Specific Aims

Rett Syndrome (RTT) is an X-linked neurological disorder considered to be a part of the Autism Spectrum Disorder (ASD) and is caused by a mutation in the gene encoding the MeCP2 protein[1]. The exact contribution of MeCP2 to the RTT phenotype is unknown; however, it is hypothesized to be involved in transcriptional regulation[2,3].

Induced pluripotent stem cell (iPSC) technologies in the Moutri Lab offer a unique opportunity to investigate RTT[4]. For example, in previous studies, human stem cell models of the neurological development of the condition using iPSCs from RTT patients revealed that there is reduced synapse number and altered neuronal morphology[5]. Moreover, in MeCP2-knockout mice, astrocytes and oligodendrocytes seemed to play a critical role in the pathogenesis of RTT phenotypes[5]. However, while *in vitro* RTT modeling is a powerful tool for diagnosis and drug screening, the underlying causes and drivers of RTT have not been fully determined.

Fortunately, the Moutri lab's novel technology of synthesizing brain organoids allows us the ability to observe RTT in a human neurodevelopmental model. Brain organoids are three-dimensional models of fetal human brain development that are created using iPSCs from patients. Advantages of this 3D model include a better anatomical organization of the brain tissue and the dynamic development of cell types and neuronal maturation.This opportunity to investigate underlying causes and potential treatments for RTT in a more accurate human model provides promising possibilities.

In previous studies, human iPSC-derived glial-restricted neural progenitor cells (GRNPCs) have been shown to improve the outcomes of mouse modeled congenital hypomyelination[6], Huntington Disease[7], and Schizophrenia[8]. Moreover, there is evidence that glia has a crucial role in synaptic and cognitive impairments in neurological diseases[9]. Furthermore, preliminary data provide evidence that, *in vivo*, transgenic re-expression of MeCP2 in astrocytes partially rescues multiple known RTT phenotypes[10]. To follow suit, we plan to test whether these findings can be replicable using brain organoids.

Thus, we propose to transplant GRNPCs as a treatment for RTT using human brain organoid and mouse models. These models will receive a single injection of progenitor cells and be evaluated for efficacy.

Aim 1: Determine the impact of human GRNPCs in MeCP2-deficient brain organoids.

Aim 2: Determine the impact of human GRNPCs in MeCP2-deficient mouse models.

Background and Significance

People with RTT undergo regression during their first years of life such as losing the ability to speak and use their hands[11]. In addition, autistic characteristics, such as antisocial tendencies and difficulty of eye contact, can be very severe. Since the behavioral features of RTT develop around the same time as and share phenotype similarity to Autism Spectrum Disorder, it suggests that both disorders share a common biological etiology. Mouse models of RTT reproduce many of the clinical symptoms observed in people with RTT which has led to many insights about the condition[12]. However, there are numerous interspecies differences that result in large gaps of knowledge about unique aspects of the condition in humans. The Moutri Lab was the first to create a human model for RTT. These models have previously shown[5,13] that RTT-derived neurons have several phenotypes, including morphology alterations (i.e., reduced branching and smaller soma size) and functional deficits (lower number of excitatory synapses, abnormal calcium transients and decreased postsynaptic current frequency).

The effect of astrocytes carrying MeCP2 mutations was first described *in vitro*[14], then *in vivo*[10], and recently from human cells[15]. However, how MeCP2 regulates molecular mechanisms in astrocytes is still unknown. Using RTT-iPSCs to re-create cultures of human neurons and astrocytes in a dish, we can observe the contribution of astrocytes to neuronal function. In addition, we will use a novel protocol to create human glial-restricted neural progenitor cells (GRNPCs) from iPSCs in order to study the relationships between astrocytes and neurons *in vitro*. Healthy neurons co-cultured atop healthy astrocytes show dendritic arborization, a feature that was lost when the neurons were co-cultured with RTT-derived astrocytes. Interestingly, RTT neurons co-cultured with healthy astrocytes reverted those neuronal alterations which indicates the possibility that transplantation of healthy astrocytes can be a therapeutic treatment for RTT. Therefore, we propose to perform a series of experiments to support the idea that transplant of glial progenitor cells, derived from human pluripotent stem cells, can rescue abnormal neuronal morphology and activity in both RTT human and mouse models. The idea that cell transplants could cure or relieve RTT symptoms has never been tested before (**Fig 1**). Our hypothesis is supported by preliminary data and aims to prove that contribution from healthy astrocytes could benefit MeCP2-mutant neurons.

Fig. 1. A proposed roadmap for RTT. The use of cell transplantation for a neurodevelopmental disorder has never been proposed before. In this schematic roadmap, stem cells are placed as a resource tool (modified from RSRT).

Preliminary Results

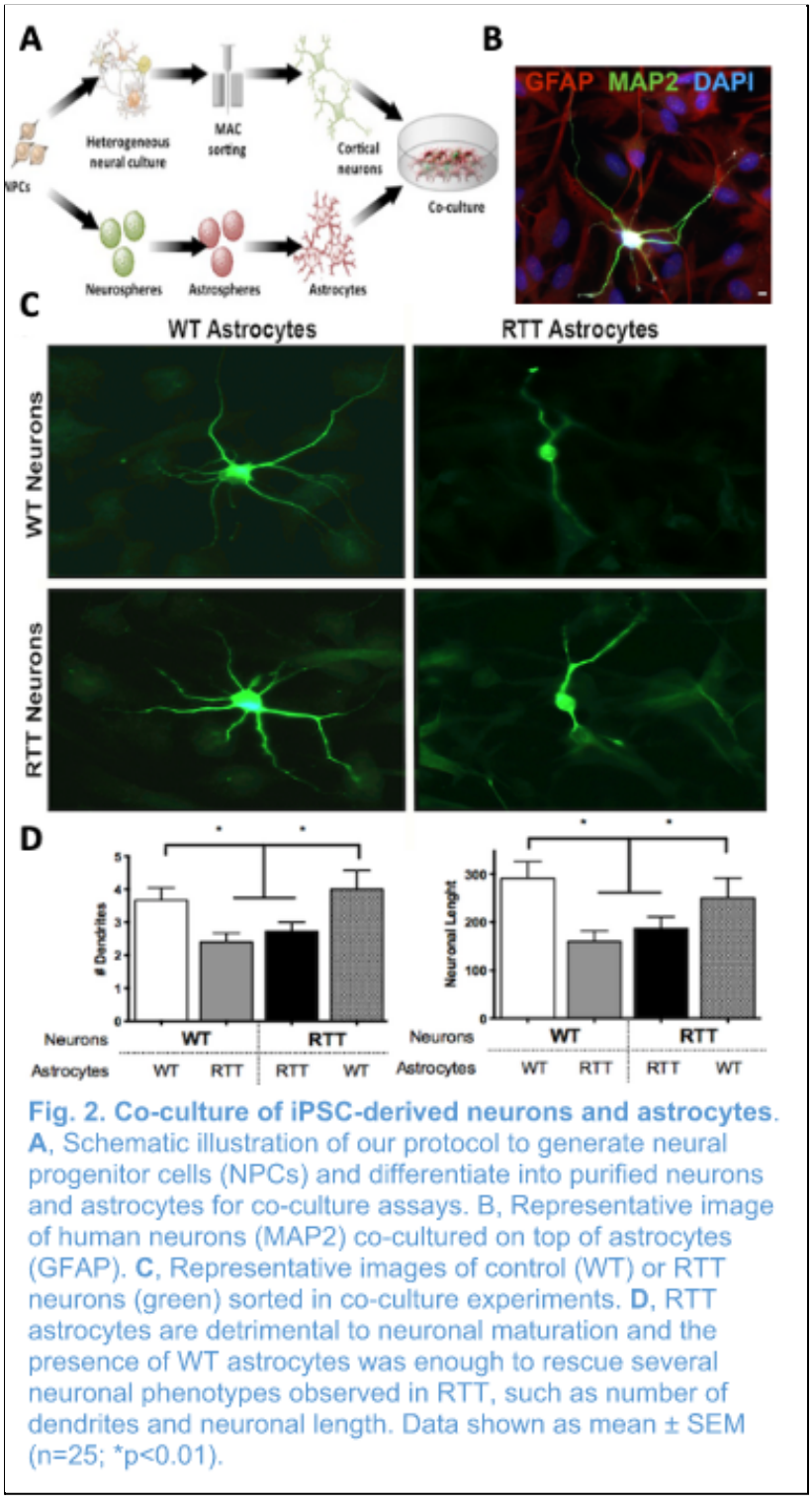

Fig. 2. Co-culture of iPSC-derived neurons and astrocytes. **A**, Schematic illustration of our protocol to generate neural progenitor cells (NPCs) and differentiate into purified neurons and astrocytes for co-culture assays. B, Representative image of human neurons (MAP2) co-cultured on top of astrocytes (GFAP). **C**, Representative images of control (WT) or RTT neurons (green) sorted in co-culture experiments. **D**, RTT astrocytes are detrimental to neuronal maturation and the presence of WT astrocytes was enough to rescue several neuronal phenotypes observed in RTT, such as number of dendrites and neuronal length. Data shown as mean ± SEM (n=25; *p<0.01).

Astrocytes from non-affected individuals can rescue RTT neurons.

Based on our previous data, we hypothesized that RTT astrocytes would negatively impact co-cultured human neurons, while non-mutant, control astrocytes may help RTT neuronal functions. We plated iPSC-derived neurons on top of a monolayer of astrocytes (positive labeling for GFAP) (**Fig. 2A, B)**. The neuronal characterization was based on dendritic length, dendritic spine number, spine density, soma area, tree number and segments. It was shown that RTT astrocytes can dramatically affect the morphology of control neurons. In contrast, by plating RTT-derived neurons with control astrocytes, we were able to rescue most of these morphological phenotypes (**Fig. 2C, D**).

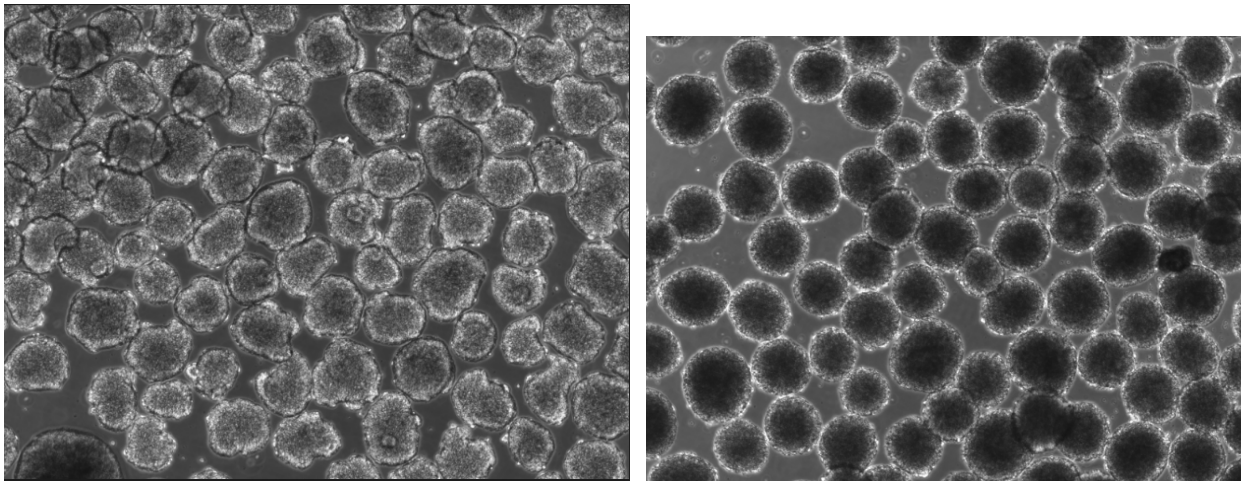

Fig. 3. Human astrocytes (left) and cortical organoids (right)

Brain Organoids lacking MeCP2.

We knocked out the MeCP2 gene in one of our control iPSC lines using CRISPR/Cas9 and used these cell lines to create brain organoids, a three-dimensional human neurodevelopmental model that closely resembles aspects of human fetal neurodevelopment. We confirmed the morphological alterations in neuronal cells (soma size, dendritic arborization, etc), and also observed defects in neural networks (lower number of excitatory synapses, abnormal calcium transients, etc).

GRNPCs transplantation in mouse brains.

As proof that our progenitor cells can integrate, migrate, differentiate and survive in the mouse brain for long periods, 80,000 GRNPCs were transplanted via bilateral injection into the corpus callosum of newborn mouse brains. At 3 months of age, transplanted mice were anesthetized with isoflurane, then perfused with cold PBS. Brains were

extracted and fixed for 24 hours in cold 4% paraformaldehyde. Our initial experiments with wild-type mice indicated that transplanted cells could successfully differentiate into astrocytes and be found widespread in the mouse brain after about 3 months. Similarly to what was previously described, we observed that human astrocytes outcompete the mouse astrocytes *in vivo* over time, taking over almost the entire mouse brain[16]. A human-specific GFAP antibody was used to visualize human cells in the mouse brain (**Fig. 4**). We did not detect any other cell fates (NPCs, neurons, microglia or oligodendrocytes) from the transplantation experiments after a month (data not shown).

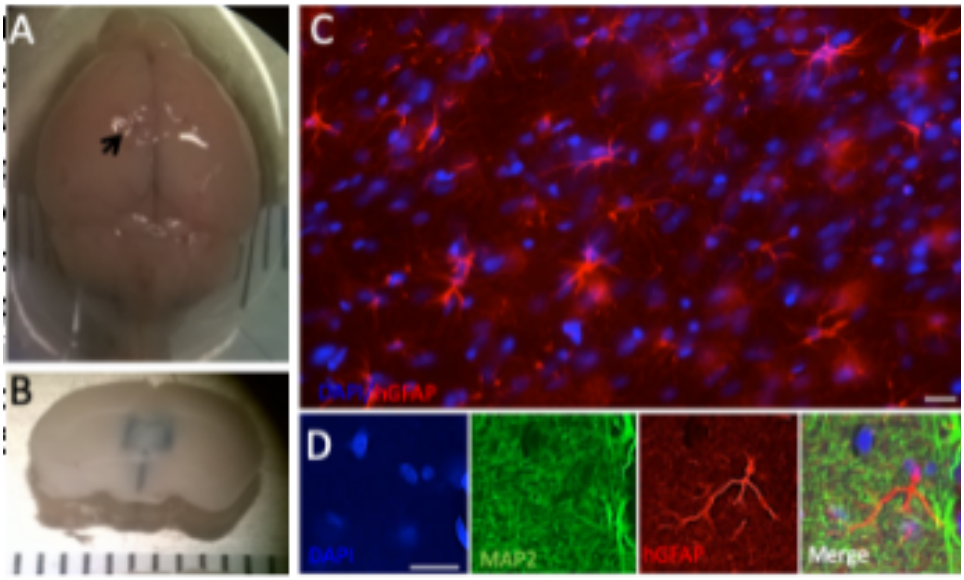

Fig. 4. Human GFAP-positive astrocytes transplanted into mouse brains. A, Isolated adult mouse brain with intact anatomy 3 months after cell transplantation. Arrow point to the original site of injection. **B**, Distribution of the transplanted cells visualized with Trypan blue dye, 15 minutes after surgery showing correct injection site localization. **C**, Widespread human astrocytes after 1-month post-transplantation of GRNPCs. **D**, Detailed human astrocyte morphology in the mouse brain.

GRNPCs transplantation in brain organoids.

To show that our GRNPCs can also integrate, proliferate, and differentiate in a human brain development, we added 20,000 dyed GRNPCs to single unaffected (WT) and MeCP2 KO brain organoids at 1 month of age. At this stage, brain organoids are composed mainly of NPCs and neurons[17]. Therefore, all astrocytes present are from transplants. 30 days after transplantation, the brain organoids were fixed in cold 4% paraformaldehyde for 48 hours and sectioned for immunohistochemical analysis. Our

preliminary results show successful survival of GRNPCs. The transplanted cells were also able to integrate and differentiate into mature astrocytes (**Fig. 5**).

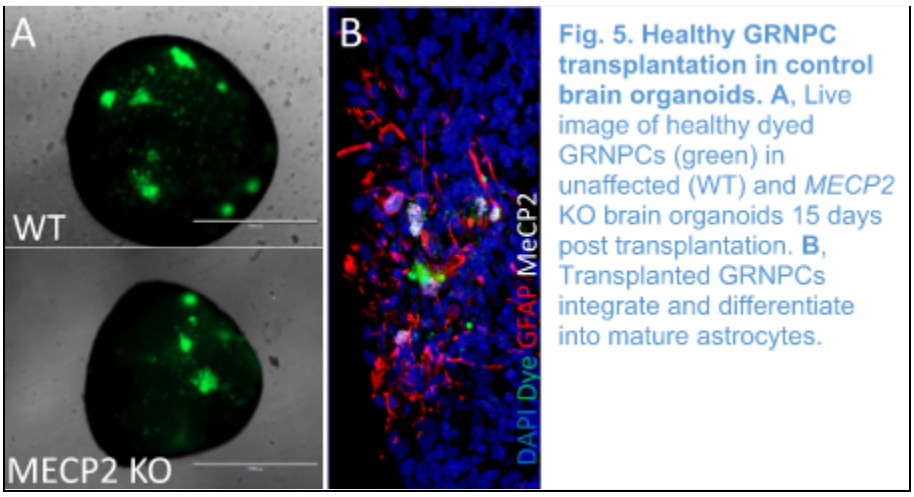

Fig. 5. Healthy GRNPC transplantation in control brain organoids. A, Live image of healthy dyed GRNPCs (green) in unaffected (WT) and *MECP2* KO brain organoids 15 days post transplantation. **B**, Transplanted GRNPCs integrate and differentiate into mature astrocytes.

Research Design and Methods

Our preliminary data clearly showed that healthy astrocytes can positively impact neurons and neural networks composed of MeCP2-mutant cells in co-culture 2D experiments. Human brain organoids are scaled down, 3-dimensional models of the brain that recapture several molecular and cellular aspects of human embryonic and fetal stages[18]. Brain organoids closely mimic the early stages of human neurodevelopment and network formation[17]. MeCP2-mutant brain organoids reproduce the neuronal morphological alterations, reduction of synaptogenesis and network deficiency observed in traditional cell monolayers. Since this model more accurately mimics the human brain environment *in vivo,* we will be employing this technique for cell transplantation.

In this project, we will use CRISPR-generated isogenic hiPSC lines that differ only by the MECP2 mutation[19]. We confirmed the expression of the corrected allele by sanger sequencing and immunoassaying with anti-MeCP2. We will transplant control cells into MECP2-KO brain organoids. We can clearly visualize the identity of the transplanted cells using the anti-MeCP2 antibody and co-localization with other cellular markers. Parallel GRNPCs generated from MECP2-KO isogenic iPSCs will be used as a negative control.

GRNPC Production. Feeder-free control healthy hiPSCs were fed daily with mTeSR1. hiPSCs were passaged when they reached 80% confluence in colonies of 250-300

μm diameter. To generate embryonic bodies (EB), hiPSC cultures were dissociated using Accutase in PBS (1:1) for 10 minutes at 37°C and centrifuged for 3 minutes at 150xg. The EBs were cultured in suspension in 6-well plates in mTeSR1 for 5 days; then switched to SM1 neural induction medium (DMEM/F12 supplemented with Neurocult SM1 Neuronal supplement) with bFGF (20 ng/ml) for 7 days. Thereafter, the EBs were plated onto laminin/poly-ornithine coated 6-well plates and cultured in SM1 media supplemented with bFGF. The plated spheres are then passaged 2-3 times with Accutase onto laminin/poly-ornithine coated 10-cm plates. At this stage, the NPCs (Neural Progenitor Cells) can be continuously passaged or frozen for future use. Once the NPCs reached 80-90% confluency they were dissociated using Accutase for 5 minutes at 37°C and centrifuged for 5 minutes at 150xg. The cell pellet was resuspended in SM1 media supplemented with bFGF (20 ng/ml). Approximately 3x10^6 cells were transferred to one well of a 24-well Aggrewell 800 plate and centrifuged for 3 minutes at 100xg. After 24 hours, the cell aggregates were mechanically lifted via trituration and transferred to one well of a 6-well plate and kept in suspension under rotation (95 rpm) to form free-floating spheres. After 48 hours, the media was changed to SM1 media with 5 mM ROCK inhibitor. After another 48 hours, the media was changed to SM1 media for 10 days, replacing the SM1 media 2-3 times per week. Media was then changed to Astrocyte Growth Media (AGM, Astrocyte Growth Medium Bulletkit) for 2 weeks, changing media 2-3 times per week. Each well of spheres were then plated on laminin/poly-ornithine coated 10cm plates and allowed to grow out onto the plate. After one week, the resulting GRNPCs were dissociated with Accutase for 5 minutes at 37°C and used for transplantation.

Cortical Organoid Co-Culture. Feeder-free hiPSCs were fed daily with mTeSR1 for 7 days. Colonies were dissociated using Accutase in PBS (1:1) for 10 minutes at 37°C and centrifuged for 3 minutes at 150xg. The cell pellet was resuspended in mTeSR1 supplemented with 10 mM SB431542 and 1 mM Dorsomorphin. Approximately 3x10^6 cells were transferred to one well of a 24-well Aggrewell 800 plate and centrifuged for 3 minutes at 100xg. After 24 hours, the cell aggregates were mechanically lifted via trituration and transferred to one well of a 6-well plate and kept in suspension under rotation (95 rpm) in the presence of 5 mM ROCK inhibitor for 24 hours to form free-floating spheres. After 3 days, mTeSR1 was substituted by Media 1 [Neurobasal supplemented with GlutaMAX, 1% Gem21 NeuroPlex, 1% N2 NeuroPlex, 1% NEAA, 1% PS, 10 mM SB and 1 mM Dorso] for 7 days. Then, the cells were maintained in Media 2 [Neurobasal with GlutaMAX, 1% Gem21 NeuroPlex, 1% NEAA and 1% PS] supplemented with 20 ng/mL FGF2 for 7 days, followed by 7 additional days in Media 2 supplemented with 20 ng/mL of FGF2 and 20 ng/mL EGF. Next, cells were transferred to Media 3 [Media 2 supplemented with 10 ng/mL of BDNF, 10 ng/mL of GDNF, 10

ng/mL of NT-3, 200 mM L-ascorbic acid and 1 mM dibutyryl-cAMP to promote maturation, gliogenesis and activity]. After 7 days, cortical organoids were maintained in Media 2 for as long as needed, with media changes every 3-4 days. After 14 days, individual cortical organoids are transferred to single wells of a 96-well round bottom plate in Media 2. 20,000 GRNPCs in single cell suspension (as described in GRNPCs production) are added to each well and the plate is kept under rotation for four days, changing media once after two days. After four days, cortical organoids are transferred back to a 6-well plate for up to two months, changing media every 3-4 days.

Brain Organoid Readouts. *Immunofluorescence and imaging analyses:* brain cortical organoids will be fixed with 4% paraformaldehyde for 48 hours at 4°C. Next, samples will be permeabilized in 1xPBS containing 0.1% (v/v) Triton X-100 for 10 minutes. Fixed cultures and sliced organoids will be incubated with blocking solution for 1 hour [3% Bovine Serum Albumin (BSA); in 1xPBS]. Primary antibodies will be diluted with blocking solution and incubated with sections overnight at 4°C. Sections will then be washed twice with 1xPBS and incubated with the secondary antibody for 30 minutes at room temperature. Secondary antibodies will be used at a 1:1000 dilution. After the 30 minutes incubation, samples will be washed twice (1xPBS), incubated for 5 minutes with fluorescent nuclear DAPI stain (VWR; 1:10000), and mounted with Slow fade gold antifade reagent. Images will be blindly collected using an Axio Observer Z1 Microscope with ApoTome and analyzed with ImageJ software. Neuronal Soma size: we will quantify neuronal soma size by tracing neurons in Neurolucida followed by unbiased counting using the Imaris software. Synaptogenesis: we will quantify the number of synapses by immunostainings of post and pre-synaptic markers (Synapsin1, Psd-95, Homer1 and VGlut1). Network analyses: neural connectivity will be quantified after evoked focal electrical stimulation and burst synchronization, spike frequency and pharmacology after plating brain organoids on MEAs. The total number of bursts and its synchronization patterns data will be extracted by the Mobius software followed by Neuroexplorer to create raster plots.

Specific Aim 2: To determine the impact of human GRNPCs in a MeCP2-deficient mouse model.

Mouse models of RTT reproduce many of the clinical features observed in people with RTT[12], providing a great opportunity to better understand the pathophysiology, define the contribution of specific molecular and cellular changes, and develop therapies for RTT. In order to test the impact of human GRNPCs on RTT phenotypes *in vivo*, we will transplant these cells in the brains of animals with a knockout of MeCP2. Transplanted animals will be subjected to a set of animal physiological and behavioral tests, and a subset will be used for tissue collection. In addition, we will collect fixed

brain tissue to characterize soma size and synaptic puncta, two cellular phenotypes found in RTT mice and also corrected by MeCP2 expression in astrocytes[10].

Neonatal Xenograft in MeCP2-null Mice. Heterozygous Mecp2-null females will be crossed with WT males to generate hemizygous male Mecp2-/y mice. The hiPSC-derived GRNPCs are prepared for transplantation as described for in vitro co-culture. Neonatal pups were transplanted bilaterally in the corpus callosum with a total of 80,000 cells. At 3 months of age, transplanted mice were anesthetized with isoflurane, then perfused with cold PBS. Brains are then extracted and fixed for 24 hours in cold 4% paraformaldehyde.

Behavioral and Physiological Testing. The primary outcome here will be significant effects on survival, with secondary effects on the behavior and physiological measures using a phenotypic score procedure that is common for RTT mouse models[12]. RTT mice have hyperventilation, apneas, and increased breathing irregularity[10, 24-26]. RTT mice are also uncoordinated and hypoactive[24-25]. *Phenotypic Score:* To monitor the specific features of the RTT-like mouse phenotype, we perform observational tests for inertia, gait, hind-limb clasping, tremor, irregular breathing, and poor general condition. Each symptom was scored weekly as absent, present, or severe (scores of 0, 1, and 2, respectively). Each score is then summed to produce an aggregated phenotypic score[27-29] (**Fig 6**). *Weight:* Animals are weighed and assessed weekly. *Blinding:* The experimenter will be blinded to the treatment group. A distinct member of the Muotri lab will determine the treatment of each animal, allocate the animals to a cage, determine the treatment allocation, and prepare the cells for the investigator who will administer the injections and perform all behavioral, physiological, and histological analysis. Data will be analyzed in a blinded fashion. *Randomization:* Cages will be prepared to include animals from different litters. Cages will be randomized to treatment arms using simple randomization based on a random number generator.

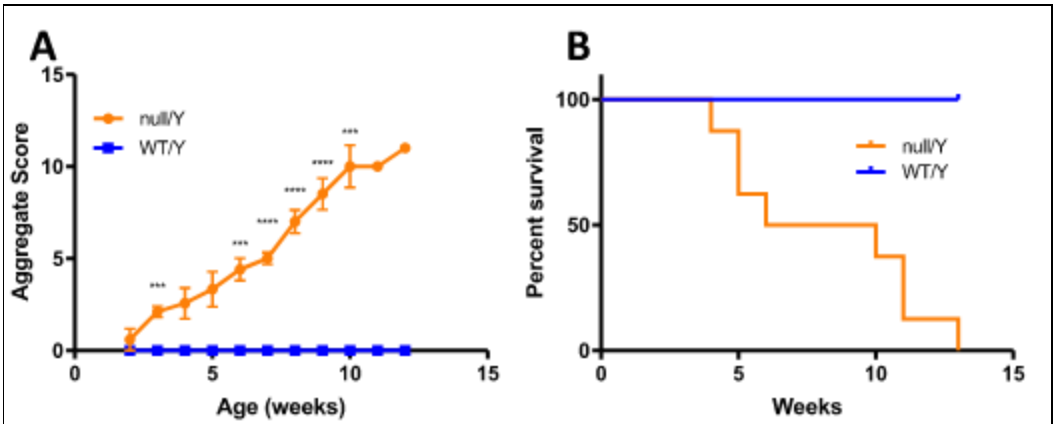

Figure 6. Phenotypic analysis of *Mecp2*-null mice performed in the Muotri lab. A, Aggregated phenotypic score for *Mecp2*-null (Orange, n=9) males, and WT (Blue, n=4) littermates. **B**, Kaplan-Meyer plot showing survival of *Mecp2*-null (Orange, n=8) males compared to WT (Blue, n=6) **littermates**. *P < 0.05, **P < 0.01, ***P < 0.001, ****P<0.0001, unpaired Student's *t*-test.

Histological Characterization. Fixed tissue collected after perfusion will be analyzed. Nissl staining will be used to define soma size in cortical regions. Anti-MeCP2 will be used to track control cells. Synaptic puncta will be identified using sheep anti-VGLUT1, and will be assessed in the hippocampus and cortex.

Results

Astrosphere-Organoid Assembloid Co-culture Fusion. Astroglial populations were derived from neural precursor (NPC) lines (**Fig. 3**), and generated as described (8). NPCs were cultured in adhesion to PO/Laminin plates with medium containing fibroblast growth factor (FGF) for 4-6 weeks. Further differentiation of NPC lines into astrosphere populations was accomplished with the coapplication of EGF and LIF in prolonged cultures which promotes astroglial specification and the selection of NPC colonies with a higher content of astroglial progenitors.

Cortical organoids are derived from mature iPSC cultures undergoing rapid differentiation in proximity wells. At the beginning of differentiation, proliferative neural progenitor cells (NPCs) self-organize into a polarized neuroepithelium-like structure. Over time, the organoids increase in size and proliferate more mature neurons to develop into concentric multilayer structures composed of NPCs, intermediate progenitors, and lower and upper cortical layer neurons.

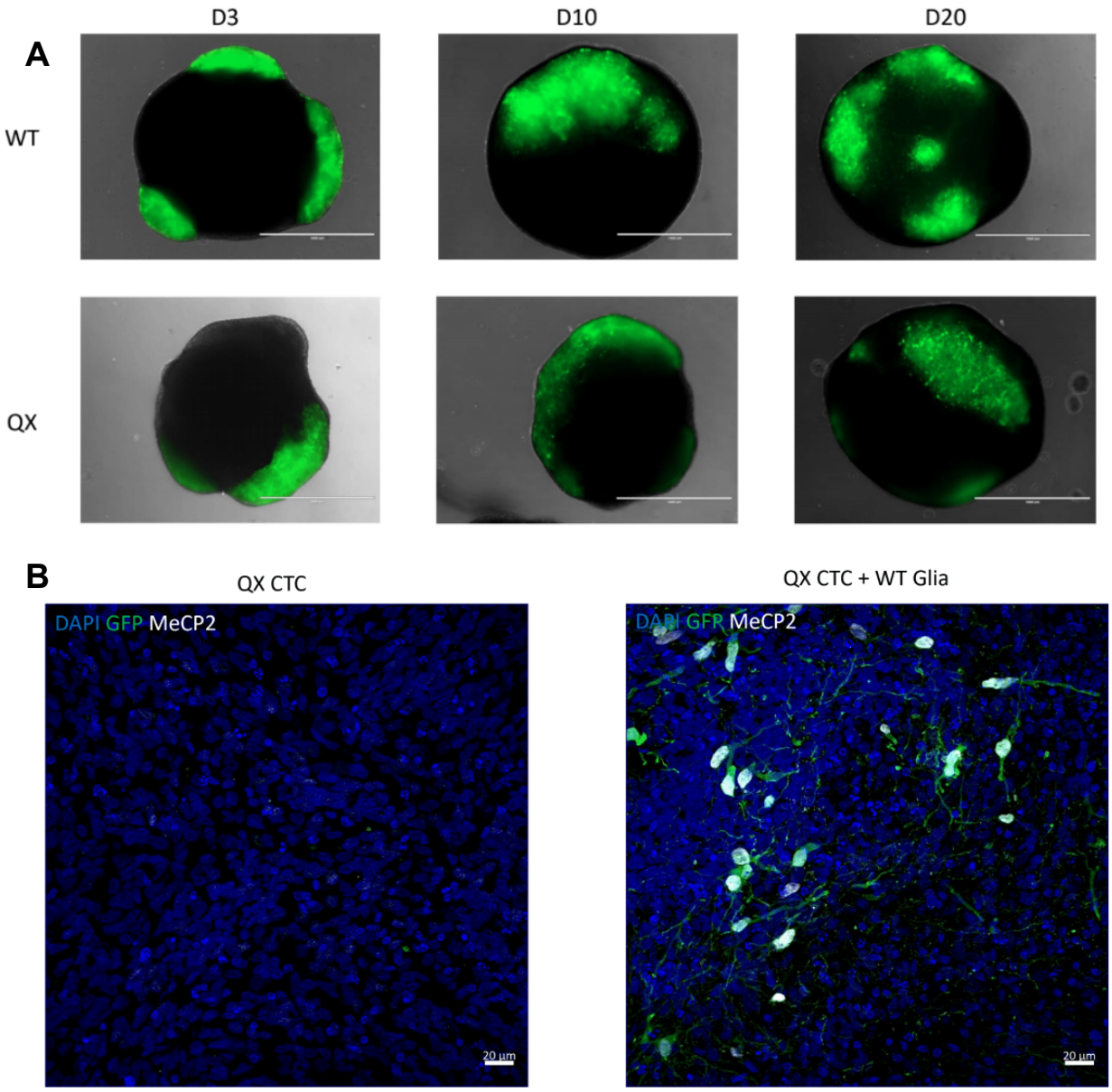

Fig. 7. Assembloid co-culture fusion. A, assembloid fusion of GFP infected astrospheres in QX and WT organoids at Day 3, Day 10, and Day 20 respectively. **B,** 20 um view of QX and QX +WT Glia organoid with GFP infected astropheres, neural progenitor marker (DAPI), and MeCP2 marker.

In a 1.5 mL tube, a D30 cortical organoid is introduced to 4 D20 GFP-infected astrospheres which incubate for 48-72 hours in M2 media until fusion. Astrospheres remain separated from organoid tissue in the early stages and slowly integrate into the tissue over time (**Fig. 7A**). We observed successful fusion of the introduced astrospheres into the cortical organoids by D30. Looking more closely at the tissue (**Fig. 7B**), we further prove that GFP-infected astrospheres effectively dispersed into the organoid tissue.

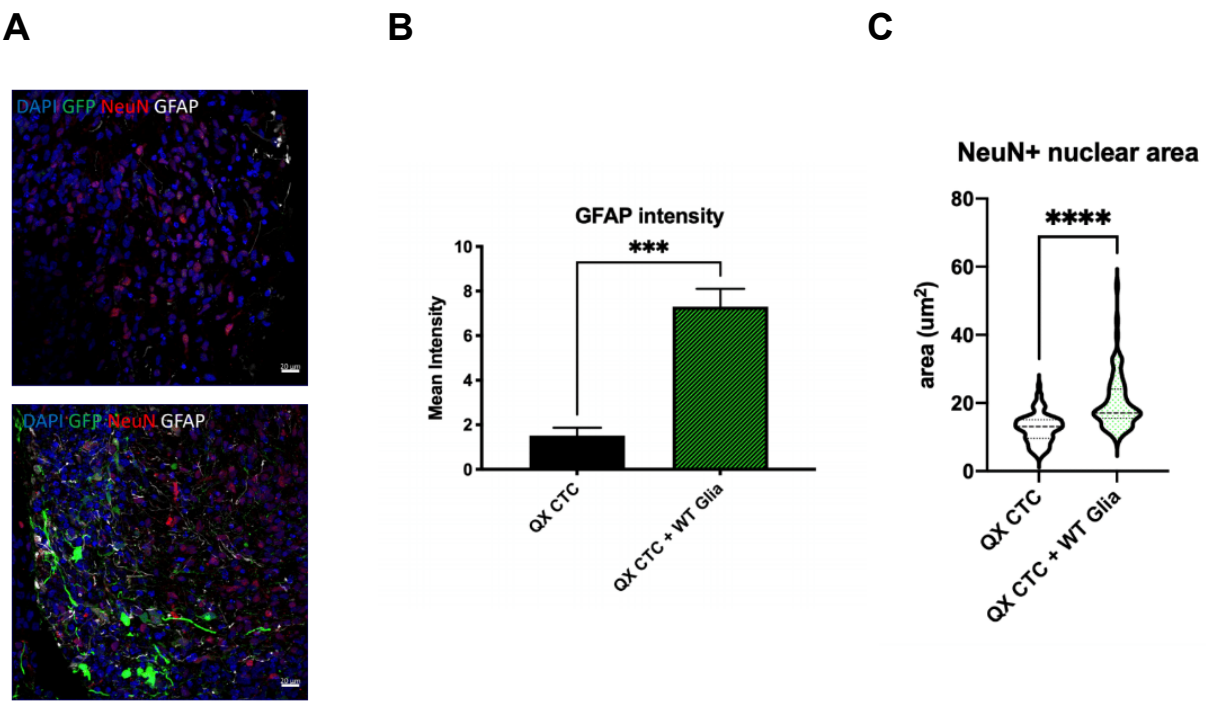

Fig. 8. GFAP and NeuN Immunofluorescence. A, 20um view of Qx organoid (top) and Qx + WT Glia organoid (bottom) with GFP infected astropheres, neural progenitor marker (DAPI), nuclear area marker (NeuN), astrocyte marker (GFAP). **B,** Qx + WT Glia organoids showed a significantly higher GFAP mean intensity compared to Qx organoids. **C,** Qx + WT Glia organoids showed a significantly larger NeuN+ nuclear area compared to Qx organoids.

GFAP and NeuN Immunofluorescence. Immunomarkers GFAP and NeuN were released into the tissue of Qx organoids (control) and Qx + WT glia organoids (treatment group) to bind to respective targets in order to visualize these fluorescent-marked molecules under a light microscope (**Fig. 8A**). GFAP tracks the maturation of astrospheres into astrocytes while NeuN dyes the nuclear area of neurons. By imaging the fluorescence dyed nuclear areas of neurons, we can measure the soma size of neurons in Neurolucida. Quantitative analysis revealed a strong significant difference in mean GFAP intensity of Qx + WT glia organoids compared to Qx organoids (**Fig. 8B**). Unbiased counting using the Imaris software showed that there was also a highly significant difference in nuclear area between Qx + WT glia organoids compared to Qx organoids (**Fig. 8C**).

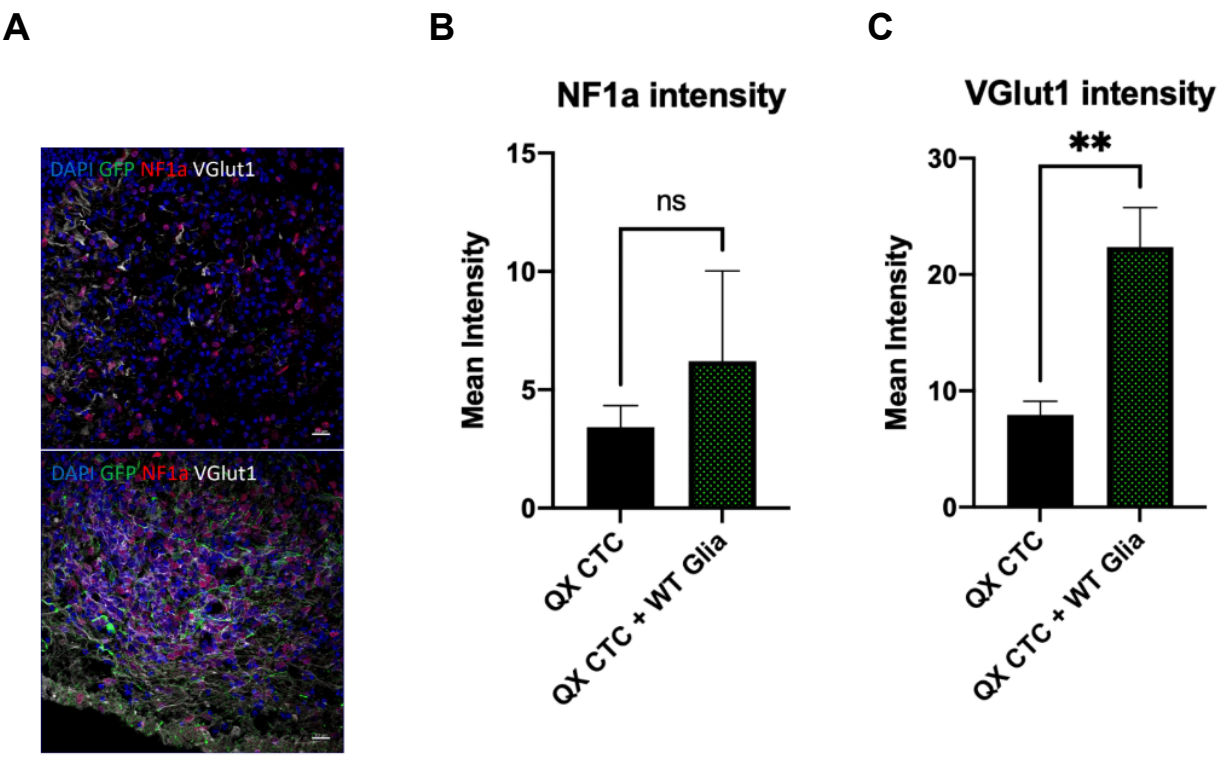

Fig. 9. NF1a and VGlut1 Immunofluorescence. A, 20um view of Qx organoid (top) and Qx + WT Glia organoid (bottom) with GFP infected astropheres, neural progenitor marker (DAPI), gliogenesis marker (NF1a), neurotransmitter docking protein marker (VGlut1). **B,** Qx + WT Glia organoids showed a nonsignificant difference in NF1a mean intensity compared to Qx organoids. **C,** Qx + WT Glia organoids showed a significantly higher VGlut1 mean intensity compared to Qx organoids.

NF1a and VGlut1 Immunofluorescence. We then sought to investigate levels of gliogenesis and presynaptic-postsynaptic activity using immunomarkers NF1a and VGlut1 respectively (**Fig. 9A**). Immunofluorescence analysis showed that there was no significant difference in the NF1a mean intensity levels of Qx + WT glia organoids compared to Qx organoids (**Fig. 9B**). In contrast, there was a significant difference in VGlut1 mean intensity between Qx + WT glia organoids compared to Qx organoids (**Fig. 9C**).

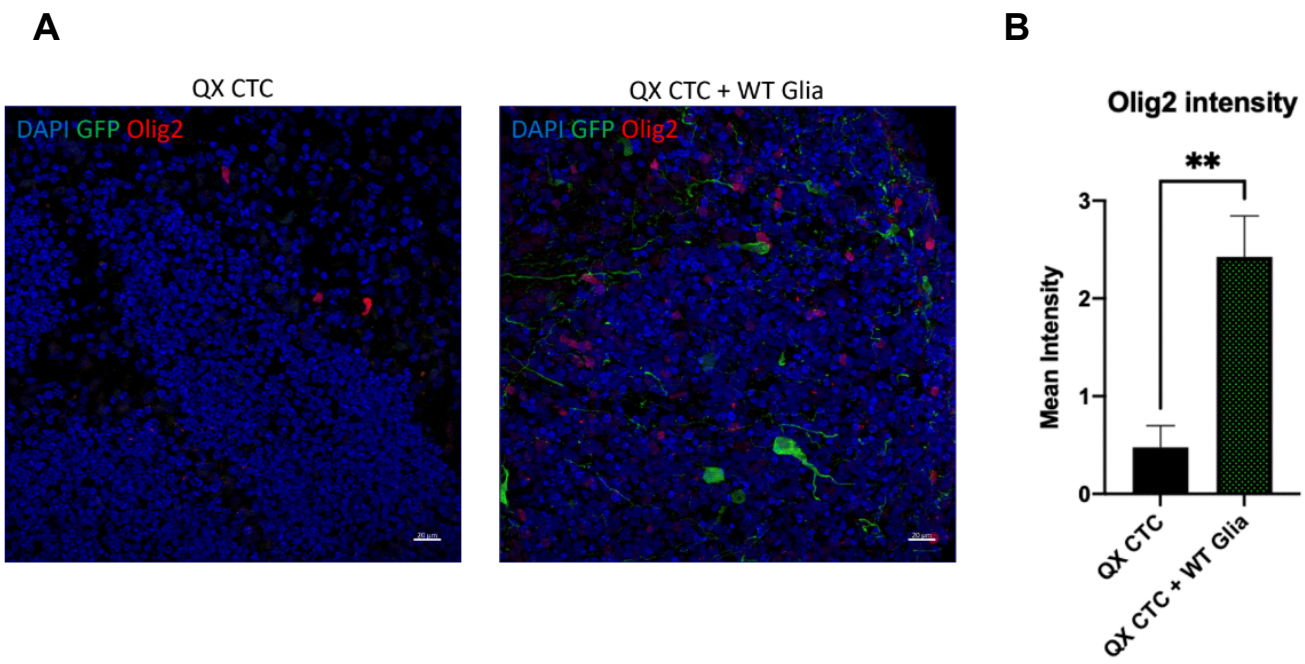

Fig. 10. Olig2 Immunofluorescence. A, 20um view of Qx organoid (left) and Qx + WT Glia organoid (right) with GFP infected astropheres, neural progenitor marker (DAPI), and oligodendrocyte marker (Olig2). **B,** Qx + WT Glia organoids showed a significant difference in Olig2 mean intensity compared to Qx organoids.

Olig2 Immunofluorescence. We next asked whether the fusion of astrospheres into organoids leads to increased proliferation of oligodendrocytes in addition to the growth of astrocytes we have observed previously (**Fig. 8**). We used the oligodendrocyte immunomarker Olig2 to visualize oligodendrocytes in organoids (**Fig. 10A**). Quantitative analysis revealed that there is a significant difference in Olig2 mean intensity of Qx + WT glia organoids compared to Qx organoids (**Fig. 10B**).

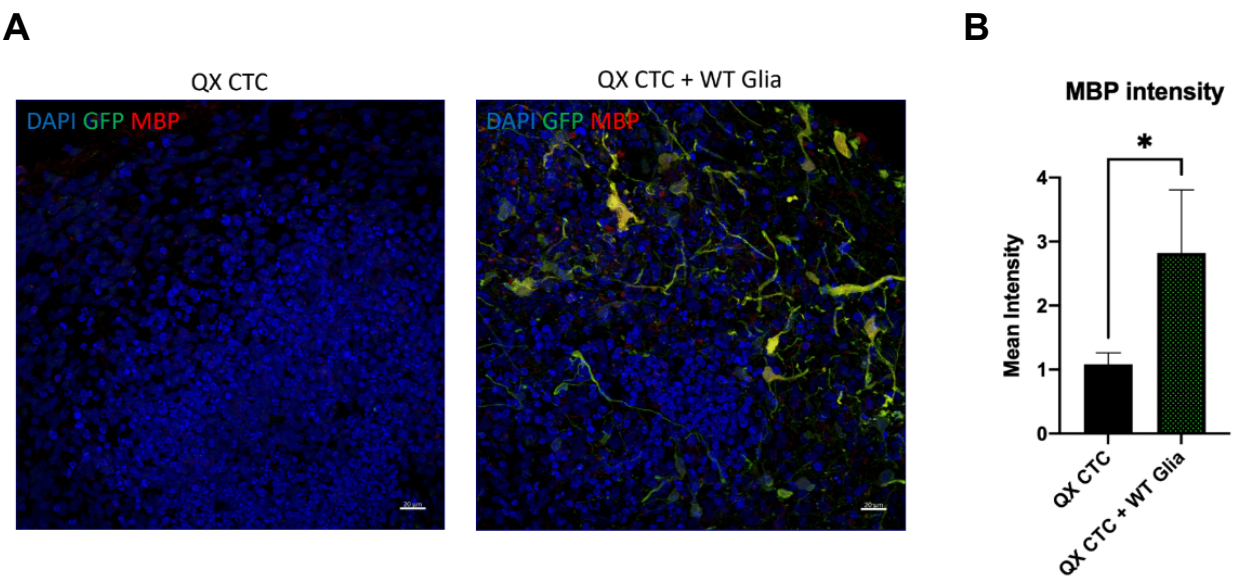

Fig. 11. MBP Immunofluorescence. A, 20um view of Qx organoid (left) and Qx + WT Glia organoid (right) with GFP infected astropheres, neural progenitor marker (DAPI), and myelin marker (MBP). **B,** Qx + WT Glia organoids showed a significant difference in MBP mean intensity compared to Qx organoids.

MBP Immunofluorescence. We next sought to determine whether that increase of oligodendrocytes in Qx + WT glia organoids leads to an increase in myelination of neurons. We used an immunofluorescence marker (MBP) that binds to a protein found in myelin to compare myelination levels in Qx organoids and Qx + WT glia organoids (**Fig. 11A**). Our analysis uncovered that there was a significant difference in MBP mean intensity of Qx + WT glia organoids compared to Qx organoids.

Discussion

Rett Syndrome is a debilitating condition that severely affects the bodily health, well-being, and every day functioning of people with the disorder. Children with Rett Syndrome follow normal development of the brain until approximately 6-18 months of age where development deviates significantly from the norm and regresses. This leaves a large window of time where children diagnosed with RTT can have intervention and treatment to prevent abnormal development before it occurs. Fortunately, the discovery of the causal gene, X-linked transcriptional regulator MeCP2, has fostered significant advancement of medical diagnoses and research of RTT. Infants very early on can be screened specifically for the MeCP2 mutant to determine whether they will have RTT far ahead of the deviation from normal brain development. With this opportunity opened up for prevention, researchers took advantage of MeCP2 KO models to further observe and understand RTT as well as trial new treatments for efficacy.

Previous scientific literature has studied the effects of re-expression of MeCP2 in glia in MeCP2 KO 2D cell cultures and mouse brain models. These studies observed that this re-expression successfully improved RTT neuronal and behavioral phenotypes. These findings display the promise of astrocytes as a potential therapeutic target for treating RTT-associated symptoms. However, these models are not very translatable to a complex human brain. 2D cell assays are lab-controlled and have limited neuronal interactions and development in the tissue. Additionally, mouse models suffer from significant interspecies differences that provide little knowledge of the integration, efficacy, and safety of glial cell transplantation in a human brain. Therefore, we have exploited the novel, robust organoid model that closely mimics the complexity of early fetal brain development to determine whether these results can be reproduced in a 3D human model.

Our study demonstrated that the assembloid protocol successfully integrated WT astrospheres into Qx organoids with images of the dispersion of GFP-expressing astrospheres into the tissue. Heightened levels of GFAP intensity in Qx + WT glia organoids displayed that these astrospheres were able to effectively proliferate and mature into astrocytes. Using nuclear area immunomarker NeuN, we were able to measure the soma size of neurons with NeuroLucida, and subsequent ImageJ quantitative analysis showed that the soma size of neurons in Qx + WT glia organoids were significantly larger. This suggests that the introduction of WT astrospheres improved the RTT pathology of soma size shrinkage. In contrast, the gliogenesis marker NF1a mean intensity showed no significant difference which demonstrated that further generation of astrocytes was not prevalent in the Qx + WT glia organoids. We next sought to determine whether RTT debilitation of synaptic connections and communication was improved by the WT astrospheres. VGlut1 immunofluorescence binds to the docking proteins responsible for successful neurotransmitter release from presynaptic to postsynaptic neurons and serves as an effective synaptic activity marker. The significantly heightened levels of VGlut1 intensity in Qx + WT glia organoids revealed that the introduction of WT astrospheres ameliorated the weak synaptic connections seen in RTT and increased synaptic activity. In addition to astrocytes, we sought to investigate whether there is also an increase in the proliferation of oligodendrocytes, glial cells responsible for more rapid and efficient transmission of signals between neurons. Oligodendrocyte biomarker, Olig2, showed significantly higher levels of mean intensity in Qx + WT glia organoids which suggests that the proliferation of oligodendrocytes was increased in the presence of WT astrospheres which, in turn, improves efficient communication between neurons. To further confirm this data, we verified whether these oligodendrocytes were functioning properly with the immunomarker, MBP. Oligodendrocytes speed up transmission between neurons by insulating the axon fibers in a myelin sheath; therefore, MBP, which binds to a protein

found in myelin, was a fitting choice. Quantitative analysis showed that MBP mean intensity levels were significantly higher in Qx + WT glia organoids which validates that WT astrospheres cause an increase in oligodendrocytes that facilitates better communication and synaptic transmission between neurons.

Our data has shown that the transplantation of healthy human glia has been able to rescue and improve RTT phenotypes of soma shrinkage and decreased synaptic connections and activity. Therefore, we were able to replicate the findings of previous 2D cell tissue and mouse studies that proved that the introduction MeCP2+ astrocytes ameliorates RTT pathology. Our study further validates the potential of glial cell transplantation as an effective therapeutic treatment for RTT by replicating previous results in a model that more closely resembles a human brain.

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