Mutant astrocytes differentiated from Rett syndrome patients-specific iPSCs have adverse effects on wild-type neurons

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The disease mechanism of Rett syndrome (RTT) is not well understood. Studies in RTT mouse models have suggested a non-cell-autonomous role for astrocytes in RTT pathogenesis. However, it is not clear whether this is also true for human RTT astrocytes. To establish an in vitro human RTT model, we previously generated isogenic induced pluripotent stem cell (iPSC) lines from several RTT patients carrying different disease-causing mutations. Here, we show that these RTT iPSC lines can be efficiently differentiated into astroglial progenitors and glial fibrillary acidic protein-expressing (GFAP+) astrocytes that maintain isogenic status, that mutant RTT astrocytes carrying three different RTT mutations and their conditioned media have adverse effects on the morphology and function of wild-type neurons and that the glial effect on neuronal morphology is independent of the intrinsic neuronal deficit in mutant neurons. Moreover, we show that both insulin-like growth factor 1 (IGF-1) and GPE (a peptide containing the first 3 amino acids of IGF-1) are able to partially rescue the neuronal deficits caused by mutant RTT astrocytes. Our findings confirm the critical glial contribution to RTT pathology, reveal potential cellular targets of IGF-1 therapy and further validate patient-specific iPSCs and their derivatives as valuable tools to study RTT disease mechanism.

INTRODUCTION

Mutations in the X-linked methyl CpG-binding protein 2 (MECP2) gene cause Rett syndrome (RTT) (1), which is a devastating neurological disease that predominantly affects females (2). To fully understand the disease mechanism and to develop effective treatments, it is necessary to determine what cells/tissues express MeCP2 and study the contributions of each MeCP2-expressing cell/tissue to the overall disease. Earlier studies showed that MeCP2 is highly expressed in mature neurons throughout the mammalian brain (3,4), suggesting that the loss of function or malfunction of MeCP2 in neurons plays a key role in RTT pathogenesis. This hypothesis is strongly supported by several later studies demonstrating that specific genetic deletion of MeCP2 in different neuronal subtypes leads to distinct subsets of RTT-like phenotypes in mouse models (5–7). More recently, it has been discovered that another major cell type in the brain, astrocytes, also express MeCP2 and that the loss of MeCP2 in astrocytes has a non-cell-autonomous influence on neuronal morphology (8,9). Furthermore, the restoration of normal MeCP2 expression only in astrocytes is sufficient to rescue RTT disease symptoms in mouse models (10). However, it is unclear whether human astrocytes also express MECP2 and whether human RTT astrocytes also adversely affect neuronal morphology and function. GPE (a peptide containing the first 3 amino acids of IGF-1) has been shown to partially rescue RTT-like phenotypes in Mecep2 mutant mice (11), which has led to the ongoing clinical trials of IGF-1 and GPE in RTT patients. Yet, the cellular targets of these IGF-1 related therapies remain unclear.

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We previously generated and characterized isogenic pairs of wild-type and mutant induced pluripotent stem cell (iPSC) lines from RTT patients carrying the V247X (valine 247 to stop codon, nonsense, rare in RTT patients), R294X (arginine 294 to stop codon, nonsense, present in 5–6% of RTT patients) and R306C (arginine 306 to cysteine, missense, present in 4–7% of RTT patients) mutations (12). Since the reprogramming process does not reactivate the silent X-chromosome in somatic cells (13), each of these RTT iPSC lines inherited and stably maintained the somatic X-chromosome inactivation (XCI) status of the fibroblast cell they originated from. Therefore, the isogenic RTT iPSC lines clonally express either the wild-type or the mutant copy of MECP2. While it has long been feasible to differentiate human embryonic stem cells (hESCs) into various subtypes of neurons, methods to efficiently differentiate hESCs and iPSCs into functional astrocytes have only recently become available (14–16). Using one of these methods (14), we have differentiated isogenic pairs of RTT iPSC lines carrying the V247X, R294X and R306C mutations into astrocytes and examined their influence on the morphology of wild-type neurons. We show here that astrocytes differentiated from RTT iPSCs maintained the non-random XCI status of their parental RTT iPSC lines, clonally expressed either the wild-type or mutant, but not both, allele of MECP2, that the mutant RTT astrocytes adversely affected the morphology and function of wild-type mouse neurons, that similar negative influence also applied to human neurons co-cultured with these astrocytes and that such a non-cell-autonomous influence was partially mediated by factors secreted from the astrocytes. Furthermore, using different combinations of GABAergic interneurons (IN) and astrocytes differentiated from the same isogenic pair of RTT iPSC lines, our in vitro co-culture system was able to clearly separate the neuronal and glial contribution to the observed neuronal deficits. Finally, both IGF-1 and GPE were effective in rescuing neuronal deficits caused by mutant RTT astrocytes. Our findings provide the first direct evidence that human astrocytes express detectable level of MECP2 and confirm the significant glial contribution to RTT pathology in human cells. In addition, our results demonstrate that the isogenic RTT iPSC lines and their derivatives as a valid in vitro system for studying RTT disease mechanisms and testing drug candidates.

RESULTS

The isogenic RTT iPSC lines can be efficiently differentiated into GFAP⁺ astrocytes

Using isogenic pairs of RTT iPSC lines to study disease mechanisms may reduce phenotypic variation across different individuals with diverse genetic backgrounds. However, it has been reported that female iPSC lines can undergo erosion of XCI (17) in culture over time. Thus, we reconfirmed the XCI status of all isogenic RTT iPSC pairs by examining the methylation profile of the androgen receptor (AR) locus, the allele-specific transcription of a single-nucleotide polymorphism (SNP) from the XIST locus, and the allele-specific transcription of the MECP2 gene. Our results clearly demonstrated that all the RTT iPSC lines used for astroglial differentiation in this study maintained their original XCI pattern (Supplementary Material, Fig. S1), likely because they were still at relatively early passages (passages 13–38, details on each line provided in Materials and Methods). We first generated astroglial progenitors from each of the RTT iPSC lines by propagating neurospheres in the presence of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) in alternating suspension and adherent culture. To carefully monitor the astrogenic potential of each RTT astroglial progenitor line, terminal differentiation was initiated by the removal of the growth factors (EGF and FGF2) coupled with the supplementation of ciliary neurotrophic factor (CNTF); this terminal differentiation was performed every month from 90 days to over 300 days after the start of differentiation (Fig. 1A). While all RTT astroglial progenitor cells efficiently differentiated into S100B-positive cells, the number of glial fibrillary acidic protein (GFAP)-positive cells continued to increase when terminal differentiation was performed on early phase (90–150 days), intermediate phase (150–270 days) and late phase (270–300 + days) astroglial progenitors (Fig. 1B). Stereological counting determined that 70–85% of cells terminally differentiated from late phase RTT astroglial progenitors were positive for GFAP (Fig. 1C). Two to 5 independent differentiations from each RTT astroglial progenitor line were analyzed. There was no significant difference in proliferation rate and differentiation efficiency between the wild-type and mutant RTT astroglial progenitor cells. Consistent with the increase of S100β and GFAP levels throughout astrocyte differentiation, we observed increased expression of the astroglial genes NFIX, GLT-1 and AQP4 and the marked decrease in expression of OCT4, a pluripotency transcription factor (Supplementary Material, Fig. S2A–D).

Astroglial progenitors differentiated from isogenic RTT iPSC lines maintain their original isogenic status

To examine the maintenance of isogenic status, we next assayed the XCI status and allelic MECP2 expression of all astroglial progenitors differentiated from the iPSC lines. The methylation status of the AR locus has been established as a surrogate marker of XCI status (18). In this assay, without digestion with methylation sensitive enzymes (uncut traces), both parental alleles of the AR locus were readily detected and unequivocally distinguished by the presence of two different-sized peaks (165 and 180 bp for the V247X lines, 165 and 171 bp for the R294X lines, 173 and 176 bp for the R306C lines, as indicated by the red and blue color) in all the RTT astroglial progenitors throughout the early, intermediate and late phases (Fig. 2A). The non-skewed ratio between the red and blue peaks in the uncut samples (ranging from 33:67 to 63:37) indicated similar amplification efficiency of the two parental alleles. After digestion with methylation sensitive enzymes (cut traces), only the hypermethylated AR allele (on the inactivated X-chromosome) could be amplified (Fig. 2A). In all RTT astroglial progenitors across the early, intermediate and late phases, the ratio between the red and the blue peaks was highly skewed (Fig. 2A), suggesting XCI was not random in these RTT astroglial progenitors. Moreover, the RTT astroglial progenitors had the same AR allele hypermethylated as the RTT iPSC lines they were differentiated from (compare Supplementary Material, Fig. S1; Fig. 2A). Allele-specific transcription of the MECP2 gene in all of the late phase RTT astroglial progenitors was also examined and
showed expression of either the wild-type or the mutant, but not both, alleles of the MECP2 gene (Fig. 2B). Finally, we performed western blot to directly examine MECP2 protein level in astrocytes terminally differentiated from late phase RTT astrogial progenitors. The MECP2 band was detected at \( \approx 75 \) kDa in astrocytes differentiated from H9, V247X-WT, R294X-WT and R306C-WT astrogial progenitors, but not in astrocytes differentiated from V247X-MT and R294X-MT astrogial progenitors (Supplementary Material, Fig. S2E and F). Because the antibody cannot distinguish the R306C missense mutation, an MECP2 band was also detected in the R306C-MT astrocytes. Taken together, our data suggest that all RTT astrogial progenitors/astrocytes maintain isogenic status throughout the differentiation procedure and clonally express either the wild-type or mutant MECP2.

**Mutant RTT astrocytes have adverse effects on the morphology and function of co-cultured wild-type mouse hippocampal neurons**

To determine whether human RTT mutant astrocytes have non-cell-autonomous influence on wild-type neurons, we isolated primary hippocampal neurons from postnatal day 0 (P0) mice and co-cultured them with either wild-type or mutant astrocytes differentiated from late phase isogenic astrogial progenitors. We chose the P0 hippocampal neurons for our first co-culture experiment because they are easier to obtain, more mature and more homogenous in their morphology than hESC- or iPSC-derived neurons. After 24 and 72 h of co-culture with astrocytes of various genotypes, a detailed morphological analysis of MAP2-positive neurons was performed with the Neurolucida tracing tool and Neurolucida Explorer software package (Supplementary Material, Fig. S3). After 24 h of co-culture, the wild-type P0 mouse hippocampal neurons had significantly smaller soma size, shorter total neurite length and a smaller number of terminal ends when cultured on mutant V247X, R294X and R306C astrocytes when compared with those cultured on their respective isogenic wild-type astrocytes (Fig. 3A). The only exception to this trend was a lack of significant difference in the number of neuron terminal ends between co-cultures performed with the R294X isogenic pair. One hundred to 200 MAP2-positive neurons (pooled from two biological replicates) from co-culture experiments with astrocytes of each genotype were included for analysis at this time point. When the morphological features were analyzed after 72 h of co-culture, it appeared that neurons co-cultured with either the wild-type or mutant astrocytes experienced a certain amount of growth. This is indicated by an increase in soma size, total neurite length and number of terminal ends of neurons when compared with their respective values at the 24-h time point. More importantly, the neurons co-cultured with the mutant V247X, R294X and R306C astrocytes exhibited a continued defect in this growth, as indicated by significantly smaller soma size, shorter total neurite length and a smaller number of terminal ends when compared with neurons co-cultured with their respective isogenic wild-type astrocytes (Fig. 3B). One hundred to 200 MAP2-positive neurons (pooled from two biological replicates) from co-culture experiments with astrocytes of each genotype were included for analysis at this time point. When the morphological features were analyzed after 72 h of co-culture, it appeared that neurons co-cultured with either the wild-type or mutant astrocytes experienced a certain amount of growth. This is indicated by an increase in soma size, total neurite length and number of terminal ends when compared with their respective values at the 24-h time point. More importantly, the neurons co-cultured with the mutant V247X, R294X and R306C astrocytes exhibited a continued defect in this growth, as indicated by significantly smaller soma size, shorter total neurite length and a smaller number of terminal ends when compared with neurons co-cultured with their respective isogenic wild-type astrocytes (Fig. 3B). One exception to this trend was a lack of significant difference in the neuron soma size between co-cultures performed with the R306C isogenic pair. Ninety-seven to 188 MAP2-positive neurons (pooled from two biological replicates)
Figure 2. Maintenance of the isogenic status in astroglial progenitors differentiated from isogenic RTT iPSC lines. (A) Human AR methylation profiles for isogenic wild-type (WT) and mutant (MT) RTT astroglial progenitors carrying the V247X, R294X and R306C mutations at the early, intermediate and late stages of astroglial differentiation. Uncut traces show the presence of both parental AR alleles: 165 and 180 bp for the V247X lines, 165 and 171 bp for the R294X lines and 173 and 176 bp for the R306C lines. Cut traces of iPSCs verified the presence of only one AR allele due to skewed, non-random XCI, which remained constant throughout the directed differentiation. The source of genomic DNA is labeled to the left of each trace. The ratio between the two AR alleles is at the bottom of each trace. (B) Representative sequencing traces of allele-specific MECP2 expression in each of the isogenic astroglial progenitor pairs. The shaded area marks the position of nucleotide that is different between WT and MT.
Figure 3. Morphological and functional defects of wild-type mouse hippocampal neurons co-cultured on mutant RTT astrocytes. (A and B) Quantification of neuronal soma area, total neurite length and total number of terminal ends of the wild-type neurons co-cultured for 24 (A) and 72 (B) hours with either WT or MT RTT astrocytes. Black bars indicate neurons co-cultured with wild-type astrocytes, whereas white bars indicate neurons co-cultured with mutant RTT astrocytes. Error bars = SEM, *P < 0.05, **P < 0.005. The number embedded in each bar represents the number of neurons analyzed for that condition. (C) Representative sample traces of mEPSCs recorded from wild-type mouse hippocampal neurons (8 DIV) co-cultured on either wild-type (WT) or mutant (MT) astrocytes differentiated from the R306C isogenic RTT pair. (D and E) Quantification of mEPSC amplitude (D) and frequency (E) recorded from the wild-type neurons co-cultured with either WT (black bars) or MT (white bars) RTT astrocytes. The number embedded in each bar represents the number of neurons analyzed for that condition. Error bars = SEM, *P < 0.005.
from co-culture experiments with astrocytes of each genotype were included for analysis at this time point. Similar morphological deficits were observed in wild-type hESC(H9)-derived forebrain neurons co-cultured with mutant RTT astrocytes (Supplementary Material, Fig. S4). To determine if the observed neuronal morphological deficits lead to functional changes, we co-cultured wild-type hippocampal neurons with either wild-type or mutant astrocytes from the R306C isogenic pair for 8 days and subsequently recorded the miniature excitatory postsynaptic currents (mEPSCs) in these neurons. While the average mEPSC amplitude was similar between neurons co-cultured with either wild-type or mutant astrocytes, the average mEPSC frequency of neurons co-cultured with mutant astrocytes was significantly lower than that of neurons co-cultured with wild-type astrocytes (Fig. 3C–E). To reveal whether the decreased mEPSC frequency was caused by a decrease in the density of synapses, we stained our mouse neuron/human astrocyte co-culture with MAP2 (to label neuronal dendrites), vGlut1 (to label presynaptic terminals) and PSD95 (to label postsynaptic terminals), and quantified the density of synapses defined as the number of co-localized puncta of vGlut1 and PSD95 over unit length of MAP2-labeled neurite. To our surprise, no significant difference was observed between the WT (0.32 ± 0.06 µm⁻¹) and MT (0.34 ± 0.04 µm⁻¹) co-cultures. Additional studies in the future are needed to better understand the mechanism of the decreased mEPSC frequency. Together, results in this series of experiments revealed both morphological and functional deficits in wild-type neurons co-cultured with RTT mutant astrocytes.

Media conditioned by mutant RTT astrocytes have adverse effects on the morphology of wild-type mouse hippocampal neurons

Having observed the adverse influence of mutant RTT astrocytes on both mouse and human wild-type neurons, we decided to test whether such a non-cell-autonomous effect was mediated by factors secreted from the astrocytes. In this series of experiments, we cultured wild-type primary P0 mouse hippocampal neurons in astrocyte-conditioned medium (ACM) collected from each isogenic RTT astrocyte pair, and examined neuronal morphology after 24 and 72 h of culture (Supplementary Material, Fig. S5). At the 24-h time point, neurons grown in ACM from mutant V247X, R294X and R306C astrocytes had significantly smaller soma size, shorter total neurite length and a smaller number of terminal ends compared with those grown in ACM from their respective isogenic wild-type astrocytes (Fig. 4A). Forty-nine to 122 neurons (pooled from two biological replicates) cultured in ACM from astrocytes of each genotype were included in analysis for the 24-h time point. At the 72-h time point, neurons grown in ACM from mutant R294X and R306C astrocytes had significantly smaller soma size than those grown in ACM from their respective isogenic wild-type astrocytes, yet no difference was found when neurons were grown in ACM from the V247X pair; neurons grown in ACM from mutant V247X, R294X and R306C astrocytes had significantly shorter neurites than those grown in ACM from their respective isogenic wild-type astrocytes; neurons grown in ACM from mutant R294X astrocytes has significantly fewer terminal ends than those grown in ACM from wild-type R294X astrocytes, yet no difference was found when neurons were grown in ACM from the V247X pair and the R306C pair (Fig. 4B). Forty-three to 123 neurons (pooled from two biological replicates) cultured in ACM from astrocytes of each genotype were included in analysis for the 72-h time point.

Mutant RTT astrocytes negatively influence the morphology of co-cultured wild-type human interneurons, while wild-type RTT astrocytes positively influence the morphology of co-cultured mutant human interneurons

The brain of a female RTT patient has a mixture of both mutant and wild-type neurons and astrocytes due to random XCI, thus there are four possible interactions concerning these two cell types with two different genotypes. To model each interaction, we differentiated the isogenic pair of R294X iPSC lines into GABAergic interneurons (19) and astrocytes, generated co-cultures of all four possible combinations (WT interneuron/WT astrocyte, WT interneuron/MT astrocyte, MT interneuron/WT astrocyte and MT interneuron/MT astrocyte), and analyzed neuronal morphology after 72 h of co-culture (Supplementary Material, Fig. S6A and B). To ensure sensitivity and consistency, only GABA-positive interneurons were included in our analysis. For both the total neurite length and the number of terminal ends, mutant astrocytes had a significant negative effect on wild-type interneurons (Fig. 5A, WT interneuron/WT astrocyte versus WT interneuron/MT astrocyte, P = 0.000004 for neurite length, P = 0.02 for terminal ends), while wild-type astrocytes had a significant positive effect on mutant interneurons (Fig. 5A, MT interneuron/WT astrocyte versus MT interneuron/MT astrocyte, P = 0.0002 for neurite length, P = 0.0009 for terminal ends). In addition to the non-cell-autonomous effect of astrocytes on neuronal morphology, MECP2 mutant interneurons also displayed cell-autonomous-negative effects in morphology (Fig. 5A, WT interneuron/WT astrocyte versus MT interneuron/WT astrocyte, P = 0.0001 for neurite length, P = 0.01 for terminal ends; WT interneuron/MT astrocyte versus MT interneuron/MT astrocyte, P = 0.02 for neurite length, P = 0.006 for terminal ends). Moreover, the astroglial effect and the neuronal effect on neuronal morphology appeared additive (no significant interaction between interneuron and astrocyte was detected by two-way ANOVA). Finally, no significant difference was observed in neuronal soma size across the four combinations (Fig. 5A). Neurons pooled from two to five biological replicates were analyzed for each category presented in Figure 5.

The effects of IGF-1 and GPE treatment on neuronal morphology in RTT interneuron/astrocyte co-cultures

Currently, both IGF-1 and a modified form of GPE are on clinical trial for treating RTT patients. Yet it is not clear what either reagent may do to RTT human neurons/astrocytes at the cellular level. Thus we examined the effect of short-term treatment with either 100 ng/ml of IGF-1 or 300 ng/ml of GPE on neuronal morphology in our co-culture system (Supplementary Material, Fig. S6A, C and D). Despite the fact that no difference in neuronal soma size was observed among all four co-culture combinations in the absence of any treatment (Fig. 5A), both IGF-1 and GPE significantly increased the neuronal soma size over the untreated condition in almost all combinations (Fig. 5B).
total neurite length, GPE had a significant benefit for both the WT interneuron/MT astrocyte combination and the MT interneuron/MT astrocyte combination (Fig. 5C). The beneficial effect of IGF-1, however, was more modest, reaching statistical significance in the MT interneuron/MT astrocyte combination and barely missing the cut-off in the WT interneuron/MT astrocyte combination ($P = 0.052$). As for the number of terminal ends, except in the GPE treatment of the MT interneuron/MT astrocyte combination, no significant improvement over untreated condition was observed for either IGF-1 or GPE treatment in any co-culture combination (Fig. 5D). Surprisingly, IGF-1 negatively affected the total neurite length and the number of terminal ends in both the WT interneuron/WT astrocyte combination and the MT interneuron/WT astrocyte combination; while GPE negatively affected the total neurite length and the number of terminal ends in the WT interneuron/WT astrocyte combination (Fig. 5C and D). Thus, the direction of IGF-1/GPE treatment effect appears to depend on the genotype of RTT astrocyte:

IGF-1 and GPE had positive influence on neuronal morphology in co-cultures involving mutant astrocytes, but had negative morphology influence in co-cultures involving wild-type astrocytes. Since expression of IGF-1 was undetectable in both WT and MT astrocytes (data not shown), it is unlikely that the IGF-1 treatment was directly rescuing a deficiency in astrocyte-secreted IGF-1. Nonetheless, as the first step to identify the cellular targets of IGF-1/GPE in our co-cultures, we examined the expression/localization of the known receptor of IGF-1 (IGF-1R). While there appeared to be diffuse IGF-1R immunoreactivity in both MAP2-positive neurons and GFAP-positive astrocytes in the co-culture, punctate IGF-1R immunoreactivity was only found in GFAP-positive astrocytes (Supplementary Material, Fig. S7A and B). Such punctuate pattern of the IGF-1R signal was more obvious when astrocytes were cultured alone (Supplementary Material, Fig. S7C). To better quantify the potential difference in IGF-1R levels between WT and MT astrocytes, we performed qPCR analysis. Comparing with wild-type
Figure 5. Morphological effects of WT and MT RTT GABAergic IN co-cultured with WT and MT RTT astrocytes (AS) and the effects of IGF-1/GPE (a peptide containing the first 3 amino acids of IGF-1) treatment. (A) Quantification of neuronal soma area, total neurite length and number of terminal ends of WT or MT RTT IN after 72 h of co-culture with WT or MT RTT AS in the absence of any treatment. (B–D) Comparison of neuronal soma area (B), total neurite length (C) and the number of terminal ends (D) after 72 h of co-culture with the addition of vehicle (Veh), 100 ng/ml IGF-1 or 300 ng/ml GPE. Black bars = WT IN co-cultured with WT astrocytes. Dark gray bars = MT IN co-cultured with WT astrocytes. Light gray bars = WT IN co-cultured with MT astrocytes. White bars = MT IN co-cultured with MT astrocytes. The number embedded in each bar represents the number of neurons analyzed for that condition. Error bars = SEM, *P < 0.05, **P < 0.005.
level, IGF-1R transcript was significantly decreased in the mutant astrocytes (Supplementary Material, Fig. S8). More detailed analysis of other IGF-1 signaling pathway components in these co-cultures in the future will help understand how IGF-1 signaling may influence the disease progression.

DISCUSSION

Previous mouse studies have implicated a potential glial contribution to the disease progression of RTT (8–10). However, it is critical to validate observations made in Mecp2 null mice in human cells with actual RTT mutations because significant differences in biology exist between mice and humans. In addition, the complete removal of a gene in mouse is not always the same as expressing a mutant gene in human. In the current study, we have efficiently differentiated RTT iPSCs into GFAP+ astrocytes, demonstrated that wild-type human astrocytes express detectable levels of MECP2, and that mutant human astrocytes carrying three different RTT mutations have an adverse influence on the morphology and function of wild-type neurons. The phenotypes of small neuron, shorter total neurite length and fewer terminal ends observed in our study are consistent with the hallmark pathologies observed in RTT human and fewer terminal ends observed in our study are consistent with the hallmark pathologies observed in RTT human autopsy samples (20) and in RTT mouse models (21–24). With this agreement between our human data and the previous mouse data, there should be little doubt left that astrocytes indeed express MECP2 and that loss of MECP2 in astrocytes contributes to neuronal abnormalities in addition to cell-autonomous dysfunctions caused by MECP2 deficiency in neurons. This conclusion has important implications for developing treatments for RTT, as strategies aimed at neutral defects in the absence of astrocytes may not work efficiently if glial defects are left untreated.

Since the initial discovery, the iPSC technology has been expected to become a powerful tool in modeling human diseases. Our study adds to the growing number of examples that demonstrate cell types of interest differentiated from patient-specific iPSCs may recapitulate certain disease phenotypes and therefore provides a valid in vitro model to study disease mechanisms. In spite of the obvious variation for all three morphological features from neurons exposed to the three wild-type astrocytes, our genotype-dependent phenotypes are still readily detectable when comparing the effects of wild-type and mutant astrocytes within an isogenic pair. Thus, our study suggests that, as long as the XCI status in female iPSC lines and their derivatives is continuously and carefully monitored, using isogenic controls will significantly increase the sensitivity and reliability in identifying characteristic disease pathologies. Moreover, because our results are mostly consistent among astrocytes carrying three different RTT mutations, they strongly suggest that the phenotypes observed in our study were caused by mutations in the MECP2 gene, but not due to variations inherent to the reprogramming process or the in vitro culture/differentiation of the iPSCs and their derivatives. As for the few phenotypic inconsistencies observed across different isogenic pairs, they could be either real differences caused by different RTT mutations or inherent experimental variations. Future studies involving human astrocytes differentiated from additional RTT iPSC lines, either carrying the same or other RTT mutations, will help distinguish these possibilities.

Because the negative influence of mutant RTT astrocytes detected in the co-culture experiments is largely recapitulated in the ACM culture, cell–cell contact independent mechanisms, namely factors secreted by astrocytes, are at least partially responsible for the observed phenotypes. The negative effect on neuronal growth could be caused by either the presence of growth-inhibiting factors or the absence of growth-promoting factors in the medium conditioned by mutant RTT astrocytes. Future identification of these factors may facilitate the development of treatments for RTT patients. Moreover, it will be interesting to test directly whether exogenous IGF-1 can antagonize the negative effect on neuronal morphology caused by ACM from mutant astrocytes.

By systematically comparing the same morphological features across all four possible combinations of interneurons and astrocytes differentiated from the same isogenic pair of RTT iPSCs, we were able to not only distinguish the contribution from each cell type to the same phenotype, but also to determine that the contributions were additive. Such a clear understanding would have been difficult to achieve from studying the intact brain wherein all four types of interneuron–astrocyte interactions are intermingled. However, it should be noted that progenitors are present in our GABAergic neuron/astrocyte co-culture and could potentially influence the phenotypic outcomes. After technology become available to purify GABAergic neurons without compromising their health, it will be necessary to revisit this experiment to conclusively rule out the effect of progenitors and other cell types. In contrast to the clear-cut results from the untreated conditions, the IGF-1/GPE treatment effects are much more complex. On one hand, consistent with their general function as growth factors, both IGF-1 and GPE had positive effects on neuronal soma size (which appeared not affected by the R294X mutation in this specific neuronal type in the absence of IGF-1/GPE) in almost all co-culture combinations. On the other hand, when the total neurite length was compared, the direction of effects of both treatments showed a strong dependency on the genotype of astrocyte. One potential explanation is that, as suggested by the punctate localization pattern, functional IGF-1Rs are only present on the cell surface of astrocytes in our co-culture system. Thus, the IGF-1 treatment indirectly influences neurons through direct effects on the astrocytes. Although the transcript level of IGF-1R is significantly decreased in mutant astrocytes, it alone cannot fully explain our observation. Alternatively, the opposite treatment effects could be caused by inherent variations in the assay, the choice of drug dosage and treatment time, the maturation status of the interneurons and astrocytes, or a combination of these factors. Although more detailed studies are certainly necessary to distinguish these possibilities, our results nonetheless provide a cautionary note that the outcome of IGF-1 therapies may be complex at the cellular level.

MATERIALS AND METHODS

Media composition for all tissue culture

Pluripotent stem cell media (PSCM): DMEM/F12 with 20% KOSR, 1 × NEAA, 1 × pen/strep, and 1 mM l-glutamine (all...
from Invitrogen) and 0.1 mM β-mercaptoethanol (Sigma). Neuronal media (NM): DMEM/F12 with 1% N2, 1× NEAA, 1× pen/strep and 2 μg/ml heparin (Sigma H3149). Supplemented with SHH (1000 ng/ml; 1845-SH; R&D System) or purmorphamine and 2% B27, 0.5% glutamax and 1× pen strep (all from Invitrogen) which was supplemented with 100 ng/ml IGF-1 (PeproTech) or 300 ng/ml GPE (Bachem) where noted.

Maintenance of iPSC and hESC cultures
RTT patient-specific iPSCs used in this study have been previously described in (12). iPSC and hESCs were propagated with PSCM supplemented with 4 ng/ml recombinant human FGF (Waisman Center Biomanufacturing) and passage with 1 unit/ml dispase (Invitrogen) upon a layer of irradiated mouse embryonic fibroblasts (WiCell Research Institute). iPSC lines at the following passage numbers were used to derive the astroglial progenitor lines presented in this study: passage 27 (P27) and P21 for V247X-WT, P23 and P25 for V247X-MT, P13-14 for R294X-WT, P25 and P39 for R294X-MT, P34 for R306C-WT and P38 for R306C-MT.

Astroglial differentiation
Astroglial progenitors were differentiated from iPSCs as previously described (14). Briefly, RTT iPSCs and hESCs(H9) were first differentiated to neural progenitors. At Day 14 of differentiation, adherent neural progenitors were expanded in a suspension culture with NM. On Day 21, media was switched to AM. These neural progenitor spheres were propagated first by trituration with a flamed polished Pasteur pipette (Days 21–90) followed by dissociation with Accutase (Innovative Cell Technologies), resulting in a monolayer culture that formed astroglial progenitor spheres. Terminal differentiation into astrocytes from astroglial progenitors was achieved by dissociation of progenitors into single cell with Accutase, and plating at a density of 2 × 10⁴ cells per coverslip in ADM with 10% FBS (Gibco). The following day, fresh ADM without FBS was added. Differentiated progenitors were then fed every other day and used for experimentation 7 days after the start of terminal differentiation. Several astroglial progenitor lines were derived from each isogenic iPSC pair to verify the similarity in astroglial specification between wild-type and mutant RTT lines.

Neuronal differentiation
The hESC line H9 at P41–P47 was used for forebrain neuron differentiation. hESCs(H9) were differentiated to neurons as previously described in (25). Day 21 neurospheres were subjected to a differentiation. hESCs (H9) were differentiated to neurons as previously described (25). Day 21 neurospheres were subjected to a differentiation. hESCs (H9) were differentiated to neurons as previously described (25). Day 21 neurospheres were subjected to a differentiation. hESCs (H9) were differentiated to neurons as previously described (25). Day 21 neurospheres were subjected to a differentiation. hESCs (H9) were differentiated to neurons as previously described (25). Day 21 neurospheres were subjected to a differentiation. hESCs (H9) were differentiated to neurons as previously described (25). Day 21 neurospheres were subjected to a differentiation. hESCs (H9) were differentiated to neurons as previously described (25). Day 21 neurospheres were subjected to a differentiation. hESCs (H9) were differentiated to neurons as previously described (25). Day 21 neurospheres were subjected to a differentiation. hESCs (H9) were differentiated to neurons as previously described (25). Day 21 neurospheres were subjected to a differentiation. hESCs (H9) were differentiated to neurons as previously described (25). Day 21 neurospheres were subjected to a differentiation. hESCs (H9) were differentiated to neurons as previously described (25). Day 21 neurospheres were subjected to a differentiation.
dilutions were as follows: Alexa Fluor 568 Donkey-anti-Rabbit antibody (Invitrogen A10042, 1:500), Alexa Fluor 488 Donkey-anti-mouse antibody (Invitrogen A21202, 1:500) and Alexa Fluor 647 Donkey-anti-mouse antibody. DAPI was used at 3 nM for counterstaining.

**Stereology**

Terminally differentiated astroglia were immunostained with anti-GFAP antibody and counterstained with DAPI. Mouse hippocampal neuron/human astrocyte co-cultures were stained with anti-Iba1 antibody and counterstained with DAPI. Cells were viewed with a Zeiss photomicroscope at ×40. Stereology was performed with the Stereo Investigator software (MicroBrightField). At least two replicates of terminal differentiation were performed for each astroglial line for the quantification of GFAP+ cells. Performed for both the GFAP and Iba1 counting, the optimal number of counting sites was determined empirically based upon a Scheaffer CE value of <0.30. Results were quantified as the percentage of either GFAP+ or Iba1+ cells of the total DAPI+ cell count.

**Western blot analysis**

One million astroglial progenitors were terminally differentiated for 1 week and collected. Nuclear extract from each culture was prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). Nuclear extracts were resolved on 10% SDS–PAGE and transferred onto Protran BA85 nitrocellulose membranes (Whatman). The membrane was blotted with anti-MeCP2 antibody (either 1:4000, Diagenode or 1:2000, Abcam) and infrared dye-conjugated secondary antibody (1:10 000, Thermo Scientific) and was scanned with the Odyssey infrared imaging system.

**Morphological analysis of neurons co-cultured with RTT iPSC-derived astrocytes or cultured in ACM**

H9 neuron and P0 hippocampal cultures were stained with anti-MAP2 antibody while IN co-cultures were stained with anti-GABA and anti-MAP2 antibodies. All samples were imaged utilizing an Olympus BX51 microscope. For IN co-cultures, only GABA+ cells were included in analysis. MAP2-labeled neuronal soma and neurites were traced using Neurolucida (MicroBrightField) and analyzed with Neurolucida Explorer (MicroBrightField). The staining, imaging and tracing steps were performed by different researchers in the triple blind manner.

**Synapse density analysis**

The density of synapses in the 8-day mouse hippocampal neuron/human astrocyte co-culture was analyzed as previously described (26). Briefly, vGluT1 and PSD95 images were thresholded using constant settings for each experiment and converted to binary images. Thresholds were chosen such that all recognizable punctate structures were included into the analysis. vGluT1 and PSD95 puncta were identified by the “partial analysis” function in ImageJ, with the minimal size 0.1 μm² for both vGluT1 and PSD95. vGluT1 puncta with overlap or directly adjacent PSD95 puncta were scored as co-localized vGluT1/PSD95 puncta. Puncta density of primary dendrites was measured as the puncta number per micrometer of dendritic branch as labeled by MAP2.

**Allele-specific transcription of MECP2 and XIST**

Genotyping was performed for iPSC and astroglial lines as previously described (12). RNA was prepared using the SV Total RNA Isolation Kit (Promega). cDNA was made using the qScript cDNA SuperMix (Quanta Biosciences). Primers: forward GCAAAGCAGAGACATGAGAA, reverse 1 (for V247X) CAGATCGGATAGAAGACTCC, reverse 2 (for R294X, R306C) GCCAGGGCTCTTACAGGTC. XIST SNP assay was performed as previously described (12). Purified PCR products were directly sequenced.

**qRT-PCR transcription analysis**

One million astroglial progenitors were differentiated for 1 week and collected. Other cell types were collected at a density to obtain ~1 μg total RNA. cDNA was prepared as described above. At least two separate astroglial differentiations were used for transcriptional analysis. GAPDH was used as a normalization control. Primers were as follows: OCT4 (accession number: NM_002701), forward: 5′-CAGTGCCGAAACCACAC-3′, reverse: 5′-GGAGACCCAGCAGCCTCAA-3′; NFIX (accession number: NM_002501), forward: 5′-ATGTACCCTCCGTACTGCTC-3′, reverse: 5′-ACATCCGCTTTTCTGCTTTCT-3′; GLT-1 (accession number: NM_004171), forward: 5′-ATCTTTGCTGCTAGGAACCC-3′, reverse: 5′-CAGGATGACACAAACACC-3′; AQP4 (accession number: NM_001650), forward: 5′-GTGGGTAAGTGGACTTTT-3′, reverse: 5′-GCCAGAAAATTCGCTGTGAC-3′; IGF-1R (accession number: NM_00875), forward: 5′-GCCACCATCTTCAAGGGCAATTT-3′, reverse: 5′-GGAAAGACAGGGAGACCAAGG-3′; GAPDH (accession number: NM_002046), forward: 5′-GTGGACCTGACCTGCCGTCT-3′, reverse: 5′-GGAGAGTGGGTGTCGCTGT-3′.

**Electrophysiology**

Dissociated hippocampal neurons were isolated from P0–P1 C57BL/6 pups and seeded upon astrocytes differentiated from the R306C RTT iPSC isogenic pair. Whole-cell patch-clamp recording of hippocampal neurons was performed on Day 8 of the co-culture at ambient temperature. Coverslips with the cultured hippocampal neurons were put into a chamber which was continuously perfused with external recording solution containing (in mM) 140 NaCl, 3 KCl, 15 HEPES, 1 MgCl₂, 2 CaCl₂, 20 glucose (pH 7.4, 300 ± 5 mmol/kg). 1 μM TTX and 10 μM bicuculline were added to the bath solution during the whole recording process. The patch pipette (3–5 MΩ) solution contained (in mM) 140 K-glutamate, 7.5 KCl, 10 HEPES-K, 0.5 EGTA-K, 4 Mg-ATP and 0.3 Li-GTP (pH 7.4, 290 ± 5 mmol/kg). Hippocampal neurons were visualized with an Olympus Optical (Tokyo, Japan) BX51WI microscope. The neurons were held at −70 mV. Whole-cell capacitance compensation was applied. The mEPSCs recording started 3 min after the whole-cell voltage clamp configuration was established and remained stable.
lasted for 6 min on a gap free mode. Recordings with series resistance ($R_s > 20$ MΩ) were excluded. Raw data were amplified with a Multiclamp 700B amplifier, acquired with pClamp10.2 software (Molecular Devices, Sunnyvale, CA, USA). Signals were filtered at 2 Hz and sampled at 10 kHz by Digidata 1440A (Molecular Devices). Digital Gaussian filter (1 kHz) was applied and mEPSCs were analyzed using the Template Search tool of the Clampfit10.2 software (Molecular Devices). The mEPSC events were accepted manually. All group data were filtered at 2 Hz and sampled at 10 kHz by Digidata software (Molecular Devices, Sunnyvale, CA, USA).

**Statistical analyses**

For neuronal morphological measures in the RTT interneuron/astrocyte co-culture with no treatment (Fig. 5A), a two-way ANOVA analysis was performed first, followed by post hoc pairwise comparison between groups of interest. The outputs from SAS 9.3 for between-subject effects are presented in Supplementary Material, Table S1. For neuronal morphological measures in the RTT interneuron/astrocyte co-culture with IGF-1/GPE treatment (Fig. 5B–D), a three-way ANOVA analysis was performed first, followed by post hoc pairwise comparison between groups of interest. The outputs from SAS 9.3 for between-subject effects are presented in Supplementary Material, Table S2. For all other experiments in this study, Student’s two tailed t-tests were used for pairwise comparisons unless stated otherwise.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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