MECP2e1 Mutation Reduces the Dendritic Complexity of Rett Syndrome Patient iPS Cell-Derived Neurons

by

Wesley Lai

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Molecular Genetics
University of Toronto

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Abstract

The gene MECP2 that is commonly mutated in Rett Syndrome (RTT) can be alternatively spliced in neurons into MeCP2e1 and MECP2e2 isoforms. Fibroblasts from a patient with a MECP2e1 mutation were reprogrammed into induced pluripotent stem cells (iPS) and differentiated into neurons with an EB-based directed differentiation protocol. I employed methods to analyze neuronal complexity with respect to numbers of terminal dendrites, higher order dendrites, intersections by Sholl analysis, and total dendrite length in RTTe1 compared to wildtype neurons. My results show that neuronal complexity is reduced in the RTTe1 neurons, indicating that the MECP2e1 isoform is essential for healthy neuronal morphology. Wildtype and RTT iPS cells from a MECP2 null and R306C patient were also differentiated into neurons with a one-step protocol by exogenous expression of Ngn2 in iPS cells. RTT neurons did not recapitulate dendritic defects, suggesting that these phenotypes may be masked by Ngn2 overexpression.
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Chapter 1
Introduction

1 Introduction

1.1 Rett Syndrome diagnosis and current treatments

Rett Syndrome (RTT) is a neurodevelopmental disorder that affects mostly girls at a prevalence of 1 in 10,000 (1). The core features of RTT are normal development until 6-18 months of age, after which the child loses acquired abilities of speech, purposeful hand movements, motor abilities, and develops stereotypical hand movements. There are additional supportive diagnostic features that are commonly present in RTT children. Some examples include: microcephaly, seizures, breathing abnormalities, autonomic dysfunction, and reduced cognitive ability (2). The presence of the core symptoms alone in the absence of exclusion factors, which are disease caused by secondary brain injury or clinical manifestations before 6 months of age, is sufficient for the diagnosis of RTT. After a period of developmental regression, the condition of a child usually stabilizes and can improve in some cases (3).

There is currently no cure for RTT. The majority of treatments aim to manage symptoms such as anxiety and seizures but have limited success. An ongoing Phase 2 clinical trial is testing the efficacy of measermin (recombinant human IGF-1) in ameliorating RTT phenotypes. In the Phase 1 clinical trial, measermin was well tolerated by treated participants. 9 out of 12 patients had RTT causing MECP2 mutations. Treatment with measermin appeared to moderately reduce breathing abnormalities and partially ameliorate behavioural symptoms associated with RTT such as anxiety (4).

A randomized, double-blinded, placebo-controlled trial of L-Carnitine was conducted and though it did not show significant advantages to improving hand apraxia, L-Carnitine did improve some aspects of motor function and behavior as well as overall patient wellbeing. The trial reported reduced constipation in treatment groups and increased energy, awareness, and physical activity demonstrating its potential utility in improving the lifestyles of both patients and caregivers. The authors noted that the long-term effects of L-Carnitine were not studied and that it may be beneficial for only a small cohort of patients who have low plasma L-Carnitine levels (5).
The use of bromocriptine, a dopamine agonist, in the clinic had been explored in a double-blind trial. Ten RTT girls with a mean age of 8 years and 1 month were partitioned into two groups, one receiving bromocriptine and the other placebo. Treatments lasted for 8 weeks and the developmental progression of children was assessed before treatment, 4 weeks into treatment, and 8 weeks after treatment. Two of the 5 girls receiving the drug benefited significantly from bromocriptine, and 4 of 5 girls receiving placebo did not. This study demonstrated that a subgroup of RTT children might benefit from dopamine agonist treatment though its mechanism remains unknown (6).

Naltrexone, an opiate antagonist, has also been explored in clinical trials but only improved breathing abnormalities found in RTT patients. 6 out of 10 girls treated with naltrexone worsened after 4 months of treatment, advancing to higher stages of disease and showing poorer motor function and no changes in the frequency of characteristic hand movements associated with RTT (7).

Few therapies have been explored in clinical trials. Identifying novel therapeutics that may improve patient symptoms or overall condition will greatly help them to achieve greater autonomy while improving not only the patients’ lifestyle, but also the lifestyle of their caregivers. It is evident that some drugs are effective in improving a patient’s condition. Therefore, efforts to identify better compounds, small molecules, or drugs that can maintain, improve, or restore the patients’ health should not cease.

1.2 The genetic etiology of RTT: MECP2 mutations

Genetically, the most common causes of RTT are mutations in the gene methyl-CpG-binding protein 2 (MECP2) (8). MECP2 encodes the ubiquitously expressed protein MeCP2, which was first shown to be a repressor of transcription (9,10). Over 200 RTT causing MECP2 mutations have been described including: whole deletions, missense, nonsense, and synonymous mutations (11-14). The majority of MECP2 mutations are single C>T transitions that localize to mutational hotspots in functional protein domains. The methyl-CpG-binding domain (MBD), inter-domain region (ID), transcriptional repression domain (TRD), nuclear localization signal (NLS), and C-terminal domain of MECP2 are particularly affected. Genetic mutations occurring in the 5’UTR, 3’UTR, N-terminus, and intronic regions of MECP2 are rare (Figure 1) (15,16).
MECP2 contains four exons. Exons 3 and 4 encode functional domains including the methyl-CpG-binding domain (MBD), inter-domain (ID), transcription repression domain (TRD), and C-terminal domain. Mutations commonly occur in domains indicated with asterisks. Examples of the most common mutations and their localization to domains are indicated (mutations with $\geq 1\%$ prevalence were included based on RettBASE).

1.3 General properties of MeCP2 and its functions on methylated DNA

MeCP2 is a ubiquitously expressed protein but is most abundant in the brain, where expression is highest in neurons and increases throughout neurogenesis (9,17). Quantification of MeCP2 levels in neuronal nuclei estimates the abundance of MeCP2 to be near histone octamer levels (18). In this same study, it was demonstrated that MeCP2 tracks methylated DNA across the genome and might modulate chromatin structure to regulate transcription.

Earlier studies aiming to elucidate the function of MeCP2 suggested that it was as a transcriptional repressor that binds methylated-CpG-dinucleotides in vitro (19). A follow up report showed that the Sin3A-HDAC corepressor complex interacts with a region of MeCP2 now called the transcriptional repression domain (TRD), when bound to methylated DNA, to facilitate the repression of transcription (20). Its mechanism of repression was further delineated by a study showing that MeCP2 interacts with the NCoR/SMRT co-repressor complex, which interacts with HDAC3 for transcriptional repression (21). Evidence for the repressive function of MeCP2 was demonstrated by microarray experiments that showed that MeCP2 subtly repressed gene expression (22-25,27).
An important function of MeCP2 in post-mitotic neurons was defined when it was shown that MeCP2 could play a role in gene regulation in response to neuronal activity. Activity dependent transcription is important for converting short-term stimuli into long-term changes in brain function. Using a candidate gene approach, it was discovered that MeCP2 participates in activity dependent transcription of brain-derived neurotrophic factor (BDNF) (28). Membrane depolarization of neurons increased calcium influx, which resulted in the phosphorylation of MeCP2 and subsequently reduced its affinity for methylated-CpG-dinucleotides. This correlated with Bdnf promoter activation (28). The resultant induction of Bdnf would facilitate neuronal remodeling in response to activity that could promote cell survival and brain plasticity. This is an example of the functional utility of MeCP2 in the brain.

However, the notion that MeCP2 functions as a transcriptional repressor only was questioned when discordant data from microarray experiments were published suggesting that MeCP2 might act as a transcriptional activator (22-27,29). It was later shown in neurons that MeCP2 binds to 5-hydroxymethylcytosine (5hmC) and strongly correlated with active transcription (30). Further evidence supporting the role of MeCP2 in activating transcription was reported when a global down-regulation of gene expression was observed in a study conducted on MECP2-deficient human embryonic stem (ES) cell-derived neurons (31). It is unclear how MeCP2 functions as both a repressor and activator of transcription. Further research needs to be conducted to understand the multi-faceted role of MeCP2.

1.4 MeCP2 isoforms, properties, and domains

An additional layer of complexity to MeCP2 function is added when the different isoforms are considered. MECP2 can be alternatively spliced into two isoforms: MECP2e1 and MECP2e2, (Figure 2) (32,33).

MeCP2e1 is expressed at much higher levels than MeCP2e2 in neurons (33). Two reasons account for this difference in protein isoform levels. The first reason is that there are higher levels of MECP2e1 transcripts than MECP2e2. The second reason is because MECP2e2 contains an upstream open reading frame (upORF) in exon 1 that interferes with the translation of the down stream ORF beginning at exon 2. Mutating the ATG of the upORF increases MeCP2e2 levels (Figure 2) (33).
Figure 2. Splice map of MECP2e1 and MECP2e2. MECP2e1 and MECP2e2 have distinct N-terminal domains because they are spliced differently. MECP2e1 excludes exon 2 while MECP2e2 includes exon 2. MECP2e1 translates from the ATG codon in exon 1. MECP2e2 is translated by initiating translation at the second ATG site. It is hypothesized that the upstream open reading frame (upORF) present in MECP2e2 interferes with translation at the second ATG start site in exon 2. Both isoforms of MeCP2 include exon 3 and 4.

MeCP2e1 and MeCP2e2 have a different N-terminal domain, but share the same MBD, ID, TRD, and C-terminal sequences (Figure 3). It is uncertain whether the two isoforms share similar or distinct functions. It has been reported that MeCP2e2 but not MeCP2e1 is neurotoxic to cells when overexpressed in cerebellar granule cells (34). However, it has also been reported that the reintroduction of MeCP2e2 in MeCP2-deficient mice can fully reverse reduced brain weight, and nuclear size phenotypes, suggesting that there may be some overlapping function between the two MeCP2 isoforms (35). More experiments need to be conducted in order to uncover the unique or common functions of both protein isoforms.
Figure 3. MeCP2e1 and MeCP2e2 have different N-termini but share the same remaining domains. Both MeCP2e1 and MeCP2e2 have an N-terminus (NTD), methyl-CpG binding domain (MBD), inter-domain (ID), transcriptional repression domain (TRD), nuclear localization signal (NLS), and C-terminal domains. The two protein isoforms have distinct N-terminal domains. Numerical values represent amino acid number. MeCP2e1 is larger in size than MeCP2e2.

1.5 Cellular phenotypes in RTT post-mortem tissue

Before the development of rodent models to study RTT, clinicians and researchers relied on the study of post-mortem tissue to gain insight into the neurobiology of RTT. Studying RTT with post-mortem tissue was a useful tool to gain insight into the cellular phenotypes present during disease stabilization. By measuring the weight of brains from RTT patients and comparing them to age-matched controls, researchers discovered that RTT brains were lighter (36-38). When trying to understand the underlying mechanism for smaller and lighter brains, Golgi staining of layer III and layer V neurons in the frontal and motor cortex showed that arborization of both apical and basal dendrites was reduced (36,37). Furthermore, increased cellular density was reported in various regions of the brain such as the cerebral cortex and hippocampus (38). In this same study, the authors showed that neuronal soma size in the hippocampus was also reduced. By creating 3-dimensional reconstructions of frontal cortical neurons labeled with Lucifer yellow and imaged by laser scanning confocal microscopy, Belichenko et al., provided evidence for reduced number of dendritic spines in RTT brains (39). This study suggested that patients had
reduced number of synapses, which was later supported when a reduced number of synaptophysin puncta was observed after immunocytochemical staining of neurons from layer II, III, and V of the frontal, temporal, and motor cortices of RTT patients (40). In summary, these studies seemed to provide preliminary evidence to support the hypothesis that RTT was a neurodevelopmental disorder with defects in neural circuitry and function.

There were a few limitations to using post-mortem tissue to study disease. One limitation that was acknowledged by researchers was that post-mortem tissue reflected more closely the stage of RTT where disease had stabilized and did not offer a representation of disease progression (38,40). Other limitations included the inability to closely match age and gender, and acquire the appropriate controls. Some studies used brains from patients with existing medical conditions such as Trisomy 21 or lupus erythematosus as healthy controls (36-38,40). The issue of tissue accessibility prompted the need for an alternative method to study RTT.

1.6 The most common RTT mouse models: behavioural deficits and cellular phenotypes of neurons and glia

The earliest attempts to generate Mecp2-deficient mice were unsuccessful when researchers engineered the loss of Mecp2 in mouse embryonic stem cells and injected them into blastocyst stage embryos for in utero development. Mecp2^{+/y} chimeric mice were highly abnormal and the majority of chimeras did not survive beyond weaning age. In contrast to Mecp2^{+/y} chimeric mice, control mice developed normally (41).

To circumvent this obstacle, the same research group created a conditional knockout mouse model of RTT. To do this, mice with exon 3 and 4 of Mecp2 flanked by loxP sites were crossed with mice, containing ubiquitous Cre or nestin-Cre. This created Mecp2-deficient mice with a ubiquitous deletion of Mecp2 or targeted deletion of Mecp2 in neurons and glia, respectively. For simplicity of nomenclature in my thesis, I called these mice Mecp2^{B+/y} for male mice and Mecp2^{B+/B-} or Mecp2^{B+/+} mice for homozygous and heterozygous female mice, respectively (42).

When these Mecp2-null mice were studied for aberrant phenotypes, abnormal gait, reduced spontaneous movement, hind limb clasping, and irregular breathing was observed after a period of normalcy. The aberrant phenotypes were also observed in Mecp2-null mice with targeted deletions in neurons and glia. Since RTT is a disorder predominantly affecting girls with a
heterozygous MECP2 mutation, the authors rationalized that studying heterozygous female mice would be the ideal genotype ($MeCP2^{B-/+}$). Interestingly, it was reported that heterozygous female mice showed symptoms much later (9 months) than viable male mice (3 months). This developmental timeline of RTT did not correspond with what has been observed in humans and suggested that mouse models may be an underrepresentation of disease phenotypes (42).

Another research group conducted a similar study using the Cre-loxP system to generate $MeCP2$-deficient mice harboring a deletion in exon 3 (43). For simplicity of nomenclature in my thesis, these mice are called $MeCP2^{J-/+}$ male, $MeCP2^{J-/-}$ female homozygous, and $MeCP2^{J-/+}$ female heterozygous deficient mice. $MeCP2$-deficient mice had behavioural defects: increased anxiety, body tremors, pila erection, and respiratory irregularities. These deficits were accompanied by neuroanatomical defects such as lighter brains, increased cellular densities in different regions of the brain that were accompanied by reduced cellular size and nuclear size. These observations have been previously described in studies conducted on human RTT post-mortem brain samples (36,38). To determine if these phenotypes were caused by $MeCP2$ deficiencies in the brain, Chen et al. targeted the loss of $MeCP2$ in neurons and glia, and neurons only and found that their observations from null-mice were recapitulated in mice with targeted deletions of $MeCP2$. This suggested that RTT was a neurological disorder and that the loss of MeCP2 in post-mitotic neurons was sufficient to cause disease (43).

The behavioural phenotypes of RTT in mice were more carefully studied when a different research group generated another mouse model of RTT that maintained partial function of MeCP2 (44). Mice were engineered to translate truncated MeCP2 that contained its functional MBD and TRD domains but lost a significant portion of its C-terminal tail. The authors reasoned that in order to observe behavioural phenotypes in RTT-male mice, a less severe mutation should be introduced to ameliorate the premature death phenotype that was observed in $MeCP2$-null male mice (42,43). Male mice expressing truncated MeCP2 was termed $MeCP2^{308/}$ mice and demonstrated general phenotypes such as tremors that were readily visible at 4 months of age and seizures by 8 months of age, and paw wringing when suspended by their tail. The motor ability of $MeCP2^{308/}$ mice was tested through a number of assays. $MeCP2^{308/}$ performed poorly on the rotarod experiment, where mice are challenged to maintain their position on an elevated rotating rod. $MeCP2^{308/}$ mice also fell down more during the vertical pole test, where mice are positioned at the top of a vertical pole and need to climb down on their own. They also
performed poorly on the wire suspension test, falling down more frequently in comparison to control mice (44).

In the three aforementioned mouse models of RTT, all authors reported delayed onset of disease progression that did not correspond to the RTT developmental timeline in humans. Specifically, female *Mecp2*-deficient mice had a tendency to develop disease symptoms in adulthood while male *Mecp2*-deficient mice manifested symptoms early in development that corresponded with the human condition (42-44). Additionally, *Mecp2*-null mice had increased incidences of obesity depending on the selected background (42,43). These observations were unexpected since male RTT patients do not usually survive into adulthood, female RTT patients develop symptoms early in their childhood, and RTT children are usually underweight. Despite these limitations, it was evident that *Mecp2*-deficient mice could recapitulate certain disease phenotypes and were a valid model for RTT. This created an opportunity to study the cellular phenotypes associated with *Mecp2*-deficiency in mice as well understand the biological mechanisms underlying RTT.

Chen et al., was the first research group to show that *Mecp2*-deficient mice had the neuronal phenotype of reduced soma size and nuclear size (43). Since then, other research groups have used *Mecp2*-deficient mice to determine if other neuronal morphological phenotypes are present. Using postnatal 3 week old *Mecp2*-deficient male mice harbouring a deletion of exon 3 and 4 (*Mecp2*<sup>B/-y</sup> mice) (42) and an exon 3 deletion (*Mecp2*<sup>L/-y</sup> mice) (43), Belichenko et al. demonstrated that variability in the distribution of dendritic spines on dendrites was present in both mouse models. The observed variability in the distribution of dendritic spines along dendrites was quantified as a greater number of spineless dendrites in both *Mecp2*<sup>B/-y</sup> and *Mecp2*<sup>L/-y</sup> mice in all compartments of the brain examined (45). *Mecp2*<sup>B/-y</sup> and *Mecp2*<sup>L/-y</sup> mice both had a greater number of dendrites with swollen segments in comparison to control mice. Interestingly, overall dendrite width was measured to be thinner than controls. Axonal organization was also examined in *Mecp2*<sup>B/-y</sup> mice and was visibly disorganized when compared to wildtype mice (45).

Dendrite and dendritic spine morphology was also examined in *Mecp2*<sup>B</sup><sub>+/+</sub> female mice at postnatal 6-7 months of age and male mice at postnatal 3 weeks of age (46). Reduced dendrite width and dendritic spine number, increased frequency of spineless dendrites and dendrites with long spines in cortical neurons was reported. Interestingly, this report provided preliminary
evidence to suggest that neurons expressing MeCP2 within the cortex could exert a positive non-cell autonomous effect on MeCP2 deficient neuronal growth (46).

Previous studies on post-mortem brain tissue had provided evidence for reduced dendrite branching and complexity (36,37). Dendritic phenotypes in layer V pyramidal neurons from the motor cortex of $Mecp2^{−/−}$ mice were identified to have reduced total dendrite length and branching (47). This finding was congruent with an earlier report demonstrating reduced dendrite branching and soma size in layer II/III pyramidal neurons in $Mecp2^{B−/−}$ and $Mecp2^{J−/−}$ mice (17). This defect was influenced predominately by cell-autonomous means but weakly influenced by a non-cell-autonomous mechanism. This was observed when the distal complexity of dendrites was reduced in wildtype neurons injected into Mecp2-deficient brains but not when wildtype neurons were injected into the brains of wildtype mice (48).

It was commonly held that MeCP2 was absent in astrocytes and that their contributions to RTT were unknown (17,49). Greater clarity into the contributions of non-cell-autonomous effects on disease was gained when various research groups independently showed that MeCP2 is endogenously expressed in astrocytes and that mutant astrocytes can exert a negative influence on wildtype neurons through the reduced secretion of soluble factors such as BDNF (50-52).

The targeted deletion of Mecp2 from oligodendrocytes has some consequences as well. Oligodendrocytes are generally known as the myelinating cells in the brain. This function is critical for proper neuronal signaling and function. Mice with a targeted deletion of $Mecp2$ in oligodendrocytes and oligodendrocyte precursors cells have more severe hind limb clasping compared to control mice. It was noted that the $Mecp2$-deficient mice in this study had dull fur and that some exhibited piloerection. These results demonstrated that the non-cell autonomous effects of other cells might partially influence disease phenotypes (53).

The reported changes in dendritic spine number and dendritic morphology and cellular soma size support the hypothesis that MeCP2 regulates neuronal circuitry and function since these structures process electrical information for communication between neurons (54). Supporting this hypothesis is evidence of electrophysiological measurements conducted on layer V pyramidal cortical neurons from the somatosensory cortex of $Mecp2^{J−/−}$ mice, which showed reduced spontaneous action potential firing in brain slices from symptomatic $Mecp2$-deficient mice (postnatal 3-4 weeks of age). This reduction in spontaneous action potential firing was
shown to be a result of shifted synaptic balance towards greater inhibition and reduced excitation. The overall net effect was reduced excitatory synaptic activity, which in part accounts for the observed altered electrophysiological properties in Mecp2-null neurons (55).

1.7 Rescue of disease models and limitations of the model

The first rescue experiments were performed by Luikenhuis et al. where the reintroduction of Mecp2 in post-mitotic neurons of developing mice increased longevity, brain weight, and improved behavioural phenotypes observed in Mecp2-deficient mice (56). Early administration of BDNF could partially abolish RTT phenotypes (57). In this study, Mecp2-deficient mice overexpressing BDNF had delayed onset of disease, improved locomotor ability and partially restored brain weight, which is usually reduced. Of particular interest was the functional restoration of spontaneous action potential firing deficits observed in mutants. These two studies demonstrated that RTT was reversible so long as Mecp2 was reintroduced early in the developing embryo. What remained unknown at the time was the reversibility of RTT after disease onset and progression.

The principle of RTT reversibility was demonstrated in an elegant study where the reexpression of MeCP2 in symptomatic Mecp2-deficient mice resulted in the amelioration of RTT phenotypes. Male and Female symptomatic Mecp2-deficient mice (containing a floxed STOP-Neo cassette in intron 2 of Mecp2 and ubiquitously expressing Cre-ER transgene had irregular breathing, hind limb clasping, abnormal gait, and electrophysiological changes in long term potentiation. Tamoxifen treatment resulted in the excision of the floxed STOP-Neo cassette, which reactivated Mecp2 postnatally and could rescue changes in electrophysiological properties (58). This study was reproduced soon after when cellular reexpression of Mecp2e2 isoform postnatally partially reversed the RTT mouse body weight phenotypes, reduced brain weight phenotype, and reduced nuclear size defect (35). These genetic rescue experiments demonstrated the revocable nature of RTT by reintroducing wildtype Mecp2 in Mecp2-deficient mice after disease onset and progression. These studies led to future attempts at aiming to reverse RTT phenotypes in symptomatic mice by using small molecules, drugs, and compounds such as: TrkB agonists, Insulin growth factor 1 (IGF-1), and Fingolimod (59-61). Interestingly, the compounds listed above targeted and activated the PI3K/AKT and MAPK pathways, which are critical for
neuronal development, synaptic maturation, and plasticity. Evidence for reduced PI3K/AKT signaling in Mecp2-deficient mice had been described previously (59,62).

*Mecp2*-deficient mice have demonstrated their utility for studying RTT. Since RTT is a complex disease that alters behaviour and motor ability, a clear read-out can be obtained from mice that is not be possible from post-mortem studies. However, it was clear from the initial use of Mecp2-deficient mice that they may be an underrepresentation of disease phenotypes since male mice reflect the human condition of girls more accurately than female mice. In addition to this caveat, another limitation is that mouse models may display supplemental phenotypes that are not commonly present in patients (42,43). One example of this scenario is that Mecp2-deficient mice are sometimes overweight, which is not a condition seen in patients who are usually underweight (43). Therefore, to overcome these obstacles, some researchers have turned to the application of induced pluripotent stem (iPS) cell and embryonic stem (ES) cell technology to study RTT disease phenotypes (63-67).

### 1.8 iPS cells in studying disease

iPS cells were discovered by Shinya Yamanaka’s research group (68). His research group demonstrated that embryonic-like cells could be generated from murine and human somatic cells such as fibroblasts by the exogenous expression of four transcription factors OCT4, SOX2, KLF-4, and c-MYC (68,69). One advantage of this technology is its ability to generate any cell type in the body, which may be useful for studying disease mechanisms, identifying disease phenotypes, drug discovery, and cell transplantation (70).

Soon after the discovery of iPS cells, several research groups were able to generate disease specific iPS cells but were unable to identify disease phenotypes (71-74). A group studying familial dysautonomia (FD) obtained FD-iPS cells and was the first group to successfully show that disease phenotypes can be elucidated from iPS derived cells and that they are useful for drug discovery (75). FD is a hereditary disease characterized by the degeneration of sensory and autonomic neurons. FD iPS cell-derived neural crest precursors had distinct gene expression profiles in comparison to control neural crest precursor cells. By comparing multiple cell lines from different FD patients, they showed that these changes in gene expression were correlated with both a differentiation defect and a migration defect. They also demonstrated that this technology could be used for drug discovery and reported a candidate compound that was able to
partially rescue some disease phenotypes. This study identified novel disease phenotypes and a potentially beneficial therapeutic compound that had not been previously identified in FD mouse models. This validated the use of iPS cells for studying early onset disease and drug discovery (Figure 4) (75).

**Figure 4. Studying neurological diseases with iPS cell technology.** Patient fibroblasts can be reprogrammed into iPS cells by the viral expression of four transcription factors: OCT4 (O), SOX2 (S), KLF-4 (K), and c-MYC (M). iPS cells can be differentiated *in vitro* to generate an affected cell type to study disease. The efficacy of drugs, small molecules, or compounds can be tested for their ability reverse disease phenotypes.

### 1.9 Pertinent neuronal differentiation techniques and subtypes made

For the effective identification of disease phenotypes with iPS cell derived neurons, *in vitro* differentiation protocols must be carefully crafted so that the specific affected cell type of interest is obtained (76). Zhang et al., was one of the earlier groups to successfully differentiate human ES cells into neurons (77). ES cells were detached from their adherent culture conditions and cultured in suspension for four days. This gave rise to embryoid bodies (EBs), which were then cultured in adherent conditions for 7 days in the presence of FGF-2. This yielded neural rosettes with neural progenitor cells (NPCs) localized at the core of rosettes and contaminating cells at the periphery. Zhang et al., isolated neural precursors by differential enzymatic treatment and adhesion and expanded neural precursors in suspension cultures with the continued presence of FGF-2. To induce the differentiation of NPCs into neurons, NPCs were dissociated with trypsin and seeded onto ornithine/laminin coated plates and cultured in the absence of FGF-2 but in the presence of N2 supplement, BDNF, and cAMP (Figure 5).
Figure 5. Schematic overview of the neuronal differentiation protocol described by Zhang et al., 2001. hES cells were differentiated into EBs by withdrawing FGF-2 from ES cell media and detaching them from adherent plates. EBs were transferred to ornithine/laminin coated plates and cultured in suspension with media containing insulin, transferrin, putrescine, sodium selenite, heparin, and FGF-2 for 10 days to generate neural rosettes. Neural progenitor cells were enzymatically isolated and cultured as neurospheres with neural progenitor media containing FGF-2. NPCs were differentiated into neurons by culturing cells with media containing N2 supplement, cAMP, and BDNF. Neurons were studied at approximately 1-2 weeks after differentiation from the NPC stage.

This research group conducted immunocytochemistry experiments and determined that the majority of cell types generated were positive for the neuronal marker MAP2 or βIII-tubulin. They also determined that the majority of cells were glutamatergic neurons with fewer GABAergic neurons present. Astrocytes could also be made with this differentiation protocol. Most importantly, the authors demonstrated that NPCs could incorporate into various regions of neonatal mice brains and differentiate into both neurons and astrocytes (77).

Li et al., also used an EB-based directed differentiation protocol that was based on the protocol developed by Zhang et al., but with a few modifications (77,78). hES cells were differentiated into EBs by detaching ES cells and culturing them in suspension with ES cell media without
FGF-2 for 4 days. EBs were transferred to plastic surface plates and cultured in neural media without FGF-2 for 10-12 days to obtain neural rosettes. NPCs were isolated from neural rosettes and expanded in suspension cultures as neurospheres with neural media lacking FGF-2. To induce the differentiation of NPCs into neurons, NPCs were dissociated into single cells with Accutase and transferred to ornithine/laminin coated plates so that NPCs could grow in adherent conditions with nutrient rich neuronal media containing BDNF, GDNF, IGF-1, N2 supplement, and B27 supplement without FGF-2 (Figure 6) (78).

**Figure 6. Schematic overview of the neuronal differentiation protocol described by Li et al., 2009.** The protocol described by Li et al., was based on the protocol reported by Zhang et al., 2001 with a few modifications. ES cells were induced to form EBs by withdrawing FGF-2 from ES cell media and detaching ES cells from adherent plates. EBs were plated after 4 days of suspension culture to plastic surface plates and cultured for 10 more days under adherent conditions in neural media lacking FGF-2. NPCs were obtained from neural rosettes and maintained in suspension cultures. To obtain neurons, NPCs were seeded onto ornithine/laminin coated plates and cultured in neuronal differentiation media consisting of neurobasal media supplemented with N2, B27, BDNF, GDNF, and IGF1 without FGF-2.
EB-based methods can be protracted, inefficient, and generate heterogeneous population of cells (79). To overcome these limitations, Chambers et al., provided evidence that the use of dual SMAD inhibitors, Noggin and SB431542, can increase the efficiency of neural rosette formation, reduce the time needed to obtain neurons, and remove the need for transient formation of EBs (79). Noggin is a bone morphogenic protein (BMP) signaling inhibitor and SB431542 is a Lefty/Activin/TGF-β signaling inhibitor. For the induction of hES or iPS cells into neural rosettes, ES cells were plated at high density and cultured in knockout serum replacement (KSR) media supplemented with Noggin for 11 days. Cells were cultured with SB431542 for the first 5 days of neural induction and withdrawn for the remaining days. Starting on day 5, KSR media was supplemented with sequential increases of N2 (25, 50, 75, 100%) with feedings and increasing dosages of N2 occurring every other day. By day 11, robust neural rosette formation was observed (Figure 7A). It was reported that neural rosettes were positive for PAX6, OTX2, and FOXG1B, which are markers for anterior fate neural precursors. This research group was interested in generating dopaminergic neurons and motor neurons. To generate dopaminergic neurons, ES cells were cultured in KSR media with Noggin and SB431542 for the first 5 days, then switched to N2 media containing BDNF, AA, sonic hedgehog (SHH). On day 9, FGF8 was added to culture media for an additional 3 days, after which neurons were cultured in N2 media supplemented with BDNF, GDNF, cAMP, ascorbic acid, TGF-β1 without SHH and FGF8. To generate motor neurons, identical culture conditions to generating dopaminergic neurons were used, but after day 9, neurons were cultured in N2 media containing BDNF, ascorbic acid, SHH, and retinoic acid (RA) for two weeks (79) (Figure 7B).
Figure 7. Schematic overview of the neuronal differentiation protocol described by Chambers et al., 2009. (A) The differentiation of ES cells into neural rosettes. The differentiation of ES cells was induced by dual SMAD inhibition in KSR media. SB431542 Lefty/Activin/TGF-β inhibitor was withdrawn after 5 days of treatment and levels of N2 supplement was sequentially increased in culture media. (B) The culture conditions for obtaining dopaminergic neurons and motor neurons were identical until day 12 of differentiation. ES cells were induced by dual SMAD inhibition and then cultured in N2 media supplemented with BDNF and ascorbic acid (AA) after 5 days of culture. SHH is added to media on day 5 and FGF8 is introduced to media on day 9. Both SHH and FGF8 were maintained in culture until day 12. To obtain dopaminergic neurons, neural rosettes were switched to neuronal differentiation media containing BDNF, GDNF, AA, cAMP, and TGF-β1. To obtain motor neurons, neural rosettes were switched to BDNF, AA, SH, and retinoic acid (RA) rich media.
One of the challenges to the aforementioned neuronal differentiation protocols is synchronizing the time course of differentiation for multiple cell lines for disease modeling. This is because the induction of iPS cells into neurons may often fail and result in incomplete experiments that lack important controls. The expansion and passaging of NPCs as neurospheres is also cumbersome and technically demanding. Brennand et al., created a neuronal differentiation protocol that addressed some of the limitations to the aforementioned protocols (77-80). iPS cells were detached with collagenase and cultured as EBs for 7 days in N2 media then transferred to polyornithine (PORN)/Laminin coated plates and cultured in N2 media with laminin for another 7 days. Neural rosettes were manually isolated and then cultured in NPC media with N2, B27-RA supplement, laminin, and FGF2. Neural rosettes were dissociated into single cells when cultures became confluent and grown on PORN/Laminin coated plates in NPC media. At this point of differentiation, these cells could be frozen so that the neuronal differentiation of multiple cell lines could be synchronized or seeded immediately for neuronal induction of NPCs into neurons. For the differentiation of NPCs into neurons, NPCs were seeded on PORN/Laminin coated plates and cultured in neural differentiation medium containing N2, B27-RA, BDNF, GDNF, cAMP, and ascorbic acid. For their study, Brennand et al. differentiated neurons for 1-3 months before performing their experiments (Figure 8) (80).

The advantages of this protocol are that NPCs can be expanded in adherent monolayer culture and frozen for future use (80). Freezing NPCs for future use is advantageous especially if multiple cell lines need to be obtained for one experiment. After NPCs for all cell lines have been acquired, NPCs can be seeded simultaneously and differentiated into neurons. Generating neurons from the iPS cell stage is very difficult and inefficient, but generating neurons from the NPC stage is much easier, more efficient, and more reliable.
Figure 8. Schematic overview of the neuronal differentiation protocol described by Brennand et al., 2011. iPS cells were detached from plates and induced to form EBs by culturing iPS cells with media supplemented with N2. EBs were cultured for 7 days then transferred to PORN/laminin coated plates and cultured with N2 media supplemented with laminin for an additional 7 days. Neural rosettes were manually isolated and dissociated into single cells and maintained as monolayer cultures with N2 media supplemented with B27 without vitamin A (B27-RA), laminin, and FGF2. To induce NPC differentiation into neurons, NPCs were cultured with N2/B27 media with BDNF, GDNF, cAMP, and ascorbic acid (AA).

To improve the protocol described by Brennand et al., so that neural rosettes could be obtained more efficiently, our lab modified the Brennand protocol to include dual SMAD inhibitors that were similarly used in the protocol described by Chambers et al. (79,80). EBs were cultured in suspension for 7 days in vitro with dual SMAD inhibitors then transferred to PORN/laminin coated plates and cultured in adherent conditions for an additional 7 days to generate neural epithelial cells that cluster together to form neural rosettes. NPCs were manually isolated from neural rosettes and propagated as monolayer cultures on PORN/laminin coated plates or cryopreserved until needed. To obtain neurons, NPCs were seeded onto PORN/laminin coated plates and cultured in neuronal differentiation media rich with BDNF, IGF-1, GDNF, cAMP, and
ascorbic acid. In our hands, this protocol required approximately 8-9 weeks of culture to obtain electrophysiologically active neurons for experiments (Figure 9).

![Directed differentiation protocol of iPS cells into neurons used in my thesis.](image)

**Figure 9. Directed differentiation protocol of iPS cells into neurons used in my thesis.** iPS cells were induced to form embryoid bodies (EBs) that were cultured in suspension conditions with media containing BMP and Lefty/Activin/TGF-β signaling inhibitors (Dorsomorphin (DSM) and SB431542, respectively). After 1 week of culture, EBs were plated onto PORN/laminin coated plates and neural rosettes were cultured in adherent conditions for 1 to 2 weeks before NPCS were manually isolated and cultured in the presence of FGF2. NPCs at this stage can be frozen for later use, expanded as monolayer NPCs, or seeded for neuronal differentiation. Supplementing media with BDNF, GDNF, IGF-1, cAMP, and ascorbic acid facilitates neuronal differentiation. Neurons can be identified as early as two weeks after differentiation but do not have robust electrophysiological read outs until 6 weeks of differentiation.

These protocols are technically challenging and require a lot of time before neurons are ready for experimentation. Another limitation to these protocols is the heterogeneity in neuronal cultures. Experiments conducted by another member of our lab observed through single cell expression analyses that our lab’s modified Brennand protocol (described below) generates glutamatergic
and GABAergic neurons, and upper layer and lower layer cortical neurons (81). Alternative methods that could obtain functionally active neurons with greater efficiency and higher purity would be favourable.

The one-step induction protocol can yield a homogenous population of excitatory, electrophysiologically active neurons within 3 weeks of induction from the iPS cell stage (82). Neurons are generated directly from iPS cells to make iNeurons through the overexpression of transcription factor neurogenin-2 (Ngn2). To obtain iNeurons, iPS cells or ES cells are infected with lentiviruses containing Ngn2 gene, a GFP reporter, and puromycin selection cassette (Figure 10A). A tetracycline inducible promoter controls transgene expression. Treating iPS cells with doxycycline induces gene expression and only cells expressing the lentiviruses are selected for by puromycin selection. Resistant cells are cultured with glial cells to facilitate neuronal differentiation and synapse formation. This protocol can yield electrophysiologically active neurons within 2-3 weeks of differentiation (Figure 10B). This method yields neurons much faster and is less technically demanding than EB-based methods. Unlike EB-based methods, this protocol has not been used to study RTT phenotypes. It is unclear whether neurons derived from this protocol can recapitulate disease phenotypes.
Figure 10. One-step induction protocol can generate functional neurons after 2-3 weeks of differentiation. (A) iPS cells were infected with viruses expressing rtTA and viruses expressing Ngn2/EGFP/puromycin fusion protein linked by P2A and T2A sequences. A TetO inducible promoter that is sensitive to doxycycline treatment controls the expression of Ngn2. (B) Lentiviruses were infected into iPS cells. Cells were treated with doxycycline to induce transgene expression. Doxycycline treatment remained for the duration of the experiment. Cells were treated with puromycin for 1 day only to select for resistant cells. Mouse astrocytes were added to neuronal cultures after puromycin selection to facilitate synapse formation and neuronal maturation. Neurons were cultured for 2-3 weeks in vitro.
1.10 Existing RTT iPS and ES cell models and their limitations

The first RTT iPS cells were described by Hotta et al., who obtained iPS cells from Mecep2<sup>308</sup> mice and an 8-year-old RTT girl harboring a MECP2<sup>R306C</sup> mutation (74). No disease phenotypes were reported, but it demonstrated that iPS cells could be isolated from both murine and human patients. Follow up studies conducted on Mecep2<sup>308/y</sup> iPS cells demonstrated the utility of mouse iPS cell technology for recapitulating disease phenotypes that have been previously described from post-mortem tissue and Mecep2-deficient mouse models (65). miPS cells were differentiated using the retinoic acid-mediated differentiation protocol that generates glutamatergic neurons. In this study, our lab demonstrated that iPS-cell derived neurons from mice with disease relevant Mecep2 mutations had reduced soma size and changes in electrophysiological properties (65).

Another research group used mouse ES-cell derived neurons with an engineered MECP2 exon 3-4 mutation to study disease phenotypes. This research group identified reduced nuclear size in MECP2-deficient ES cell-derived neurons (67). These studies validated the use of neuronally differentiated iPS and ES cells from Mecep2-deficient mice for studying RTT.

Marchetto et al., was the first research group to show that human RTT iPS cell derived neurons have disease phenotypes and can be used for studying the efficacy of drugs at reversing disease related phenotypes (63). Fibroblasts from four female patients with distinct mutations were reprogrammed into iPS cells and differentiated into neurons for morphological analysis. Marchetto et al., first determined whether X chromosome-inactivation was erased after reprogramming patient fibroblasts and found that their iPS cell lines either had two active X chromosomes or retained an inactive X chromosome. They chose to model the disease using only the reactivated iPS cell lines because they were randomly inactivated upon differentiation to produce a mixture of wildtype and mutant neurons for phenotyping. An EB-based method of neuronal differentiation was used to obtain neurons from iPS cells. Treatment with retinoic acid induced neuronal differentiation. The neuronal subtypes made were not studied extensively but glutamatergic neurons were identified by immunocytochemistry. RTT neurons had reduced soma size and fewer synapse numbers, which was quantified by the number of VGLUT1 puncta on MAP2 positive dendrites. The reduced number of synapses was correlated with altered activity-dependent calcium transients that reflected functional deficits present in RTT neurons. Remarkably, the synapse deficit phenotype was reversed by IGF-1 treatment. This seminal
research paper demonstrated that iPS cell derived neurons from RTT patients can effectively reveal RTT phenotypes and be applied to drug discovery (63).

Our lab obtained human fibroblasts from a female 6-year-old RTT patient with a deletion in exon 3 and 4 of MECP2 (RTTΔ3-4). The fibroblasts were reprogrammed into iPS cells for neuronal induction and morphological phenotyping (64). When fibroblasts were reprogrammed into iPS cells, the inactive X-chromosome was retained in a non-random pattern that was also maintained after neuronal differentiation. This allowed for the isolation of an isogenic wildtype control (RTTΔ3-4#37) that shared an identical genetic background to a RTT cell line (RTTΔ3-4#20), but differed by the deletion of exon 3 and 4 of MECP2. The isolation of isogenic cell lines is advantageous since variability between cell lines due to genetic background is lost. iPS cells were differentiated into neurons using the protocol described by Li et al., and the soma sizes of iPS cell derived neurons from both cell lines were measured (78). Reduced soma size was reported for RTT neurons. Our study signified the potential to generate isogenic pairs of cell lines from RTT patients, which can be utilized to study disease phenotypes (64).

To study the impact of MECP2 mutations in its various domains, Kim et al., obtained fibroblasts from 5 different patients with point mutations affecting the three main domains of MECP2: MBD, TRD, and CTD. RTT patient fibroblasts were obtained and reprogrammed into iPS cells. The X-inactivation status of iPS cells was also studied and this research group isolated some iPS cell lines with two active X-chromosomes and some with only one active X-chromosome. They differentiated iPS cells into neurons using an EB-based differentiation protocol and studied the expression level of candidate genes and found that the expression level of both TuJ and markers for sodium channels were reduced. The authors concluded that RTT neurons had a neuronal maturation defect, which they alluded to as in agreement with the study conducted by Marchetto et al. (63,66).

Ananiev et al., obtained fibroblasts from a RTT patient with a MECP2 R294X mutation and reprogrammed them into iPS cells (83). This research group also studied the X-inactivation status of iPS cells and neuronal derivatives and showed that the X-inactivation mark was not erased upon reprogramming of patient fibroblasts into iPS cells. When these cells were differentiated into neurons they retained the inactive X chromosome status in a nonrandom manner. This explains why isogenic cell lines could be isolated from this one patient. The iPS
cell lines were differentiated into neurons using a slightly modified version of the protocol described by Zhang et al., for morphological analyses (77). It was reported that R294X RTT neurons had reduced nuclear size compared to isogenic wildtype controls. This study supported previous findings that Mecp2-deficient mice and neurons from post-mortem RTT patients had reduced nuclear size (83). This reaffirmed the notion that RTT iPS cell derived neurons can exhibit clinical features observed in post-mortem tissue and Mecp2-deficient mice.

Pluripotent stem cell derived systems have great utility for studying the cellular morphology of specific cells that may be otherwise difficult to obtain. In the context of RTT, further optimizations of differentiation protocols to yield highly homogenous cultures of neurons will facilitate more accurate characterizations of affected cells. However, this same advantage may be a disadvantage since the brain itself is a highly heterogeneous organ. Glial cells are required for proper neuronal development in living organisms, and the in vivo environmental niche of the brain may not be easily recreated in vitro. Neurons can be cocultured with glia, but it is still challenging to recreate an in vivo context that can faithfully reconstruct the disease environmental niche. Cell line variability is also a concern for iPS cell-based studies, and can be countered by evaluating multiple cell lines obtained from the same patient (84). The genetic background of cell lines can also affect the phenotypic read out of interest. Therefore, if comparisons are made between disease cells and healthy cells, where possible, the employment of isogenic controls will reduce artifacts of genetic background (84).

1.11 Establishment and characterization of the RTTe1 cell lines

Since the discovery of MECP2 isoforms e1 and e2, there has been greater interest in identifying their functions and relevance to RTT. At the beginning of my graduate studies in 2012, it was unclear whether the two isoforms had unique or similar function. Work from Rudolph Jaenisch’s research group suggested that they had overlapping function and that RTT phenotypes could be partially rescued by the delayed overexpression of MeCP2e2 in symptomatic Mecp2-deficient mice, and fully rescued if reintroduced early in the neural development of Mecp2-deficient mice (35,56). Patients with MECP2e1 mutations have been previously described, whereas patients with MECP2e2 mutations have not (32,85-88). This suggests that MECP2e1 is the disease relevant isoform of RTT.
No one has characterized the functional role of MeCP2e1 in humans. To study the function of this isoform, our lab acquired and reprogrammed fibroblasts into iPS cells from a RTT patient with an 11 base pair (bp) deletion in exon 1 of MECP2 (RTTe1) (32). This deletion causes a premature stop codon resulting in truncated MeCP2e1 while leaving MeCP2e2 intact.

From the reprogramming event, a total of 4 RTTe1 iPS cell lines were extensively characterized. Three of those cell lines were included in my study. The characterized RTTe1 cell lines were karyotypically normal, and demonstrated pluripotent properties in vitro and in vivo via teratoma assay formation (Table 1). cDNA from RTTe1 iPS cells and neurons were sequenced to verify the presence of their MECP2 deletion. Androgen receptor assays were also performed to demonstrate that RTTe1 iPS cell lines and neurons were skewed towards the mutant allele.

Neurons were obtained by the modified Brennand protocol described in Figure 9. Based on single cell gene expression profiling comparing control neurons to RTTe1 neurons, RTTe1 neurons generated similar numbers of upper layer and lower layer neurons, and similar numbers of excitatory and inhibitory neurons, but fewer cells expressing neuron markers: DCX, NCAM, and MAP2 compared to controls. The soma sizes of RTTe1 neurons were also measured and found to be smaller. Electrophysiological recordings of RTTe1 neurons showed that they had reduced capacitance, and reduced frequency and amplitude of both evoked action potentials and spontaneous action potentials. These assays were performed by other members of our lab and demonstrated that the RTTe1 cell lines were pluripotent, carried MECP2 mutations, and can display RTT phenotypes. No isogenic controls were isolated from the reprogramming of fibroblasts into iPS cells (81).

In my thesis, additional wildtype and RTT iPS cell lines were employed as controls for assaying dendritic complexity. The characterizations of iPS cell lines were completed by other members of our lab and are summarized in Table 1. The additional cell lines expressed pluripotency genes and made cells from the three germ layers in vitro and in vivo. All iPS cell lines were karyotypically normal. All iPS cells were differentiated into neurons using the protocol described in Figure 9.
Table 1. Summarized characterizations of RTT and healthy control cell lines. Nine cell lines were included in my thesis. iPS and ES cell lines were characterized for pluripotency by lab members. All iPS cell lines were karyotypically normal.

### 1.12 Thesis Rationale and Hypothesis

*MECP2* mutations affecting both isoforms have been described in human patients and results in defects of neural circuitry and function (63). These findings were also reported in *MeCP2*-deficient mice containing a mutation affecting both isoforms of MeCP2 (55). Both lines of evidence strengthen the current perspective that communication between neurons in RTT is defective. MeCP2e1 is approximately 10-fold more abundant than MeCP2e2 in the brain (33). No research group has studied the effects of losing only the predominant isoform of MeCP2, MeCP2e1, on neural circuitry and function in humans. It is unclear whether a disease causing mutation affecting MeCP2e1 and leaving MeCP2e2 intact is sufficient to cause perturbed dendritic complexity, which would partially affect intercellular communication. I hypothesize that a MECP2e1 mutation in RTT iPS cell derived neurons is sufficient to cause defects in dendritic complexity.
Chapter 2
Materials and Methods

2 Materials and Methods

2.1 Cell Culture

Human patient derived iPS cells and hES cells were cultured under the approval of the SickKids Research Ethics Board and Canadian Institute of Health Research Stem Cell Oversight Committee. Pluripotent cells (iPS and ES cells) were cultured on matrigel-coated plates (STEMCELL Technologies) and maintained in iPS media [mTESR™1 media containing 5X supplement (STEMCELL Technologies) and 100X penicillin and streptomycin (Gibco)]. Spontaneously differentiating cell types surrounding pluripotent colonies were manually removed with a plastic micropipette tip under a dissection microscope. Pluripotent cells were enzymatically detached with Dispase (STEMCELL Technologies) and passaged every 7 days.

2.2 Neuronal Differentiation

Pluripotent stem cells were differentiated into neurons using the directed differentiation protocol described by Brennand et al. with a few modifications or recently acquired one-step induction protocol (80,82).

To obtain neurons by the directed differentiation protocol, pluripotent cell colonies were enzymatically detached from matrigel-coated plates using Collagenase IV (Invitrogen), transferred to 6 well low cluster plates for embryoid body formation (Corning). Embryoid bodies (EBs) were cultured in EB media [Dulbecco Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12; Gibco) supplemented with N-2 (100X; Gibco), MEM Non-essential Amino Acids (NEAA; 100X; Gibco), Penicillin and Streptomycin (100X; Gibco), Heparin (2 µg/ml; Sigma), Fibroblast Growth Factor 2 (FGF2; 10 ng/ml; R&D), Dorsomorphin (2 µM; Sigma), and SB431542 (10 µM; Stemgent)] for 7 days in vitro then transferred to Poly-L-Ornithine (0.1 mg/ml; Sigma) and Laminin (20 µg/ml; Roche) (PORN/Laminin) coated plates for the formation of primary neural rosettes. Primary neural rosettes were cultured in adherent conditions and maintained in Neural Rosette (NR) media [Dulbecco Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12; Gibco) supplemented with N-2 (100X; Gibco), MEM Non-essential Amino
Acids (NEAA; 100X; Gibco), Penicillin and Streptomycin (100X; Gibco), Heparin (2 µg/ml; Sigma), Fibroblast Growth Factor 2 (FGF2; 10 ng/ml; R&D), and Laminin (1 µg/ml; Roche) for 7 days in vitro. Primary Neural Rosettes were manually resected using a sterile needle and P20 micropipette tip, and transferred to PORN/Laminin-coated plates for an additional 7 days of in vitro culture in NR media. Secondary NRs were manually resected and enzymatically dissociated to single cells with Accutase (Innovative Cell Technologies). Dissociated Neural Progenitor Cells (NPCs) were transferred to PORN/Laminin-coated plates and were cultured in NPC media [Dulbecco Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12; Gibco) supplemented with N-2 (100X; Gibco), MEM Non-essential Amino Acids (NEAA; 100X; Gibco), Penicillin and Streptomycin (100X; Gibco), Heparin (2 µg/ml; Sigma), Fibroblast Growth Factor 2 (FGF2; 10 ng/ml; R&D), Laminin (1 µg/ml; Roche), and B27 without Vitamin A (50X; Gibco)]. NPCs were enzymatically passaged with Accutase every 7 days and were discarded after 10 passages.

For assays performed on 2-week-old neurons, NPCs were seeded together at a density of 5.0x10⁴ cells/well of 24-well plates with PORN/Laminin-coated glass cover slips. NPCs were cultured in complete Neuronal Differentiation (cND) media [Neurobasal (Gibco) supplemented with N-2 (100X; Gibco), MEM Non-essential Amino Acids (NEAA; 100X; Gibco), Penicillin and Streptomycin (100X; Gibco), Laminin (1 µg/ml; Roche), B27 without Vitamin A (50X; Gibco), BDNF, GDNF, IGF-1 (all 10 ng/ml; Peprotech), ascorbic Acid (200ng/ml; Sigma) and cAMP (1 µM; Sigma)] for two weeks in vitro.

For 6-week-old neuronal morphology assays, RTT neurons were seeded at equivalent densities (10x10⁴ cells/well of 24-well plate) and maintained in cND media with the addition of N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester (DAPT) to promote neuronal induction and manage cell density. Cells were treated with DAPT for the first 10 days of NPC induction into neurons and then maintained in cND without DAPT for the remaining 32 days. For western blot experiments performed on neurons differentiated for 6 weeks, NPCs were seeded at a density of 6.0x10⁵ cells/well of 6-well PORN/Laminin-coated plates and maintained as described above.

Neurons derived from the one-step induction protocol were obtained from Dae-Sung Kim, a post-doctoral fellow in our lab (82). Briefly, pluripotent cells were infected with Ngn-2 lentiviruses while being maintained in iPS media. One day after infection, cells were treated with
doxycycline to induce transgene expression. Ngn2 expressing cells were isolated by puromycin selection then cocultured with mouse astrocytes on the following day. Neurons were differentiated for two or three weeks in vitro before fixation, immunocytochemical staining, and morphological analyses.

2.3 Immunocytochemical (ICC) Staining

Neuronal cultures were fixed with 4% formaldehyde (EMD Biosciences) diluted in phosphate buffer saline (PBS) for 10 min at room temperature and permeabilized with 0.1% Triton X-100 diluted in PBS for 10 min at room temperature. Neuronal cultures were incubated in 5% normal goat serum diluted in PBS (blocking solution) for 1 hour at room temperature before incubating neuronal cultures in primary antibodies diluted in blocking solution overnight at 4°C. Excess primary antibody was removed by washing with PBS and neuronal cultures were incubated with the appropriate fluorescence conjugated secondary antibody for detection. After staining nuclei with DAPI (2 µg/ml), coverslips were mounted onto glass slides and imaged with a Leica DMI 4000B microscope equipped with a Leica DFC340 Fx camera. Images were acquired using Leica Application Suite V3.6 software. Information regarding primary antibodies and their pairings with secondary antibodies are described in Table 2.
<table>
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<tr>
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<th>Catalog #</th>
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<th>Catalog #</th>
</tr>
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<td>×</td>
<td>×</td>
<td>×</td>
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<tr>
<td></td>
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<td>Sigma #M6818</td>
<td>Anti-Ms-488</td>
<td>1:500</td>
<td>Molecular Probes #A11001</td>
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<tr>
<td></td>
<td>Rb Anti-MAP2</td>
<td>1:500</td>
<td>Millipore #AB5622</td>
<td>Anti-Rb-555</td>
<td>1:500</td>
<td>Molecular Probes #A21429</td>
</tr>
<tr>
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<td>1:1000</td>
<td>Invitrogen #A11122</td>
<td>Anti-Rb-488</td>
<td>1:500</td>
<td>Invitrogen #A11008</td>
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<td>1:3000</td>
<td>Sigma #M1406</td>
<td>Anti-Ms-555</td>
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<td>Molecular Probes #A21422</td>
</tr>
<tr>
<td>Dendrite Morphology (One-step)</td>
<td>DAPI</td>
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<td>N/A</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td></td>
<td>Ms Anti-MAP2</td>
<td>1:3000</td>
<td>Sigma #M1406</td>
<td>Anti-Ms-555</td>
<td>1:500</td>
<td>Molecular Probes #A21422</td>
</tr>
</tbody>
</table>

Table 2. Summary of antibodies used in various experiments. All antibodies were diluted in blocking solution (5% normal goat serum diluted in PBS).

2.4 Neuronal Morphometric Analyses

Neurite morphology assays were performed on neurons differentiated for 2 weeks and 6 weeks in vitro. One day before fixation, EF1α-EGFP plasmids were transfected into neurons with Lipofectamine 2000 (Invitrogen). To obtain low efficiency transfections, cultures were exposed to the transfection mixture for 2.5 – 4 hours only and then transfection mixture was replaced with fresh cND media. Neurons were given 24 ± 3 hours for transgene expression. After immunocytochemical staining, GFP positive cells that costained with MAP2 were imaged. Grey-scaled, colour-inverted GFP images were manually traced using NeuronJ software. Since axons were not easily distinguished from dendrites at 2 weeks of differentiation, neurites were measured. Terminal neurites less than 10µm in length were excluded from tracings. In contrast to morphology assays performed on 2-week-old neurons, axons were easily distinguished from dendrites at 6 weeks of differentiation. Therefore axons were excluded from manual tracings and only dendrites were traced. Terminal dendrites less than 10 µm in length were excluded from final reconstructions.
Dendrite order was labeled in NeuronJ software. The first projection from the soma was labeled order 1 (ie. primary dendrite) and dendrites branching from primary dendrites were labeled order 2. This method of dendrite labeling continued until all dendrites were labeled. Sholl analysis was performed to measure neurite complexity through ImageJ software. Conceptually, a series of concentric circles are drawn irradiating outward in defined increments from the soma and the number of intersections between neurite and concentric circle at each increment is quantified. The Sholl radius was set to 10 µm increments and the number of intersections between reconstructed neurites and concentric circles was computed in ImageJ software.

Dendrite morphology assays were also performed on neurons differentiated by the one-step induction protocol after 2 and 3 weeks of differentiation with one modification; neurons were reconstructed based on grey-scaled, colour-inverted MAP2 images instead of GFP images since no transfections were necessary.

Soma size based on MAP2 stains of neurons differentiated for 6 weeks in vitro by the directed differentiation protocol was measured using ImageJ software. The perimeters of neuronal somas were traced and the area was determined by ImageJ.

2.5 Western Blot

Neurons differentiated for 6 weeks in vitro were lysed with Radio Immuno Precipitation Assay (RIPA) buffer [150 mM NaCl, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0)] containing protease and phosphatase inhibitors. Lysates were incubated on ice for 30 min with periodic vortexing before sonication. Protein concentration was quantified and 80 µg of whole cell extract was mixed with 4X Laemmli buffer [20% glycerol, 0.125 M Tris-HCl pH 6.8, 8% SDS, 0.02% Bromophenol blue, 20% β-mercapto ethanol] before resolving by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Resolved proteins were transferred to Hybond membranes (GE Healthcare Life Sciences). Membranes were blocked with 5% Milk in 0.05% Tween-Phosphate Buffer Saline (T-PBS) for 1 hour at room temperature and probed with rabbit anti-MeCP2 (1:1000; Millipore #07-013) primary antibody overnight at 4°C. Blots were washed several times with T-PBS and incubated with anti-rabbit-HRP conjugated (1:5000) secondary antibody for 1 hour at room temperature. Blots were washed several times with T-PBS and imaged with Chemidoc MP Imaging System and Image Lab 4.1 software. Blots were stripped twice and reprobed overnight at 4°C with anti-β-actin
(1:5000; Sigma #A5441) or anti-βIII-tubulin (1:5000; Millipore #MAB1637) loading controls. HRP-conjugated anti-mouse (1:5000) secondary antibodies were used to detect both loading controls. Densitometry was performed using ImageJ software.

2.6 Statistics

All datasets were managed in Microsoft Excel 2011 and imported into GraphPad Prism 6 for statistical analyses. For experiments where only two groups were compared, Student’s t-test was used with the significance p-value set to 0.05. When multiple comparisons had to be made between 2 groups (ie. Sholl analysis and comparing the number of dendrites per order in 6-week-old dendrite complexity assays), multiple t-tests were conducted with a false discovery rate set to 5%. When 3 or more groups were compared, Analysis of Variance (ANOVA) statistics were used with post-hoc correction.
Chapter 3  
Results  

3 Results  

3.1 RTT neurons were obtained by directing the differentiation of iPS cells into neurons  

As a first step towards phenotyping the morphology of RTT neurons, I performed test differentiations on 2 well-characterized human RTT cell lines (RTTΔ3-4#37 wildtype and RTTΔ3-4#20 isogenic null mutant). I chose to employ the well established directed differentiation technique described by Brennand et al., with a few modifications, under the guidance of Alina Piekna, a technician in our lab, because NPCs could be frozen and stored until all cell lines were acquired for experiments, NPCs could be seeded as single cells making it easier to analyze neuronal morphology after differentiating NPCs into neurons, and this protocol has been successfully used for studying disease phenotypes (Figure 10) (80). All NPCs used in my thesis were obtained from this neuronal differentiation protocol and were performed by more experienced lab members or me. I performed the induction of all NPCs into neurons for my experiments.  

I induced iPS colonies to form embryoid bodies (EBs) and cultured EBs for 7 days in suspension. After 7 days of suspension culture, EBs were transferred to PORN/Laminin coated plates and cultured in adherent conditions for an additional 7 days. During this period, neural rosettes formed and comprised the multipotent neural progenitor cell (NPC) population, which was manually isolated and expanded as monolayers to obtain a pure population of NPCs. I stained NPCs for the NPC marker, Nestin, and the neuronal marker, Microtubule associated protein 2 (MAP2), and observed an abundance of Nestin positive cells and few MAP2 positive cells. I seeded RTTΔ3-4#37 healthy and RTTΔ3-4#20 null NPCs into separate vessels with media rich in BDNF, IGF-1, GDNF, cAMP, and ascorbic acid for NPC differentiation into neurons. Neuronal cultures were stained for MAP2 and DAPI after two weeks of culture. MAP2 positive neurons were visible by this time (Figure 11).
Figure 11. Test RTT neurons were differentiated into MAP2 positive neurons. RTTΔ3-4#37 healthy isogenic control and RTTΔ3-4#20 null iPS cells were induced to form embryoid bodies (EBs) and then cultured in adherent conditions. Neural progenitor cells (NPCs) within neural rosettes were manually isolated and stained for Nestin and MAP2. NPCs were differentiated into neurons for two weeks in vitro and stained for MAP2. All images were obtained from my own differentiation. Scale bar = 100 µm.
3.2 Establishing neuronal morphometric analyses to quantify neurite complexity

To visualize an individual neuron and its neurites from adjacent neurons for morphological analyses, I tested a sparse labeling technique developed in the lab by Dr. Joel Ross, a post-doctoral fellow in our lab. In brief, 50,000 NPCs were seeded in one well of a 4-well or 24-well plate and cultured in neuron differentiation media for 2 weeks in vitro. One day before fixing neurons, neuronal cultures were lipofected with 0.5 µg of EF1α-EGFP plasmid for four hours to obtain low efficiency transfection (ie. sparse labeling). The transfection mix was replaced with regular neuron differentiation media and transfected neurons were given 24 hours for transgene expression before being fixed. I optimized this transfection protocol so that the neurites from an individual neuron can be distinguished from neurites of an adjacent neuron (Figure 12).

![Figure 12. Schematic represents sparse labeling technique for the visualization of single neurons.](image)

NPCs were seeded at a defined density and cultured in neuronal differentiation media for 2 weeks in vitro. One day before fixing neuronal cultures, cells were lipofected with EGFP plasmids controlled by an EF1α promoter. Neurons were transfected for 4 hours to obtain low efficiency transfection so that after 24 hours for transgene expression, single neurons and its neurites can be easily distinguished from adjacent neurons.

After twenty-four hours post-transfection, neuronal cultures were fixed and stained for the neuronal marker microtubule-associated protein 2 (MAP2) and GFP. Staining for GFP was used to amplify its signal. Staining for MAP2 was employed to determine if GFP positive cells had neuronal identity (Figure 13-arrow) or did not (Figure 13-arrowhead), since GFP was expressed in neuronal and non-neuronal cells as expected for an EF1α promoter reporter.
Figure 13. GFP positive cells counterstained with MAP2 verified the identity of sparse labeled neurons. Twenty-four hours after transgene expression, neuronal cultures were fixed, permeabilized, and stained for anti-GFP (green) and anti-MAP2 (red). GFP staining was essential for signal amplification. The purpose of MAP2 staining was to verify that GFP positive cells were neurons (ie. MAP2+; Arrow) and to distinguish them from non-neuronal cells (MAP2--; arrowhead). Nuclei were stained with DAPI. Scale bar = 50 µm.

To examine neuronal complexity, I manually reconstructed MAP2 positive and GFP positive cells with NeuronJ software (Figure 14A). Grey-scaled, color-inverted GFP images were imported into NeuronJ software and traced using the tracing tool. After tracings were completed, the “measure tracings” option was selected to calculate total neurite length. Tracings were exported into ImageJ software for Sholl analysis, which measures neurite complexity as distance increased from the soma. In principle, Sholl analysis is conducted by drawing a series of concentric circles irradiating outwards at defined increments from the soma, and the number of intersections between concentric circles and traced neurites are quantified (Figure 14B). The Sholl radius was set to 10 µm for my experiments. From the exported tracings into ImageJ software, the number of terminal neurites was manually counted. In total, 3 measures of neurite complexity were employed: Total neurite length, number of terminal neurites, and number of intersections per 10 µm away from the soma (ie. Sholl analysis).
Figure 14. GFP positive neurons can be reconstructed to assay neurite complexity. (A) A GFP positive cell that was positive for the neuronal marker MAP2 was reconstructed with NeuronJ software. The neurites from a grey-scaled, colour-inverted GFP image was manually traced. (B) Schematic illustrates the principle of Sholl analysis. To perform Sholl analysis, a series of concentric circles were drawn irradiating outward from the soma at defined increments of 10 µm. To measure neurite complexity, the number of intersections between concentric circle at each increment and neurites was quantified.
3.3 RTT neurons differentiated for two weeks in vitro have reduced neurite complexity

To test whether the RTT neurons I derived recapitulate RTT phenotypes, I analyzed the morphology of 2-week-old neurons from two RTT patients (RTTΔ3-4#20 null and RTTe1#27) and two healthy controls (SK0186_001#2.14 and RTTΔ3-4#37). RTTΔ3-4#20 null and its RTTΔ3-4#37 isogenic control NPCs were obtained from my differentiation while RTTe1#27 affected patient and SK0186_001#2.14 unrelated female healthy control NPCs were obtained from experienced technicians in our lab (Table 3).

<table>
<thead>
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<th>Genotype</th>
<th>Cell Line</th>
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<th>NPC → Neuron differentiation by:</th>
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<td></td>
<td>SK0186_001#2.14</td>
<td>Technician</td>
<td>WL</td>
</tr>
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<tr>
<td></td>
<td>RTTe1#27</td>
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</tr>
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</table>

Table 3. Summary of cell lines included in experiment to determine if RTT neurons I derived recapitulate the disease phenotype of reduced neurite complexity. Two wildtype and two RTT cell lines were selected for this experiment. The RTTΔ3-4#37 and RTTΔ3-4#20 NPCs were obtained from my own differentiation (highlighted in orange). SK0186_001#2.14 and RTTe1#27 NPCs were acquired from a differentiation performed by different technicians. I differentiated all NPCs into neurons (highlighted in orange). WL are initials for Wesley Lai.

Healthy control and RTT NPCs were differentiated for two weeks in vitro for morphological analysis. I manually counted the number of terminal neurites and observed a reduced number of terminal neurites in RTT null neurons and RTTe1 neurons compared to healthy control neurons (Figure 15A). Differences in total neurite length were quantified by NeuronJ software from morphological reconstructions and a shorter overall neurite length was observed from both patients and one healthy control cell line (SK0186_001#2.14). Conclusions could not be made because of inconsistencies between the two healthy control cell lines (Figure 15B). I observed a
similar inconsistency by Sholl analysis. Neuronal reconstructions were exported into ImageJ software and Sholl analysis was performed. The Sholl radius was set to 10 µm, so that a series of concentric circles irradiating outward at defined increments from the soma were drawn and the number of intersections between neurite and circle at each level were quantified. RTT neurons had fewer intersections compared to one healthy control (SK0186_001#2.14) but not the other (RTTΔ3-4#37) healthy control, thus making my results inconclusive (Figure 15C). These results suggest that the WT neurons that I derived from my NPCs (RTTΔ3-4#37) behave differently than the WT neurons that I derived from NPCs given to me by a technician (SK0186_001#2.14).
Figure 15. Two-week-old RTT neurons have reduced neurite tip number compared to healthy control neurons. (A) Two-week-old RTT neurons have reduced number of neurite tips. (B) Results for differences in total neurite length were inconclusive because of inconsistencies between control cell lines. (C) Results for number of intersections per 10 µm away from the soma were inconclusive due to inconsistencies between healthy control cell lines. All values are mean ± SEM, *p<0.05. This experiment was performed in triplicate.
3.4 Experimental set up for neuronal morphometric assays performed on neurons differentiated for 6 weeks *in vitro*

I was interested in testing whether RTTe1 neurons had defects in dendritic complexity and morphological assays conducted on neurons differentiated for 2 weeks *in vitro* did not provide clear results that addressed my hypothesis. This was because dendrites and axons were not easily distinguished at the 2 week time point. I rationalized that because other members of our lab have electrophysiology data suggesting that functional neurons can be obtained after 6 weeks of differentiation, the axons and dendrites of neurons should be easily identified. Indeed, axons were easily identified from dendrites by their long morphology (*Figure 16*). Therefore, I chose to repeat my neuronal morphometric analyses on neurons differentiated for 6 weeks.

*Figure 16. The axon of a neuron could be easily identified by its long morphology.* Neurons were differentiated for 6 weeks, sparse labeled with GFP, and imaged. The axon of a neuron (arrows) was much longer than the dendrites of a neuron. Scale bar = 50 µm.
Inconsistencies between the two healthy controls were present in neuronal morphology assays conducted on neurons differentiated for 2 weeks. Two possible reasons for the difference between the WT neurons is that the RTTΔ3-4#37 cells expressed less MeCP2 than the other or were mislabeled null cells. To test these hypotheses, I performed immunocytochemistry on 6-week-old neurons and stained them for MAP2, MeCP2, and DAPI to determine if MeCP2 was present in wildtype and mutant cell lines. This experiment was performed three times and I was blinded to the identity of the cell line each time.

In parallel to the immunocytochemistry experiments performed for the detection of MeCP2, I prepared 6-week-old neurons for western blot experiments to quantify MeCP2 levels. One 3 cm dish per cell line was prepared. Whole cell lysates were obtained and western blotted. Blots were probed for MeCP2, and two loading controls: β-actin and βIII-tubulin. The β-actin loading control was used to control for total protein. The βIII-tubulin loading control was used to determine if there were large differences in the differentiation efficiency that may affect my results.

Neurons for dendritic morphology assays were simultaneously prepared with neurons purposed for MeCP2 detection by immunocytochemistry and western blots. After 6 weeks of differentiation, neurons were sparse labeled with GFP and stained for MAP2 and GFP. GFP+ and MAP2+ neurons were imaged. The dendrites of GFP+ neurons from grey-scaled, colour-inverted GFP images were traced and quantified for defects in dendritic complexity using NeuronJ software.

Since the soma sizes of the RTTΔ3-4#37 wildtype and RTTΔ3-4#20 null neurons have been characterized previously, I also performed soma size measurements on RTTΔ3-4#37 wildtype and RTTΔ3-4#20 null neurons from my experiments to determine if they were behaving as described previously (64). Soma size from grey-scaled MAP2 images obtained from sparse labeled neurons was measured by tracing the perimeter around the soma using ImageJ software. The area was automatically calculated by ImageJ software after the tracing was performed. I was blinded to the identity of the cell line.
To ensure that any differences I observed were not due to cell line differences, I obtained NPCs of two additional female WT control cell lines and two additional RTTe1 cell lines and included them in the immunocytochemistry, western blot, and dendritic complexity assays described above.

In summary, 8 cell lines were included in the assays performed after 6 weeks of differentiation (Table 4, Figure 17). I performed all differentiations of NPCs into neurons. NPCs from 8 cell lines were seeded simultaneously. Three experimental replicates were prepared one week apart.

<table>
<thead>
<tr>
<th>Cell line generated by:</th>
<th>Healthy Female Control</th>
<th>RTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPC generated by:</td>
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<td>other</td>
</tr>
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</tr>
<tr>
<td>Dendrite morphology</td>
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</tr>
</tbody>
</table>

- ✓ - Completed  
- ✗ - Not completed  
- WL - Wesley Lai

**Table 4. Summary of cell lines used and assays performed on neurons differentiated for 6 weeks.** Four WT female cell lines and four RTT cell lines (1 null and 3 RTTe1) were differentiated into neurons for 6 weeks. Neurons were prepared for immunocytochemistry to determine if MeCP2 was present, western blot to quantify MeCP2 levels, and dendritic morphology assays to test if defects were present. The soma sizes of RTTΔ3-4#37 and RTTΔ3-4#20 null neurons obtained from my differentiation were also measured to determine if they reproduced previously published results. NPCs obtained from my differentiation are highlighted in orange.
Figure 17. Overview of experimental design performed on neurons differentiated for 6 weeks. NPCs from 8 different cell lines were differentiated into neurons. Two coverslips per cell line was stained for MAP2, MeCP2, and DAPI, and then imaged. One 3 cm plate per cell line was prepared for western blot experiments. For neuronal morphology assays, 5-6 coverslips per cell line was sparse labeled with GFP and stained for GFP and MAP2. GFP+ and MAP2+ neurons were imaged. Dendrites were traced based on grey-scaled, colour inverted GFP images and analyzed for morphological defects. Soma sizes of the RTTΔ3-4#37 and RTTΔ3-4#20 neurons was measured from grey-scaled MAP2 images. Neurons within the field of view were randomly selected for soma size measurements. All experiments involving immunocytochemistry were performed in a blinded manner. Three experimental replicates were performed for each assay described.
3.5 RTTΔ3-4#37 neurons and NPCs obtained from my differentiation should be excluded

One possible explanation for the morphological difference between the two wildtype cell lines in neurite morphology assays performed on neurons differentiated for 2 weeks is that RTTΔ3-4#37 neurons were mislabeled RTTΔ3-4#20 null cells. To determine if this was true, I performed immunocytochemical staining of 6-week-old neurons for MeCP2, MAP2, and DAPI. The presence of MeCP2 in neurons was assessed in 8 cell lines (4 healthy controls, 1 RTT null, 3 RTTe1). This experiment was performed three times from separate differentiations. Nuclear MeCP2 was detected in 6-week-old neurons derived from all four healthy control cell lines and not detected in RTT null (RTTΔ3-4#20) and RTTe1 cell lines (Figure 18). Based on immunocytochemistry experiments, MeCP2 was clearly present in the RTTΔ3-4#37 neurons and absent in the RTTΔ3-4#20 null neurons. This indicates that RTTΔ3-4#37 cells were not mislabeled RTTΔ3-4#20 null cells.
Figure 18. Nuclear MeCP2 was detected in healthy control neurons but not in RTT neurons. Neurons differentiated for 6 weeks in vitro from the NPC stage were fixed and probed for anti-MeCP2 (green) and anti-MAP2 (red). DAPI stained nuclei (blue). MeCP2 was detected in healthy control neurons but not detected in RTT neurons. This experiment was performed in triplicate. Scale bars = 10 µm.

A second possible explanation for why differences were present between the two wildtype cell lines is because RTTΔ3-4#37 neurons produced lower levels of MeCP2 than the other wildtype neurons. To quantify differences in MeCP2 protein levels, whole cell lysates from RTT and healthy control neurons differentiated for 6 weeks were collected for western blot experiments. I established a reproducible western blot protocol for detecting MeCP2 protein, which was not previously reliable to our lab. Whole cell lysates were collected and the total concentration of protein was quantified. 80 µg of lysate were subject to 8% SDS-PAGE and transferred to Hybond membranes. Blots were probed for MeCP2, and then stripped twice to probe blots for β-actin and βIII-tubulin loading controls.
Using an antibody that recognizes the C-terminus of MeCP2, protein was detected by western blot in all four healthy control neurons. MeCP2 was not detected in RTTΔ3-4#20 null neurons, which was expected since these neurons harbor a MECP2 deletion spanning exon 3 and 4. Interestingly, MeCP2 was detected by western blot in neurons from all RTTe1 cell lines. This finding is consistent with our hypothesis that the MECP2e1 mutation affects MeCP2e1 only and does not alter the integrity of MeCP2e2. The presence of MeCP2 in RTTe1 neurons is likely from the continued expression of MeCP2e2. For quantification purposes, two loading controls were used: β-actin and βIII-tubulin. The βIII-tubulin loading control was also used to account for potential differences in differentiation efficiency, which may alter the levels of MeCP2. Quantification of MeCP2 protein levels normalized to loading controls were indicated by numerical values. MeCP2 levels appeared to be lower in RTTΔ3-4#37 neurons compared to the 3 other healthy control neurons regardless of loading control selected for normalization. This also indicated that the differentiation efficiencies were similar between cell lines (Figure 19). This experiment was performed in triplicate with lysates from three separate differentiations.
Figure 19. RTTΔ3-4#37 neurons had less MeCP2 than the 3 other healthy controls. Western blots were probed with an antibody that recognizes the C-terminus of MeCP2. MeCP2 was detected in healthy control neurons. MeCP2 was not detected in RTTΔ3-4#20 null neurons. MeCP2 was detected in RTTe1 neurons, which is consistent with the continued expression of MeCP2e2 though MeCP2e1 is lost. Blots were stripped twice and probed for β-actin and βIII-tubulin loading controls, respectively. MeCP2 levels in RTTΔ3-4#37 and RTTe1 neurons were visibly reduced when compared to the other unrelated healthy controls. Quantifications above loading controls reflected this observation. This experiment was performed in triplicate with lysates obtained from different differentiations.
I quantified MeCP2 levels by normalizing to β-actin loading control. This analysis showed that RTTΔ3-4#37 neurons consistently yielded approximately 3-fold less MeCP2 compared to other healthy control neurons, and that RTTe1 neurons consistently yielded approximately 8-fold less MeCP2 compared to healthy controls (Figure 20). This observation is consistent with our hypothesis that the RTTe1 mutation affects MeCP2e1 but leaves MeCP2e2 intact. This experiment was performed in triplicate.

![Graphical representation of MeCP2 protein levels normalized to β-actin loading control.](image)

**Figure 20.** Graphical representation of MeCP2 protein levels normalized to β-actin loading control. Normalized MeCP2 protein levels were compared to RTTe1 neurons. RTTe1 neurons consistently had a 8-fold decrease in MeCP2 protein levels compared to unrelated healthy controls. RTTΔ3-4#37 neurons were excluded from the other healthy control group because it consistently yielded 4-fold lower levels of MeCP2. Values are mean ± SEM from three experimental replicates. * p < 0.05.
MeCP2 protein levels were also normalized to βIII-tubulin loading control and quantified. Similar to the quantification obtained from measuring MeCP2 levels normalized to β-actin, RTTΔ3-4#37 neurons consistently yielded 3-fold lower levels of MeCP2 compared to other unrelated healthy controls. RTTe1 neurons yielded 9-fold lower levels of MeCP2 compared to healthy control neurons. This result is consistent with the continued expression of MeCP2e2 in RTTe1 neurons (Figure 21). This experiment was performed in triplicate.

![Graphical representation of MeCP2 protein levels normalized to βIII-tubulin loading control.](image)

**Figure 21.** Graphical representation of MeCP2 protein levels normalized to βIII-tubulin loading control. A 3-fold lower level of MeCP2 was detected in RTTΔ3-4#37 healthy control neurons compared to other unrelated healthy controls. Quantification of MeCP2 levels in RTTe1 neurons showed a 9-fold reduction compared to unrelated healthy controls. Values are mean ± SEM from three experimental replicates. * p < 0.05.

It was evident that RTTΔ3-4#37 neurons were underperforming in western blot experiments in addition to morphology assays performed on neurons differentiated for 2 weeks. Our lab previously published results demonstrating that isogenic RTTΔ3-4#37 wildtype neurons had larger soma sizes than RTTΔ3-4#20 null neurons. To determine whether RTTΔ3-4#37 and RTTΔ3-4#20 neurons were misbehaving, I performed soma size measurements with ImageJ software. The soma was identified by MAP2 staining and the perimeter of the soma was traced.
The area of soma was calculated automatically by ImageJ software after the tracings were performed. RTTΔ3-4#37 isogenic healthy control neurons had smaller soma sizes than RTT null neurons, demonstrating that RTTΔ3-4#37 was not performing as expected (Figure 22B). Over a hundred neurons from each cell line was measured between three independent experiments.

Figure 22. RTTΔ3-4#37 isogenic healthy control neurons to RTTΔ3-4#20 null neurons have smaller soma sizes. (A) The perimeters of neuronal somas were traced as demonstrated based on MAP2 staining obtained from neurons differentiated for 6 weeks in vitro. Scale bar = 10 µm. (B) Bar graph shows that RTTΔ3-4#37 isogenic control to RTTΔ3-4#20 null neurons had reduced soma size. The total number of neurons analyzed were 130 for RTTΔ3-4#37 isogenic control and 142 for RTTΔ3-4#20 null neurons between three experimental replicates. Values are mean ± SEM. * p < 0.05.

From the accumulation of evidence suggesting that RTTΔ3-4#37 healthy control neurons are not behaving as expected, I concluded that it would be appropriate to exclude this and its isogenic null cell line from my morphometric assays conducted on neurons differentiated for 6 weeks in vitro (Table 5). The reason for their underperformance is unknown but appears to be an isolated event of faulty NPCs from my differentiation since other members of our lab were able to reproduce the published soma size defect using their own NPCs differentiated from the same iPS cells I used.
Table 5. Summary of assays conducted and their results suggesting that RTTΔ3-4#37 and its isogenic RTTΔ3-4#20 null cell line should be excluded from dendritic complexity assays performed on neurons differentiated for 6 weeks. NPCs obtained from my differentiation are indicated in orange. MeCP2 was present in wildtype but undetected in RTT neurons by ICC. MeCP2 was present in wildtype neurons and RTTe1 neurons but undetected in RTT null neurons. Lower levels of MeCP2 were detected in the RTTΔ3-4#37 and RTTe1 neurons compared to the 3 other healthy control neurons. RTTΔ3-4#37 neurons had smaller soma size than RTTΔ3-4#20 null neurons.

3.6 RTTe1 neurons differentiated for 6 weeks in vitro have reduced dendritic complexity

Morphology assays performed on neurons differentiated for two weeks showed that cellular density was unequal amongst cell lines and that seeding the same number of NPCs at the beginning of neuronal differentiation was ineffective at equalizing culture density. In attempts to overcome this limitation in assays performed on neurons differentiated for 6 weeks, NPCs were treated with DAPT for the first 10 days of differentiation into neurons. This approach has been routinely used in our lab. DAPT promotes cell cycle exit and neuronal differentiation. It appeared to be ineffective at equalizing cellular density in my hands (Figure 23).
Figure 23. DAPT treatment was ineffective at equalizing cellular density after 6 weeks of differentiation. Control and RTTe1 NPCs were seeded simultaneously and differentiated for 6 weeks in vitro before being transfected with GFP one day before fixation. Neurons were stained for MAP2 (red) and GFP (green). NPCs were treated with DAPT for 10 days at the beginning of neuronal induction in attempts to equalize cellular density. DAPT treatment appeared to be ineffective. Only GFP positive and MAP2 positive cells were manually reconstructed. Grey-scaled, colour-inverted GFP images were used for neuronal reconstructions. This experiment was performed in triplicate. Scale bars = 50 µm.

I measured the dendritic complexity of neurons from 3 RTTe1 lines and 3 healthy control lines after 6 weeks of in vitro differentiation. Total dendrite length, dendrite tip number, and the number of intersections by Sholl analysis were quantified from reconstructed neurons as described previously. RTTe1 neurons had significantly shorter total dendrite length than WT neurons (Figure 24A). The average total dendrite length of wildtype neurons and RTTe1 neurons was 359.7 ± 10.41 µm and 266.9 ± 13.13 µm, respectively. RTTe1 neurons had reduced number of terminal dendrites that was significantly different (Figure 24B). The mean number of dendrite tips for wildtype neurons and RTTe1 neurons was 6.26 ± 0.35 and 5.09 ± 0.16, respectively. RTTe1 neurons also had reduced number of intersections by Sholl analysis that reached significance as distance increased from the soma (Figure 24C). These observations support my hypothesis that RTTe1 neurons have reduced dendritic complexity.
Figure 24. RTTe1 neurons differentiated for 6 weeks *in vitro* have reduced dendritic complexity. (A) RTTe1 neurons have reduced total dendrite length. Values are mean ± SEM. * p <0.05. (B) RTTe1 neurons have reduced number terminal dendrites. Values are mean ± SEM. * p < 0.05. (C) RTTe1 neurons have reduced number of dendrites per Sholl radii irradiating outward from the soma at 10 μm increments. Values are mean ± SEM from three experimental replicates. * FDR = 5%.
To obtain an additional measure of dendritic complexity, I quantified the number of primary dendrites and higher order dendrites from neuronal reconstructions. Primary, secondary, and tertiary dendrites were labeled following the principle of centrifugal order of branch labeling until all dendrites were labeled. The centrifugal order of branch labeling states that primary dendrites projecting immediately from the soma are labeled “order 1”, secondary dendrites branching from “order 1” dendrites are labeled “order 2”, and tertiary dendrites branching from “order 2” dendrites are labeled “order 3”. This system applies to all dendrites until all of them have been assigned an order. Dendrite order was manually labeled using the “label tracing” option in NeuronJ software (Figure 25A). No difference in the number of primary dendrites was observed, but a significant reduction in the number of higher order dendrites in RTTe1 neurons compared to healthy control neurons was observed (Figure 25B).

Figure 25. RTTe1 neurons differentiated for 6 weeks in vitro have reduced number of higher order dendrites. (A) Dendrites were labeled by following the principle of centrifugal order of branch labeling. Primary dendrites projecting immediately from the soma were labeled “order 1”. Dendrite branches from “order 1” dendrites were labeled “order 2” and dendrites branching from “order 2” dendrites were labeled “order 3” dendrites. This method of dendrite labeling was applied until all branches were labeled. (B) No differences in the number of primary dendrites were observed. RTTe1 neurons had reduced numbers of higher order dendrites. Values are mean ± SEM from three experimental replicates. * FDR = 5%.
The findings from neuronal morphology experiments performed on neurons differentiated for 6 weeks \textit{in vitro} support my hypothesis that RTT neurons with a MECP2e1 mutation have reduced dendritic complexity.

### 3.7 The overexpression of Ngn2 for induction of iPS cells into neurons may mask RTT phenotypes

Dae-Sung Kim, a post-doctoral fellow in our lab, differentiated two patient derived RTT iPS cell lines and two healthy control cell lines into neurons using the one-step neuronal induction protocol developed in the lab of Thomas Sudhof (82). Dae-Sung stained neuronal cultures for MAP2 and acquired fluorescent images. I obtained the images from him for experiments performed after 2 and 3 weeks of neuronal induction and conducted dendrite morphology assays to determine whether RTT neurons derived by the one-step induction protocol can recapitulate disease phenotypes. Preliminary experiments performed on RTT null neurons and RTT R306C patient derived neurons from the one step-induction protocol after two weeks of \textit{in vitro} differentiation did not have shorter total dendrite length or reduced number of dendrite tips (Figure 26A and B). The average total dendrite length for wildtype neurons and RTT neurons was 395.35 ± 30.19 µm and 394.05 ± 25.93 µm, respectively. The average number of dendrite tips for wildtype neurons and RTT neurons was 10.91 ± 0.86 and 10.36 ± 0.80, respectively. I observed a significant reduction in the number of second order dendrites between RTT neurons and one healthy control, which was not reproduced in a second healthy control (Figure 26C). No differences in dendritic complexity between RTT neurons and healthy control neurons were observed by Sholl analysis (Figure 26D). These data may suggest that the overexpression of Ngn2 for neuronal differentiation might mask RTT phenotypes that were previously described. This conclusion cannot be made however, since this experiment was performed once with a small sample size.
Figure 26. RTT neurons derived from the one-step induction protocol for 2 weeks in vitro do not recapitulate disease phenotypes. (A) RTT neurons do not have reduced total dendrite length. (B) RTT neurons do not have reduced number of dendrite tips. (C) RTT neurons do not have differences in the number of primary dendrites. Differences in the number of secondary dendrites were observed between RTT neurons and one healthy control but not the other. Inconsistencies preclude any conclusions. (D) No differences in dendrite complexity were observed by Sholl analysis. All values are represented as mean ± SEM from one experimental replicate.
Similar results were obtained when I analyzed 3-week-old neurons derived from the one-step induction protocol. No significant difference in total dendrite length was observed between wildtype neurons and RTT neurons (Figure 27A). The average total dendrite length of wildtype neurons and RTT neurons was 365.3 ± 37.61 µm and 405.7 ± 44.53, respectively. No significant difference in the number of dendrite tips was observed between RTT neurons and WT neurons (Figure 27B). The mean number of dendrite tips for wildtype neurons and RTT neurons was 9.75 ± 0.75 and 10.22 ± 0.86, respectively. Differences in the number of primary and higher order dendrites were present when I quantified the number of dendrites per order, however, none of the differences were consistent between cell lines. Therefore, the results from this assay were inconclusive (Figure 27C). I assayed dendrite complexity by Sholl analysis, but no differences were observed between healthy control neurons and RTT neurons (Figure 27D).

These results demonstrate that the reduced dendritic complexity defect previously observed in neurons obtained from the EB-based directed differentiation protocol was not present in neurons obtained from the one-step induction protocol. I hypothesize that this is because the continued overexpression of Ngn2 in neurons obtained from the one-step induction protocol masks RTT phenotypes. More experiments needed to be conducted to determine whether my observations from this first replicate can be reproduced.
Figure 27. RTT neurons derived from the one-step induction protocol for 3 weeks in vitro do not recapitulate disease phenotypes. (A) RTT neurons do not have differences total dendrite length. (B) RTT neurons do not have differences in dendrite tip number. (C) Differences in the number of dendrites per order were observed but lacked consistency. (D) No differences in dendritic complexity determined by Sholl analysis were observed. Values are mean ± SEM.
When the number of terminal dendrites was compared between neurons obtained from the EB-based directed differentiation protocol after 6 weeks of differentiation and neurons obtained from the one-step induction protocol after 2 or 3 weeks of differentiation, it was evident that neurons obtained from the one-step protocol had a greater number of tips. The mean dendrite tip number for wildtype neurons obtained from the directed differentiation protocol was 6.26 ± 0.35 compared to 10.91 ± 0.86 (2 week) and 9.75 ± 0.75 (3 week), from the one-step induction protocol. The increased number of dendrite tips might reflect a potential increase in complexity, which is likely due to the non-cell autonomous effects of astrocyte culture, which facilitate neuronal maturation. It is also possible that the overexpression of Ngn2 may increase neuronal complexity as well, but this hypothesis requires further testing.

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<th>One-step (3 weeks)</th>
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<td>9.75 ± 0.75</td>
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<tr>
<td>Total dendrite length (µm)</td>
<td>359.1 ± 10.41</td>
<td>395.35 ± 30.19</td>
<td>365.3 ± 37.61</td>
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*Table 6. Neurons obtained from the one-step induction protocol may be more complex than neurons differentiated for 6 weeks with the directed differentiation protocol.* The mean value of total dendrite length between wildtype cell lines was compared between neurons obtained from the one-step induction protocol and directed differentiation protocol. No difference in total dendrite length was observed. The mean number of dendrite tips from wildtype cell lines was also compared between neurons obtained from the one-step protocol and directed differentiation protocol. Neurons obtained from the one step induction protocol had more dendrite tips than neurons obtained from the directed differentiation protocol. All values are displayed as mean ± SEM.
Chapter 4
Discussion

4.1 Potential consequences of reduced neuronal dendritic complexity on behaviour and neuronal function

In this thesis, I provide evidence that RTTe1 neurons have reduced dendritic complexity. I employed methods to reconstruct individual neurons for morphological analysis and used four assays that demonstrate reduced dendritic complexity in RTTe1 patient neurons. RTTe1 iPS cell derived neurons have reduced total dendrite length, number of terminal dendrites, higher order dendrites, and number of intersections per 10 µm increment away from the soma by Sholl analysis. The observed reduction in dendritic complexity supports the current notion that neurons with mutant MeCP2 are less mature and form disrupted neural networks in RTT brains. What is unclear is whether the reduced dendritic complexity is a result of delayed growth where neurons continue to grow but at a slower rate or are stunted in growth. Reduced dendritic complexity has been described in previous studies conducted on post-mortem brain tissue and Mecp2-deficient mouse models (17,36,37,47,48). A recent report using a human RTT ES cell derived neuronal system tested whether or not RTT ES cell derived neurons had reduced neurite complexity by Sholl analysis (31). In this study ES cells containing an exon 3 deletion of MECP2 were differentiated into neurons and shown to have reduced number of neurite intersections compared to isogenic controls. My results are the first to document a reduced dendritic complexity phenotype in RTTe1 patient derived neurons. This complements the RTTe1 small neuron phenotype and changes in electrophysiological properties reported by Aaron Cheung in our lab and Wenbo Zhang from Mike Salter lab, respectively.

The changes in electrophysiological properties include reduced amplitude, increased time course, and reduced frequency of evoked action potentials. RTTe1 neurons also displayed lower frequency and amplitude of spontaneous action potential (miniature excitatory postsynaptic currents), and reduced ability to store up electrical charges in their cell bodies (ie. reduced capacitance) (81). These alterations in electrophysiological properties provide direct evidence that there is reduced excitability in RTT neurons as was previously described (55,65). The
electrophysiology data and my dendritic complexity assays support the notion that MECP2e1 mutations can alter brain function. Evidence for this is supported by mice lacking Mecp2e1, which show behavioural symptoms similar to Mecp2 null mice (89). Since dendrites and synapses are the functional units of information exchange between neurons, changes in dendritic arborization of cortical neurons can in part account for the behavioural phenotypes observed in RTT patients (54,90). The small neuron phenotype, the changes in electrophysiological properties, and reduced dendritic complexity demonstrate that a MECP2e1 mutation is sufficient to cause RTT.

4.2 Targeting Ras-PI3K-AKT, Ras-MAPK, and Rho-family GTPases signaling pathways may reverse defects of dendritic complexity

Mechanistically, the reason for reduced dendritic complexity is likely due to aberrant AKT signaling. Mecp2-deficient mice have defects in the AKT/mTOR signaling (62). The effects of aberrant AKT/mTOR signaling were demonstrated in human ES cell derived neurons harbouring a deletion of exon 3 of MECP2 (31). RTT ES cell derived neurons had reduced levels of phosphorylated AKT (p-AKT) and phosphorylated S6 (p-S6; a downstream effector of mTOR), which could be elevated by BDNF and IGF-1 treatment. Both BDNF and IGF-1 can rescue RTT phenotypes (57,60). In the study conducted by Li et al., the administration of BDNF and IGF-1 both elevated p-AKT and p-S6 levels to near wildtype levels and was sufficient to rescue both the soma size and neurite complexity defect that was identified in RTT ES cell derived neurons (31). Knockdown of PTEN, an upstream negative regulator of AKT signaling, effectively increased neuronal soma size and neurite complexity by Sholl analysis. Based on these experiments however, it was unclear whether PTEN knockdown completely or only partially restored neuronal morphology to wildtype standards (31). Blocking the phosphorylation of AKT using p-AKT inhibitors (LY294002) can significantly reduce total dendrite length, number of terminal tips, number of intersections by Sholl analysis, and soma size, the same phenotypes identified in RTTe1 neurons (90,91). Interestingly, in the study conducted by Kumar et al., they demonstrated that the selective activation of Ras-AKT signaling only and Ras-MAPK signaling only could modulate total dendrite length with similar efficacy (90). In this study, selective activation of MAPK signaling only increased the number of terminal dendrite tips more than the selective activation of PI3K/AKT signaling only. Soma size however, was altered only by
selective activation of PI3K/AKT signaling. These data suggest that in addition AKT signaling, the MAPK signaling pathway may be an effective target for rescuing certain RTT phenotypes like reduced number of terminal tips even though the MAPK pathway may not be affected in RTT (59). This unaffected pathway might be one reason for the lack of observed difference in the number primary dendrites in RTTe1 neurons. An upstream effecter of MAPK is GSK-3β and its phosphorylation by integrin-linked-kinase (ILK) has been shown to result in the inactivation of GSK-3β and an increase in dendrite initiation (92).

The Rho-family of GTPases: RhoA, Rac1, and Cdc42 are also regulators of dendritic complexity (93). Specifically, RhoA is a negative regulator of dendritic complexity and when mutated to be constitutively active, reduces dendrite length (94-96). RhoA activation has also been shown to reduce the number of primary and secondary dendrites by reducing the levels of its downstream effector cypin, which increases dendritic number when active (97). Rac1 and Cdc42 are positive regulators of dendrite initiation. When active, they increase dendrite outgrowth from the soma (98). The activation of both Rac1 and Cdc42 also promote dendrite branching (95,96,99)

The reason for the observed lack of difference in the number of primary dendrites in RTTe1 neuron may be due to the effects of Rac1 and/or Cdc42 signaling. It is possible that the ILK-GSK-3β-MAPK pathway may also be responsible for the lack of observed difference in the number of primary dendrites. I hypothesize that one or more of these signaling pathways is responsible for maintaining the number of primary dendrites in RTTe1 neurons and that the reason for the observed differences in the number of higher order dendrites is due aberrant AKT signaling, which has been studied before (31).

4.3 Experimental Limitations

4.3.1 A method to equalize cell density needs to be established to account for potential density-dependent effects on dendritic arborization

Cellular density is not easily maintained in neuronal cultures and can have major effects on dendritic arborization. It is known that neurite arborization can be modulated by contact-dependent activation of Notch signaling (100). In this study, the authors cultured neurons at low and high density. They showed that neurons grown at low density had reduced activation and translocation of Notch to the nucleus, whereas neurons grown at high density had greater
activation and translocation of Notch to the nucleus. They drew the correlation that interneuronal contact in high-density cultures facilitated the rapid growth of neurites through Notch activation, which stopped after 9 days in vitro in contrast to low-density cultures with lower Notch activation, which extended neurites much slower but continued to grow in total length. An additional observation made was that individual neurites of neurons grown in low-density cultures were longer and smoother in comparison to high-density cultures, where individual neurites were shorter and bushier. Another study reported that Notch1 activation inhibited average dendrite length but not the number of dendrite branches (101). Based on the raw values within individual replicates from the 6-week-old neuronal morphology assays, I examined whether there were consistent correlations between cellular density and total dendrite length but did not find a consistent cell density dependent effect. However, these are crude observations and I acknowledge that the effects of cellular density cannot be underestimated and future experiments should aim to manage this better.

Using DAPT early in the neuronal differentiation process (ie. first ten days), which has been shown to promote cell cycle exit and neuronal differentiation, was ineffective at equalizing cellular density between cell lines (102). Others members in our lab are currently exploring the use of Ara C, also known as cytarabine, which is a potent chemotherapeutic agent that kills proliferating cells. This compound was used to equalize cell density between cell lines in experiments conducted by another research group who studied RTT phenotypes using ES cell derived neurons (31). The explored approach is to differentiate neurons for a time, treat them with Ara C overnight, pass a defined number of neurons to a new dish, and allow neurons to recover for 1 week before proceeding to experimentation. This agent should remove proliferating cells and leave post-mitotic cells unharmed, which would allow us to study RTT phenotypes in culture conditions with equal densities after treatment and passaging.

4.3.2 No RTTe1 isogenic wildtype controls were isolated from reprogramming

Isogenic wildtype control cells to RTTe1 cells were not isolated when fibroblasts were reprogrammed into iPS cells. When comparing phenotypes between iPS derived mutant cells and unrelated wildtype cells, the potential effects associated with genetic background or modifier genes were unaccounted for. To compensate for unavailable isogenic controls and the potential variability due to genetic background, I included 3 unrelated wildtype controls into my
experimental design. Recently, other members of our lab were able to improve the situation by generating a rescue RTTe1 cell line by infecting NPCs with MECP2e1 lentiviruses controlled by a mouse MeCP2 promoter (MeP). The expression of MeP-MECP2e1 reversed an identified RTTe1 neuronal soma size defect, demonstrating that it would be a good control for future studies conducted on neurons obtained from the RTTe1 patient. This does not replace the advantages to having isogenic wildtype controls however. With strategies such as TALEN and CRISPR/Cas-mediated genome engineering, it may be possible to create isogenic wildtype controls from RTTe1 iPS cells.

4.3.3 RTTΔ3-4#37 healthy control neurons consistently underperformed

An observation from my experiments was that RTTΔ3-4#37 neurons did not behave as described previously (64). My colleagues and I have high confidence in this cell line since it has been characterized extensively and performed well in assays for other lab members. But for an unknown reason, NPCs obtained from my differentiations only, behaved similar to the isogenic RTTΔ3-4#20 null cell line in many assays. I provided evidence for the detection of MeCP2 in RTTΔ3-4#37 wildtype neurons by both immunofluorescence staining and western blot experiments. Based on western blot experiments, RTTΔ3-4#37 neurons had lower levels of MeCP2 relative to other unrelated healthy controls. RTTΔ3-4#37 healthy control neurons had smaller soma sizes than RTTΔ3-4#20 null neurons, which was contrary to what had been previously published by our lab (64). Since none of my colleagues have had issues with RTTΔ3-4#37 cell lines, I rationalized that something peculiar must have happened when I differentiated iPS cells into NPCs, though the precise cause of its deviation from norm is unknown. It is possible that NPCs were misidentified neural crest cells, which make peripheral neurons instead of cortical neurons. This could be tested by immunocytochemical staining of cells with AP2, PAX7, and p75 markers for neural crest. Additionally, I could stain cells for NPC markers: Sox2, Sox1, and Musashi1.

4.3.4 Loss of MeCP2e1 and continued expression of MeCP2e2 in RTTe1 neurons need to be confirmed by isoform specific antibodies

Based on western blot experiments, RTTe1 neurons have detectable levels of MeCP2. I attributed the detection of MeCP2 to the continued expression of MeCP2e2 isoform, which
should be unaffected by the 11 bp deletion in exon 1 of MECP2. To test this hypothesis however, I would need to employ the use of MeCP2 isoform specific antibodies, which do exist (103,104). I obtained these antibodies and attempted to use them to demonstrate the continued expression of MeCP2e2 but could not obtain clear results because a large number of non-specific bands were present.

4.3.5 EB-based neuronal differentiation protocol lacks heterogeneity that is often found in the brain

The selected neuronal differentiation protocol employed for my experiments lack the degree of heterogeneity found within the brain. Glial cells are important contributors to normal brain function. Several lines of evidence suggest that glia contribute to RTT pathophysiology. MeCP2 is expressed in astrocytes and oligodendrocytes albeit at lower levels than neurons (50). When hippocampal neurons from wildtype mice are cocultured in vitro with wildtype astrocytes as a source of nutrients and factors for growth, wildtype neurons remain healthy and are able to extend projections that appear normal. However, when hippocampal neurons from wildtype mice are cultured in vitro with RTT mouse astrocytes that lack MeCP2, wildtype neurons appear stunted in growth with shorter projections and compromised soma size (50). The inability of RTT astrocytes to support normal neuronal growth likely occurs because RTT astrocytes are unable to secrete soluble factors that are necessary for neuronal growth such as BDNF (51).

The loss of MeCP2 in oligodendrocytes also contributes to RTT pathology. Mice lacking MeCP2 in oligodendrocytes have a hindlimb clasp, which is a characteristic phenotype analogous to hand clasping that occurs in some RTT patients (53).

The contributions of glia in RTT disease progression is fortified by a recent study demonstrating that bone marrow transplants to RTT mice can significantly rescue RTT phenotypes (105). Wildtype and MeCP2-deficient mice were given lethal doses of irradiation to eradicate all nascent immunity and then a bone marrow transplant from wildtype donors. MeCP2-deficient mice that did not receive bone marrow transplants presented with RTT phenotypes such as reduced longevity, body weight, and brain weight, which were rescued by transplantation. The rescue effect was attributed to the transplantation of wildtype microglia and their ability to respond to immunologic stimuli and carry out phagocytosis, both of which are altered in mice lacking MeCP2 (105).
The culture system I used to demonstrate morphological deficits in neurons does not integrate supporting cell types for neuronal growth. Therefore, future experiments should seek to apply coculture systems that include both wildtype and RTT glial cells so that a more faithful representation of the brain can be obtained.

4.3.6 One-step induction neurons should be cocultured with both wildtype and MeCP2-deficient astrocytes to faithfully model disease

Our lab has begun integrating coculture systems in some of our experiments. For neuronal morphology experiments performed on neurons obtained from the one-step induction protocol, wildtype mouse astrocytes were added to culture conditions to facilitate neuronal maturation. By comparing just the Sholl analysis profiles of neurons differentiated by the directed differentiation protocol and one-step induction protocol, it was evident that neurons from the one-step induction protocol were much more complex. Astrocytes cocultured with neurons obtained from the one-step method were the likely contributors to the observed increase in dendritic complexity. Since RTT patients are mosaic for wildtype and mutant cells, a coculture system that includes both wildtype and mutant glia is ideal and methods to include representative proportions of glia in culture needs to be further developed.

It cannot be ruled out however, that the increased complexity of neurons obtained from the one-step induction protocol was also due to the overexpression of Ngn2, which can activate the AKT/mTOR pathway, to increase dendrite complexity, and/or reduce RhoA activity, which will also increase dendritic complexity (94-97,99,106,107). This hypothesis needs to be tested more thoroughly however.

4.3.7 Lack of experimental replicates for morphology assays performed on neurons obtained from the one-step induction protocol preclude biological conclusions

No conclusions could be made from experiments performed on RTT neurons derived by the one-step induction protocol. This protocol is appealing to us since it offers the potential to shorten the time required for obtaining functional neurons. Our current directed differentiation protocol commonly used in our lab requires approximately 9 weeks to obtain electrophysiologically active neurons from iPS cells. The one-step induction protocol reports the acquisition of functional neurons by 3 weeks from the iPS cell stage (82). It would be advantageous if this protocol were
successful at recapitulating disease phenotypes. However, preliminary experiments suggest that it does not. I performed neuronal morphometric analyses on neurons from two RTT patients and two healthy controls, studied at two different time points. At both time points (2 and 3 weeks), no consistent differences in dendritic complexity were observed. Experiments were performed once at both time points and require more replicates before making any conclusions about the rescue effects of Ngn2. If Ngn2 does rescue RTT dendritic morphology defects, this may be a novel pathway that might have some therapeutic value.
Chapter 5
Future Experiments

5 Future Experiments

5.1 Exploring genetic and pharmacological approaches to rescue RTT dendritic complexity defects

5.1.1 Background and Rationale

RTT phenotypes caused by MeCP2 mutations in mice can be reversed by the reintroduction of wildtype MeCP2 after disease onset (58). However, it is unclear whether the reintroduction of MECP2 in human neurons can reverse morphological defects present in patients. Our lab has evidence suggesting that reintroducing MECP2e1 in human iPS cell derived neurons obtained from the RTT e1 patient can rescue the soma size defect (81). MECP2e1 lentiviruses controlled under the endogenous mouse MeCP2 promoter (MeP) were infected into NPCs of one RTT e1 cell line and differentiated into neurons (52). RTT e1 neurons expressing MeP-MECP2e1 had larger soma sizes that resembled unrelated wildtype control neurons. This suggests that neuronal morphological defects found in human RTT patients can be rescued by reintroducing functional MECP2. No attempts to evaluate RTT e1 dendritic complexity defects in the rescued neurons have been made and it would be interesting to test whether gene therapy can effectively reverse dendritic phenotypes. If successful, this would also validate the use of rescue RTT e1 neurons as controls for future experiments aiming to identify other RTT e1 phenotypes. Rescued RTT e1 neurons would also be a good positive control for exploring pharmacological agents and their efficacy in reversing disease phenotypes.

For MeCP2-deficient mice, the administration of BDNF and IGF-1 can alleviate their behavioural symptoms (57,60). BDNF and IGF-1 can also reverse both the reduced soma size defect and reduced neurite complexity defect observed in RTT ES cell derive neurons (31). Our lab has not been able to reproduce the published results likely because our neuronal differentiation protocol uses high levels of neurotrophic factors, BDNF and IGF-1, for neuronal induction. In order for us to observe an effective rescue effect, it may be necessary to remove exogenous BDNF and IGF-1 from culture conditions.
Media lacking BDNF and IGF-1 may not be able to support neuronal induction. Therefore, coculturing both wildtype and RTT cells with wildtype mouse astrocytes may be a viable source of neurotrophic factors (50). These astrocytes are readily available in our lab since others have been using them for their own experiments. By relying on astrocytes for the secretion of BDNF, IGF-1 and other soluble factors, this will establish a baseline of neuronal complexity for wildtype and mutant neurons. To test the rescue effects of IGF-1, I would propose to supplement wildtype and RTT neurons with exogenous IGF-1 and compare them to untreated cells. I hypothesize that exogenous expression of MECP2e1 and treatment with IGF-1 can rescue RTTe1 dendritic phenotypes.

5.1.2 Rescuing RTTe1 dendritic defects with MeP-MECP2e1 and IGF-1

To test this hypothesis, I would propose to infect NPCs of the three RTTe1 cell lines used in my experiment with MeP-MECP2e1 (RTTe1 rescue neurons), which will serve as a positive control for pharmacological rescue experiments. NPCs of the 3 RTTe1 rescue cell lines, 2 unrelated iPS cell lines described in 6-week-old morphometric assays, and 3 RTTe1 lines will be differentiated with the EB-based directed differentiation protocol (80). Since DAPT was ineffective in controlling cellular density, the more potent compound Ara C will be used to eliminate proliferating cells after a period of neuronal differentiation. An equal number of NPCs will be seeded across all cell lines and cocultured with wildtype astrocytes. Neurons will be differentiated for five weeks in vitro before being treated with Ara C overnight. After overnight treatment, neurons will be passed into a new flask, reseeded with wildtype astrocytes, and cultured for an additional one-week recovery period. To test whether RTTe1 dendritic phenotypes can be rescued by exogenous IGF-1 treatment, some uninfected RTTe1 and wildtype neurons will be cultured in media supplemented with IGF-1, in addition to coculture with astrocytes (see Table 7 for summary of experimental setup).

One day before fixation, neurons will be transfected with EF1α-EGFP and analyzed for dendritic phenotypes as described in my thesis (see Section 2.4). Three experimental replicates should be performed as was done previously. The novelty of this experiment is in the combination of IGF-1 treatment and coculturing neurons with astrocytes that would represent the in vivo environment more faithfully than coculturing neurons without astrocytes. This study would also test whether
overexpressing MECP2 under the MeP promoter in RTTe1 neurons could reverse dendritic complexity defects, which has not been examined previously.

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</table>

**Table 7. Summary of proposed experimental conditions for rescue experiments.** All cell lines described above are derived from iPS cell lines obtained and reprogrammed from patient biopsies. In total there are 2 wildtype (WT) iPS cell lines, 2 WT iPS cell lines treated with IGF-1 (10 ng/ml), 3 RTTe1 cell lines, 3 RTTe1 iPS cell derived lines infected with MeP-MECP2e1, and 3 RTTe1 cell lines treated with IGF-1 (10 ng/ml). All cells are cocultured with mouse wildtype astrocytes.
5.2 Exploring RTT phenotypes in RTT neurons derived by the overexpression of Ngn2

Preliminary experiments performed on neurons derived from the overexpression of Ngn2 suggest that RTT dendritic phenotypes that have been previously identified from neurons generated from a directed differentiation protocol may be masked by transgene expression. Ngn2 is a transcription factor that mediates both RhoA and AKT/mTOR signaling pathways. The advantage to treating patients with Ngn2 is that it would activate these two pathways, both of which, have been reported to mediate dendritic complexity. However, it may not be feasible to administer Ngn2 via gene therapy. The finding of masked phenotypes was unexpected, but conclusions were inadmissible due to the lack of experimental replicates and small sample size. To improve on the experiment that was conducted previously, I would propose to introduce one more control cell line and 3 RTTe1 cell lines (See Table 8 for complete list of cell lines and experimental conditions). All cell lines would be cocultured with mouse astrocytes so that in vivo conditions could be replicated more faithfully. Experiment should be performed in triplicate.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cell line</th>
<th>Astrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>H9 (ES cell)</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>RTTΔ3-4#37</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>SK0186_001#2.14</td>
<td>WT</td>
</tr>
<tr>
<td>MUT</td>
<td>RTTΔ3-4#20</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>RTT R306C</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>RTTe1#27</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>RTTe1#39</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>RTTe1#96</td>
<td>WT</td>
</tr>
</tbody>
</table>

WT – Wildtype    MUT – Mutant

Table 8. Summary of cell lines included in study to determine whether Ngn2 overexpression masks RTT phenotypes. Three wildtype cell lines (1 ES cell line, 2 iPS cell lines) and 5 RTT cell lines from 3 different patients would be included in this study. All cell
lines would be cocultured with mouse astrocytes. This experiment should be performed in triplicate with iPS cells seeded and infected with lentiviruses one week apart.

Once neurons have matured for 3 weeks in vitro, neurons would be fixed and stained with MAP2, dendritic marker. Neurons would be reconstructed based on MAP2 staining using NeuronJ software. Total dendrite length, number of terminal dendrites, number of primary and higher order dendrites, and number of intersections by Sholl analysis would be performed as described previously (see Section 2.4).

I predict that there would be no differences in the level of dendritic complexity between RTT cell lines and healthy control cell lines and that the lack of difference would be due to a masking effect by Ngn2 overexpression. The rescue effect of Ngn2 would be tested by infecting RTTe1 NPCs with lentiviruses containing Ngn2 and comparing their neuronal morphology to RTTe1 neurons and healthy control neurons, after 6 weeks of in vitro differentiation using the EB-based differentiation protocol.

I predict that Ngn2 overexpression would mask RTTe1 dendritic morphology defects. There is evidence suggesting that Ngn2 transcription factor acts upstream of the AKT/mTOR signaling pathway (106,107). Ngn2 is a transcription factors that associates with Brn3A, which regulates the expression of TrkA, TrkB, and TrkC receptors. TrkB receptors are activated by BDNF, a small molecule that is able to reverse RTT phenotypes by stimulating the AKT pathway.

To test this hypothesis, RTTe1 cell lines overexpressing Ngn2 should have higher levels of phosphorylated AKT (p-AKT) than uninfected RTTe1 cell lines. This can be tested by western blot analysis, where cultures would be treated with Ara C to eliminate astrocytes, cellular debris washed away, and lysates obtained from remaining neurons. Western blots would be probed for p-AKT and the relative amounts would be compared between groups. The downstream effector phosphorylated S6 (p-S6) would also be probed to determine if elevated p-AKT correlates with an increase of p-S6. This study would determine whether the overexpression of Ngn2 has the potential to restore RTT phenotypes to wildtype conditions. If there is potential therapeutic value to stimulating Ngn2 expression, it may be worthwhile to seek pharmacological inducers or activators of Ngn2.
5.3 Conclusions

In this thesis, I employed morphology based *in vitro* methods to assess the complexity of neuronal dendrites from RTT e1 patient neurons with a MECP2e1 mutation. RTTe1 neurons from 3 iPS cell lines had significantly reduced total dendrite length, reduced number of terminal dendrites, reduced number of higher order dendrites, and reduced complexity by Sholl analysis compared to wildtype neurons from 3 healthy iPS cell lines. In conjunction with the small soma size phenotype of RTT e1 neurons and the genetic rescue experiments by our lab, we provide strong evidence that the MECP2e1 isoform is essential for healthy neuronal morphology. Preliminary experiments suggest that RTT neurons obtained from the one-step induction protocol do not recapitulate disease phenotypes. I hypothesize that this might be because Ngn2 overexpression masks RTT morphological defects and I propose future studies that could be done to test this hypothesis.
References


