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Systematic Three-Dimensional Coculture Rapidly Recapitulates Interactions between Human Neurons and Astrocytes

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SUMMARY

Human astrocytes network with neurons in dynamic ways that are still poorly defined. Our ability to model this relationship is hampered by the lack of relevant and convenient tools to recapitulate this complex interaction. To address this barrier, we have devised efficient coculture systems utilizing 3D organoid-like spheres, termed asteroids, containing pre-differentiated human pluripotent stem cell (hPSC)-derived astrocytes (hAstros) combined with neurons generated from hPSC-derived neural stem cells (hNeurons) or directly induced via Neurogenin 2 overexpression (iNeurons). Our systematic methods rapidly produce structurally complex hAstros and synapses in high-density coculture with iNeurons in precise numbers, allowing for improved studies of neural circuit function, disease modeling, and drug screening. We conclude that these bioengineered neural circuit model systems are reliable and scalable tools to accurately study aspects of human astrocyte-neuron functional properties while being easily accessible for cell-type-specific manipulations and observations.

INTRODUCTION

Astrocytes have numerous dynamic synapse-modulating and homeostatic functions during development, maturation, and disease (Blanco-Suarez et al., 2016; Verkhratsky and Nedergaard, 2016). While most investigations into these cellular functions have been conducted in rodent models due to ease of experimental access, human astrocytes are known to have unique gene expression, morphological characteristics (Colombo et al., 1997b), and functionality (Oberheim et al., 2009; Zhang et al., 2016) that may have consequences for higher brain circuit processing in healthy and disease conditions (Krencik et al., 2016; Oberheim Bush and Nedergaard, 2017). One strategy to examine human cellular function is to study the interplay between human pluripotent stem cell (hPSC)-derived astrocytes (hAstros) and hPSC-derived neurons (hNeurons). We previously reported a long-term protocol consisting of weekly dissociation of three-dimensional (3D) spheres of hAstros, termed astrospheres, to induce developmental maturation and purity followed by attachment onto two-dimensional (2D) substrates for coculture studies (Krencik et al., 2015; Krencik and Ullian, 2013; Krencik and Zhang, 2011). More recently, organoid approaches have been reported to mature and maintain long-term glial-neuronal interactions over many months within intact 3D spheres (Pasca et al., 2015; Qian et al., 2016; Sloan et al., 2017). Here, we detail an alternative fast and reliable protocol to recapitulate these 3D interactions mirroring the complexity found in human brain. This method has the ease and advantage of allowing precise control of both the numbers and types of human neural cells using identical or different genetic backgrounds, as well as rapidly inducing mature synapse formation.

To investigate human astroglial activity in a more native-like environment, several innovative methods have been recently devised. One approach was to engraft human fetal- or hPSC-derived glial progenitors and hAstros into immunodeficient rodents followed by in vivo and ex vivo analysis (Chen et al., 2015; Han et al., 2013; Krencik et al., 2011; Li et al., 2015). While this technique is advantageous for being able to integrate cells into intact neural networks with measurable behavioral output, there are at least two major limitations. First, the human cellular response in a xenospecies environment may be altered compared with integration within matched species (Goldman, 2016). Second, scalability and optimization of conditions is impractical in chimeric mice due to time, cost, and space limitations. To overcome these issues, many human culture studies have been recently conducted with 3D spheroids or organoids,
as an improved technique over 2D monolayers, to maintain structural integrity and tight cellular interactions (Kelava and Lancaster, 2016; Simian and Bissell, 2017).

Typically in 3D cultures of human neural cells, hAstros appear gradually over a long time period with no precise experimental control over the number or maturity of cells (Pasca et al., 2015; Qian et al., 2016; Sloan et al., 2017). The neuronal population is also not well specified as they contain a mixture of progenitor and mature neuronal subtypes. In an ideal experimental neural coculture system, the ratio of glial to neuronal cells as well as the extracellular milieu should be stable over time to better mimic the long-term nervous system environment during normal and disease conditions. Therefore, we aimed to develop such systematic and repeatable coculture methods to rapidly mimic many aspects of the endogenous cell-cell interactions. This system described here can be applied for studying cell-autonomous versus cell non-autonomous effects by combining cells from different genetic backgrounds or treatment conditions. Furthermore, it can be used as a surrogate for monitoring cellular engraftment of human neural types as a model for cellular replacement therapy.

RESULTS

High-Density Neuronal Coculture Induces Complex Human Astrocyte Morphology

In order to better recapitulate endogenous human astrocyte complexity, we compared the morphology of hAstros in the absence or presence of neurons. One particular disadvantage in using hNeurons for coculture studies is that the population will also contain a mixture of undifferentiated neuroepithelial cells and gliogenic progenitors, typically in rosette structures, that generate immature synaptogenic hAstros over time (Johnson et al., 2007). These hPSC-derived “endogenous” glial cells will confound any phenotypes observed in neuronal studies. To overcome this, we utilized a clonal hPSC line with inducible expression of a transgene to generate Neurogenin 2 (NGN2) protein, which rapidly generates homogeneous cortical iNeurons after induction in a neural supportive medium (Wang et al., 2017) (Figure 1A). We determined whether pre-differentiated hAstros (>6 months, from embryonic stem cell line WA09 and from previously characterized induced pluripotent stem cell lines; Krencik et al., 2015) were able to induce maturation of iNeurons in a 2-week conventional 2D coculture as previously reported in hNeurons (Krencik et al., 2011). As expected, hAstros induced expression of a synapse-related gene, as measured by a Synapsin 1-promoter-driven GFP reporter, and the hAstros themselves became elongated and branched (Figures S1A and 1B). However, this culture paradigm did not induce the astrocytes to exhibit the morphological complexity of astrocytes within the human brain.

We next examined if hAstro morphological complexity could be enhanced in higher density cultures that are more similar to endogenous conditions. During coculture studies, we observed that hAstros tend to extend upon neuronal processes (Movies S1 and S2). This surprisingly also occurred, although to a lesser extent, when hAstros were cultured on parallel carbon nanofibers without neurons (Figure S1B). We therefore examined the morphological response of hAstros to high densities of parallel-orientated neuronal fibers using a membrane-targeted GFP reporter (mGFP). To mimic neuronal tracts, we generated organoid spheres of hPSC-derived neural progenitors from the iNeuron line without induction of NGN2, seeded spheres separated by a few hundred micrometers onto Matrigel-coated glass, and then induced NGN2 expression for 2 weeks to promote maturation and neuronal process extension toward neighboring seeds (Figure 1C). Next, we cultured pre-differentiated (>6 months) dissociated hAstros on these tracts, or on Matrigel without neurons as a control, for an additional 2 weeks. In comparison with cultures in the absence of iNeurons, the coculture system led to a dramatic restructuring of hAstros with increased number of branches (N = 10 versus 11 technical replicates for each, branches of WA09-derived hAstros only versus hAstros + iNeurons; primary = 3.6 ± 0.7 versus 11.2 ± 1.4; secondary = 1.6 ± 0.6 versus 30.9 ± 3.6; tertiary = 0.2 ± 0.2 versus 11.2 ± 3.0). The overall area of total membrane per cell was not different, yet total territory occupied per hAstro was increased (5,379.6 ± 1,226.6 versus 10,994.7 ± 4,714.2 μm²) indicating membrane restructuring with no change in total membrane content. In support of this, the length of the maximum process was also increased (79.5 ± 35.1 versus 145.2 ± 44.2 μm) (Figure 1D).

These data suggest that high-density neuronal axons are sufficient to induce profound changes in hAstro morphology and yield hAstros morphologically similar to endogenous human white-matter-tract astrocytes.

Enhanced Astrocyte Complexity in a 3D Organotypic-like Coculture Environment

Since increased neuronal process density appears to be crucial to induce complex astrocyte morphology, we next examined the effect of coculturing hAstros with a high density of neurons in 3D tissue. We turned to the method commonly utilized to maintain neural networks of rodent or human tissue, termed organotypic cultures (Humpel, 2015). We cultured neurospheres from the iNeuron line for 2 weeks, and then seeded spheres upon polytetrafluoroethylene (PTFE) membranes to generate healthy organotypic-like cultures (with medium only in contact with the lower surface for better gas exchange)
Compared with being submerged in medium on glass coverslips coated with Matrigel, spheres readily flattened as thick layers on membranes, and both conditions led to complete neuronal differentiation with a lack of rosette structures after NGN2 induction (Figure 2B). To determine whether this environment would improve the complexity of hAstros over that of the high-density 2D culture described above, we seeded mGFP-labeled astrospheres, containing solely hAstros, onto the organotypic-like iNeurons for a duration of 4 weeks. Surprisingly, this culture system resulted in the presence of fine membranous processes and dramatic branching reminiscent of astrocytes within the human brain and not previously reported in vitro (Figure 2C). This morphological complexity was observed to continuously increase between 3 and 9 weeks post engraftment, and fine leaflet-like structures (reminiscent of perisynaptic astrocyte processes) (Khakh and Sofroniew, 2015) could gradually be observed (Figure 2D). Furthermore, hAstros distributed into territories, displayed club-like bulbous endings and produced long-projecting varicose processes through the territories (Figures S1C and S1D), similar to unique features observed within the human brain (Colombo et al., 1997a; Oberheim et al., 2009). These data suggest that in contrast to axons only, neuron cell bodies and dendrites may promote a more “gray-matter”-like appearance of hAstros.

**Asteroid Coculture System for Disease Modeling**

We sought to optimize the emerging long-term 3D organoid methods by coculturing a defined number of human astrocytes and neurons while maintaining 3D structure and extracellular components. In order to achieve this system (which we termed asteroids), we combined dissociated neural progenitor cells and pre-matured hAstros at a 1:1 cell ratio. The presence of ROCK inhibitor (Y-27632) for 24 hr...
promoted sphere formation and survival (Figure 3A). To control cell numbers, we stopped growth factor treatment of hAstros before coculturing and induced neuronal progenitors to convert to a pure population of iNeurons as observed via TUBB3 (Figure 3B). After 35 days of coculture, immunostaining and 3D imaging revealed the continued presence of hAstros evenly, yet randomly, throughout the asteroids, dispersed into distinct territories among the iNeurons (Figure 3C). In all cases, sparse mGFP labeling revealed complex morphology, including fine-membrane processes projecting from individual GFAP-positive branches (Figure 3D). To determine if this 3D environment enhances maturation of astrocytes, we compared protein expression of a well-known astrocyte maturation marker in astrospheres and asteroids. Glutamate transporter GLT1 (also known as EAAT2), which has been reported to upregulate in hAstros post-rodent engraftment (Haidet-Phillips et al., 2014), had increased labeling within the asteroids (Figure S1E). Next, as a proof of principle for synapse studies and disease modeling, we generated asteroids containing pre-matured hAstros from a Costello syndrome-specific induced hPSC line with an over-activating mutation in HRAS (HRASG12S). As previously described, HRASG12S hAstros have an increased synapticogenic potential compared with cells generated from a control line (HRASWT) (Krencik et al., 2015). First, we investigated the ability of hAstros to induce the formation of synapses within asteroids with or without induction of NGN2. We measured the density of apposed pre- and post-synaptic markers Synapsin 1 (SYN1) and PSD95, respectively, and discovered an increased density of synapses in the coculture group and furthermore with NGN2 induction at day 35 (iNeurons without hAstros, hNeurons [without NGN2 induction] with hAstros, 68.3 ± 15.4, 132.1 ± 15.1, 175.8 ± 22.1 per 100 μm², respectively; iNeurons without hAstros to hNeurons with hAstros, p = 0.007; iNeurons without hAstros to iNeurons with hAstros, p = 0.002; hNeurons with hAstros to iNeurons...
Figure 3. Analysis of 3D Asteroid Cocultures
(A) Bright-field examples and illustration of asteroids in the absence (left panel) or presence of hAstros with (right panel) or without induction of NGN2.
(B) Progenitor cells, as rosette structures, are not present with NGN2 induction (right panel).
(C) After 35 days, hAstros are dispersed throughout the asteroids.
(D) mGFP reveals the fine-membrane processes emanating from GFAP-positive hAstro branches.
(E) The addition of hAstros significantly increases the number of opposed pre- and post-synaptic markers compared with iNeurons without hAstros; the combination of iNeurons with hAstros increases synaptic density to a further extent. *p < 0.05; **p < 0.01.
with hAstros, p = 0.048; n = 3 independent replicates each) (Figure 3E). Synaptic measurements were repeated with hAstros generated from three non-related wild-type hPSC biological replicate lines at 3 weeks (Figure S2A). In these cultures, we were also able to detect perisynaptic astrocyte processes utilizing the protein marker Ezrin (Lavialle et al., 2011), which surrounded synaptic structures within asteroids, but were not observed in iNeurospheres (Figure S2B). Lastly, the addition of HRASWT hAstros to the iNeurons in 3D asteroid cocultures increased apposed pre- and post-synaptic markers SYN1 and Homer, yet, similar to the values we reported previously (Krencik et al., 2015), we detected more than a 50% increase in synapse density in the presence of HRASG12S hAstros (no hAstros, HRASWT hAstros, HRASG12S hAstro per 100 μm², 91.7 ± 5.06, 174.3 ± 12.5, 301.67 ± 11.7, respectively; no hAstros to HRASWT, p = 0.007; no hAstros to HRASG12S, p < 0.001; HRASWT to HRASG12S, p = 0.004; n = 3 independent replicates each) (Figure 3F). These data reveal the asteroid coculture system to be an efficient and innovative method to precisely investigate normal and disease-relevant roles of astrocytes upon synapse formation in a 3D setting.

Finally, we sought to generate a more systematic and large-scale bioengineering approach for 3D sphere production rather than the random self-assembly method described above (Figure S4A). We immediately induced NGN2 directly from the hPSC line in 2D for 48 hr in neural medium and then mixed these rapidly maturing progenitors after dissociation with equal numbers of mGFP-labeled post-mitotic hAstros in microwell plates with Y-27632 to generate 3D spheres of specific and consistent sizes (Figure S4B). The ratio of neurons to astrocytes could be altered as desired before sphere formation. In addition, to regulate multiple sphere assembly, we subsequently transferred the resultant asteroids to either bioreactor spinner flasks to promote long-term separation of spheres or to stationary flasks to allow fusion through close physical contact (Figure 4A). This rapid assembly method also produced hAstros with complex morphology by 3 weeks as observed by confocal microscopy and scanning electron microscopy (Figures 4B–4D). Notably, similar to techniques reported for neurosphere organoids (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017), this approach demonstrates the utility for investigating long-range migration or signaling of astrocyte-neuron circuits from assembled spheres that were specified to different brain regions, treated in different conditions, or generated from differing genetic backgrounds.

DISCUSSION

How do human astrocytes contribute to local and long-range neural circuit processing? Human explants have been used to address this question, yet the scarcity of available tissue limits the utility of such systems and has led to the emerging use of organoids and/or spheroid systems from hPSCs. Unfortunately, the techniques to date are extremely long and not yet systematic. The work presented here provides an alternative approach with a rapid and controllable technique to study the impact of human astrocyte-neuron interaction in various conditions. As 3D cultures increase background noise during fluorescent imaging compared with 2D monolayers, we plan to introduce light sheet and other emerging technologies (Bindocci et al., 2017) to achieve higher resolution during examination. With augmented complexity (e.g., addition of immune or vascular cells, oligodendrocytes, or other neuronal subtypes), these coculture systems can be further utilized for brain modeling and posing questions about precise cell-cell interactions. For example, do human astrocytes induce oligodendrocytes to myelinate or maintain blood-brain-barrier properties of endothelial cells in 3D conditions? What is the relationship between human astrocytes and microglia in synapse pruning and engulfment?

Human astrocytes have more overlap in their territorial domains, project long processes through many layers of tissue and have bulbous endings compared with their rodent counterparts. Remarkably, these distinguishing features are observed in the systems described here. Perisynaptic astrocyte processes have independent and dynamic responses to local synaptic transmission in the form of calcium fluctuations, yet the functional importance of these local phenomena is still unclear (Bazargani and Attwell, 2016). Because the coculture systems described here preserve neuronal synapses in close association with vellate astrocyte processes, this will now allow for investigation into how these long-process and fine-membrane components influence and respond to neuronal activity.

EXPERIMENTAL PROCEDURES

Neural Differentiation and Processing

hAstros and hNeurons were differentiated from hPSCs (lines H9 [WA09], 13, 162D, 165D, and CS16) and characterized for cellular identity as detailed previously (Krencik et al., 2011, 2015). iNeurons were produced from a transgenic hPSC line by NGN2 induction via
addition of 2 μg/mL doxycycline as described previously (Wang et al., 2017). Astrospheres were supplemented with EGF and FGF2 (10 ng/mL each) before coculture experiments as detailed previously (Krencik and Zhang, 2011) and not forced to be mature or reactive by the addition of exogenous factors. All coculture experiments were conducted in neural medium consisting of DMEM/F12 containing glutamax, sodium bicarbonate, sodium pyruvate, N-2 supplement, B27 supplement (Gibco), and 1 mg/mL heparin (Sigma). For 2D culture, acid-etched glass coverslips were pre-coated with 0.1 mg/mL polyornithine (Sigma) in water and 0.08 mg/mL growth factor-reduced Matrigel matrix (Corning) in medium. Alternatively, cells were cultured on Matrigel-coated chamber slides containing carbon nanofibers (Sigma, Z694576). For organotypic-like cultures, spheres were plated on hydrophilic PTFE cell culture inserts with 30 mm diameter and 0.4 μm pore size (Millicell). For sphere cultures, cells were either self-assembled and maintained in stationary culture or assembled into specific sizes utilizing a microwell plate (Stem Cell Technologies, AggreWell-800 24-well plate) followed by optional spinning in a bioreactor (Corning Proculture Spinner Flask on Dlab MS-C-S4 Magnetic Stirrer). All cultures were maintained in 5% CO2 in 37°C in room oxygen levels.

**Sample Processing**

Immunoanalysis of spheres consisted of 30 min incubation at 4°C in 4% paraformaldehyde followed by staining of intact 3D spheres or sucrose-embedded cryosectioning as 30 μm sections and attached to slides. Sections were stored at −80°C until immunostaining. For immunoanalysis, samples were blocked in 0.25% Triton X-100 with 5% goat or donkey serum and labeled with the following antibodies: S100 (Abcam, ab868), GFAP (Chemicon, MAB360), GFP (Aves, GFP-1020), TUBB3 (Covance, MMS-435P), GLT1 (a generous gift from Dr. Jeffrey Rothstein), MAP2 (Synaptic Systems, 188 004), Ezrin (Thermo Fisher, MA5-13862), SYN 1 (Synaptic Systems, 106 103), PSD95 (Abcam, ab2723), and Homer (Synaptic Systems, 160 011). hAstro morphology and synaptic colocalization was measured utilizing ImageJ software. Scanning electron microscopy was conducted at the Houston Methodist Research Institute Electron Microscopy Core. mGFP reporter was generated by infecting cells with lentiviral particles generated with a reporter construct as described previously (Addgene plasmid no. 22479; Rompani and Cepko, 2008). For calcium imaging, hAstros were infected with lentivirus generated from Addgene Plasmid no. 72303 (Dempsey et al., 2016).

**Statistics**

For quantification analysis, statistical tests were performed only when spheres were independently generated from separate starting hPSC cultures. SEM was calculated using averages of data from randomly selected fields throughout the spheres. All measurements are presented as means ± SEM. When appropriate,
significance was tested using two-tailed unpaired t tests. In all figures, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and three movies and can be found with this article online at https://doi.org/10.1016/j.stemcr.2017.10.026.

AUTHOR CONTRIBUTIONS

R.K. and E.M.U. designed the study; R.K., K.S., J.V.v.A., N.B., C.C., and S.B. conducted cell cultures, processing, and data analysis; R.C., C.L., C.W., M.E.W., and L.G. generated and provided the iNeuron line; P.J.H. and D.H.R. provided intellectual contributions; R.K. and E.M.U. co-wrote the paper.

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