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Supplementary information

Cell stress in cortical organoids impairs molecular subtype specification

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Supplementary Information

Supplementary Discussion

Organoid models experience increased cellular stress compared with primary human cortical cells. Organoids lack vascularization, limiting the distribution of nutrients and oxygen to the core of organoids, however the degree of cellular stress and its molecular consequences have not been well characterized. During *in vivo* development, increased activation of the unfolded protein response elicited by cellular stress can inhibit maturation and cell type specification[1,2]. It remains controversial whether ER stress observed in organoid models differs from primary tissue[3], or reflects endogenous cell types[4] at early stages of development[5]. However, our studies have found significant evidence that organoids reflect non-endogenous levels of stress (Extended Data Figs. 14-15).

Glycolysis in an anaerobic state is a normal requirement for the maintenance of a stem cell niche[6], and we have observed small numbers of progenitor cells in primary datasets that express this program early in development. However, glycolysis is not down-regulated during neuronal differentiation in organoids, as it is in primary cortex. Our results suggest that while *in vitro* culture systems may all promote some level of glycolytic stress, three-dimensional culture exacerbates this stress, even in transplanted primary cells and metabolic stress may in turn limit the specification fidelity of cell subtypes. The protocol that expressed the least stress included a much faster differentiation progression and less time in three-dimensional aggregates (versus two-dimensional culture)[5], further supporting this observation. Hypoxia in aggregate culture has also been shown to induce unfolded protein responses that resemble ER stress[7]. A recent study improved the reproducibility of organoid-derived cell types[8]. In our re-analysis of their data we validated the degree of correspondence between primary and organoid cell types reported in their study (~70%) and assigned subtype correspondence which was similar to our organoids (<50%) (Fig. 2A, Extended Data Fig. 6B). However, this protocol utilizes large spinning flask bioreactors, which increases O_2 and nutrient diffusion. We observed a specific increase in the number and fidelity of their intermediate progenitor cell population, which may be particularly vulnerable to hypoxic conditions typically observed in organoids (Extended Data Fig. 6B)[7]. Innovations to limit aggregate culture, including culturing organoid slices at the air liquid interface, appear to improve organoid health[9]. However, single-cell analysis indicates that glycolysis and ER stress persist in these experimental models (Extended Data Fig. 14A, Extended Data Fig.15), indicating that additional innovations are required to mitigate stress and maximize subtype specification in cortical organoids.

When primary cells are placed in culture, stress gene networks have increased expression and when organoid cells are transplanted *in vivo* their expression decreases. The organoid (or more broadly *in vitro*) environment, rather than the neural induction and differentiation trajectory in iPSC culture, appears to be responsible for stress pathway activation. Moreover, metabolic and ER stress are specifically problematic for organoid models seeking to recapitulate processes of neural development and model disease due to their impact on cellular identity. Although these studies do not conclude whether cellular stress is causative for the loss of cell subtype specification, they clearly demonstrate a relationship between these processes. Importantly, all protocols, PSC lines, and timepoints in our analysis demonstrated impaired subtype specification, suggesting that this is a deficiency across all cerebral organoid models. Therefore, to maximize the utility of organoid models to studies of disease, development, and regeneration it will require a careful comparison of cell types, maturation states and fate specification in organoids and primary samples. The analytical toolkit, resource, and current limitations presented here will provide avenues for

improvements in organoid culture, helping to realize the potential of cerebral organoids in studies of disease, development, and regeneration.

Supplementary References

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Supplementary Table Legends

Supplementary Table 1. Cell meta data, including sample, age, line or individual, cell type assignments, and sequencing metrics (primary: n= 5 biologically independent individuals, n= 189,409 cells from 5 independent experiments; organoids: n= 37 organoids, n= 242,349 cells from 4 stem cell lines across 4 independent experiments).

Supplementary Table 2. Cluster markers of primary single-cell analysis. Includes cell type annotations, with rationale for the genes that informed the cluster assignment (n= 5 biologically independent individuals, n= 189,409 cells from 5 independent experiments).

Supplementary Table 3. Cluster markers of organoid single-cell analysis. Includes cell type annotations, with rationale for how cluster identity was determined via correlation, literature or other analyses including the hierarchical clustering or key marker genes organoids: n= 37 organoids, n= 242,349 cells from 4 stem cell lines across 4 independent experiments).

Supplementary Table 4. Cluster markers of re-analysis of published single-cell datasets.

Supplementary Table 5. Cluster assignments for the 20,000 cell subset of cells used in 4 additional co-clustering approaches as well as analyzed by MetaNeighbor. Table includes the differentially expressed marker genes for each of the 4 methods in the space of any clusters with at least 20% contribution from both primary and organoid cells. For the genes that are upregulated in organoids, the full annotation of the top 20 Gene Ontology terms is provided.

Supplementary Table 6. variancePartition results for both primary and organoid single-cell datasets across relevant metadata properties. For each gene and metadata property, the percent of variation explained is indicated in the table, and any variation unexplained is indicated in the residual column (primary: n= 5 biologically independent individuals, n= 189,409 cells from 5 independent experiments; organoids: n= 37 organoids, n= 242,349 cells from 4 stem cell lines across 4 independent experiments).

Supplementary Table 7. Differential gene expression analysis results between primary and organoid clusters designated as either oRG or upper layer subtypes. Data was re-normalized jointly before differential expression was performed (primary: n= 5 biologically independent individuals, n= 189,409 cells from 5 independent experiments; organoids: n= 37 organoids, n= 242,349 cells from 4 stem cell lines across 4 independent experiments).

Supplementary Table 8. Module assignments generated with WGCNA in primary radial glia, and subsequently used for pseudoage calculation and application to organoid radial glia (primary: n= 5 biologically independent individuals, n= 189,409 cells from 5 independent experiments; organoids: n= 37 organoids, n= 242,349 cells from 4 stem cell lines across 4 independent experiments).

Supplementary Table 9. Unique area signatures resulting from area gene analysis. Each line shows the marker gene and the area it is assigned to (n= 5 biologically independent individuals, n= 189,409 cells from 5 independent experiments).

Supplementary Table 10. Cluster markers of pre- and post- transplantation single-cell analysis (n=7 biologically independent samples across 2 independent experiments).

Supplementary Table 11. Differential gene expression between pre- and post-transplantation samples (n=7 biologically independent samples across 2 independent experiments).