insulin growth factor 1, a growth factor known to ameliorate the phenotype of RTT mice, improved the RTT iPSC-neuronal phenotypes, providing evidence that synaptic defects can be rescued in neurons derived from RTT patients (14,32,146). The importance of the PI3K pathway is reflected in a number of therapies designed for RTT that aim to restore its activity through the direct application or augmented endogenous synthesis of growth factors such as BDNF or IGF-1 (146-149). These therapies target the tyrosine kinase receptors and hyper-activation of their subsequent downstream cascade that will cause increased protein synthesis in the end impact synaptic maturation and function (73). One recent unbiased, high-content, small molecule screen in primary cortical neurons derived from the Angelman syndrome mouse model revealed a possible new therapeutic avenue that may be applicable to RTT (150). This study showed inhibitors of topoisomerase I and II, enzymes that regulate DNA supercoiling, could be used to unsilence the dormant paternal copy of the Ube3a gene (the primary genetic cause of Angelman Syndrome) in several regions of the nervous system. The resulting non silencing lead to the expression of a functionally normal Ube3a protein, the level of which remained elevated in a subset of spinal cord neurons, weeks after drug treatment. These findings highlight a remarkable potential for a short-term treatment that could lead to long-term effects on gene expression and possible reestablishment of proper neuronal UBE3A function. Furthermore, recent data also suggests that immune system, whether it is adaptive (T cells) or innate (microglia), profoundly impact normal brain function and plasticity (151-153). According to Derecki *et al.*, bone marrow transplant from healthy animals into mutant Mecp2 male animals is being investigated as an approach for the amelioration of RTT symptoms (49).

Originally, RTT was considered as a disorder of early postnatal life; however, one recent study has shown that inducible deletion of MECP2 in adults recapitulates the

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germ-line knock out phenotype in mice (154). In another study, neural progenitors derived from RTT iPSCs were used to analyze mobile element regulation via MeCP2 loss of function, suggesting a new potential molecular mechanism of RTT (80).

MECP2 disorders and developing drug therapies

Currently, RTT has no effective treatment. The recent studies demonstrating that neurological deficits resulting from loss of MeCP2 can be reversed upon restoration of gene function are quite exciting. However, in RTT mice lacking *Mecp2*, reactivation of the *Mecp2* gene after the onset of disease can rescue the disease phenotype (155,156). The genetic rescue data are promising because they show that neurons have suffered the consequences of loss of MeCP2 function are poised to regain functionality once MeCP2 is provided gradually and in the correct spatial distribution. This demonstrates the possibility of RTT gene therapy strategies, where delivering *MECP2* into the affected neurons may indeed improve RTT symptoms. This provides hope for restoring neuronal function in patients with RTT. Rastegar stated that they are creating the first generation of *MECP2* isoform-specific retroviral and lentiviral gene therapy vector and showed their efficiency and long-term expression in the adult brain-derived neural stem cells, in their neuronal progenies, and in the brain microenvironment (157). However, the *in vivo* rescue effect of the gene therapy delivery of these viruses remains to be elucidated. In humans, overexpression of MECP2 caused by duplication of the MECP2 locus leads to a variety of neurological symptoms including seizures and mental retardation (158-160). Alternatively, drug treatments can be designed to target proteins, which may compensate for MeCP2 loss in neurons. However, the strategy in humans will require providing the critical factors that function downstream of MeCP2, because of the challenges in delivering the correct MeCP2 dosage only to neurons that lack it, given that the slightest perturbation in MeCP2 level is deleterious. Thus, therapeutic strategies necessitate the identification of the molecular mechanisms underlying individual RTT phenotypes and picking out the candidates that can be therapeutically targeted. Although the data are minimal, it is quite likely that there will be many genes whose expression is sufficiently altered to cause neuronal dysfunction, probably due to loss of normal homeostatic responses. While it is conceivable that some of these molecules could be investigated for potential therapeutics, it might prove challenging to restore levels of tens of targets.

In addition to the modeling considerations described herein, for the use of stem cell derivatives in human therapy, it will be particularly important to monitor cell karyotype to detect chromosomal abnormalities that could arise during prolonged cell culture. Karyotypic changes have been repeatedly reported for hESCs expanded in culture and might also be expected for hiPSCs, given that these could cause tumorigenicity in addition to teratoma formation after transplantation of derivatives into patients. Finally, factor free reprogramming in fully defined, feeder-free culture conditions will probably be a regulatory requirement for this class of cell-based therapeutics. Another potential use of iPS cells is personalisation of treatment. If scientists could overcome the hindrances to reprogramming, it would be feasible to generate iPS cells from every patient. These could be used to screen for drugs in each individual patient.

Conclusions

The way for disease treatment and prevention is through pathogenesis and physiological mechanisms that eventually lead to the phenotypic symptoms of diseases. Live and post-mortem samples, plus animal models, are the best sources for disease study. Though iPSC technology has a great potential for research and disease modeling, it is still in the initial phase. More research is necessary to determine whether iPSCs from patients with other forms of ASD share common cellular phenotypes with those of RTT patients and if those *in vitro* phenotypes are robust enough to be translated into clinically relevant drug screening. Transplantation of hiPSC derivatives into diseased lesions would probably be the first application of cell replacement therapy, although it is currently unclear whether fully differentiated cells or progenitor or stem cells would more easily reconstitute the tissues at the site(s) of disease.

Future studies with cell-type specific manipulation of MeCP2 to identify and examine the circuit-level contributions to the function which promises to elucidate the further mechanism of disease progression and provide new potential therapeutic targets for RTT. Disease models utilizing patient-specific hiPSCs will probably generate a wealth of information and data that could be combined with genetic analyses of disease. The combination of genetics and hiPSC trait information may allow early and more accurate prediction and diagnosis of disease and disease progression. Moreover, the redefinition of disease subtypes through disease modeling is likely to provide many examples of

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differential response to therapy and understanding of individual responses to drugs will have implications for their use and development by the pharmaceutical industry. Hence, the RTT story started in the clinic and today it has inspired many exciting scientific studies in neurobiology and epigenetics. It is anticipated that the next chapter in this story will involve translation of some discoveries back to the clinic to benefit patients with RTT and patients with related neurological disorders.

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Footnote

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