A forebrain organoid model of progranulin-associated frontotemporal dementia

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Background

Heterozygous mutations in the progranulin gene (*GRN*) are the cause of 5-10% of frontotemporal dementia (FTD) cases, with patients consistently showing TDP-43 pathology at post-mortem. There is evidence to suggest that progranulin (PGRN) plays an important role in lysosomal function. PGRN is localised to the lysosome and has been shown to regulate lysosomal enzyme activity. Additionally, homozygous *GRN* mutations lead to neuronal ceroid lipofuscinosis (NCL), a group of lysosomal storage disorders, further highlighting PGRN's role in lysosomal function. PGRN is expressed throughout the brain with highest expression seen in microglia. Recent evidence suggests lysosomal function is particularly impaired in PGRN deficient microglia despite TDP-43 pathology being predominantly localised to neurons, therefore further studies looking at the interactions between microglia and neurons are essential to assess the potential non-cell autonomous mechanisms at play. The main aims of this study were to 1) establish cerebral organoid model of PGRN associated FTD, 2) establish a cerebral organoid – microglia co-culture model of PGRN associated FTD and characterise potential phenotypes in these models.

Methods

For this study, iPSC lines from the Kolf 2.1J background were used and included the wildtype (WT) line, R493X heterozygous and homozygous mutation. These were obtained from the human iPSC Neurodegenerative Disease (iNDI) Initiative (1).

Forebrain organoids were differentiated using a protocol adapted from Bowles et al (2). In brief, embryoid bodies (EBs) were differentiated from iPSC lines in StemFlex media with Y-27632 in 96-well v-bottom plates. EBs were mainted in the 96-well plates on a series of media with the base being N2B27 without vitamin A for the first 70 days and then with vitamin A after. (Day 2-8 XAV939 (2uM) + SB431542 (10uM) + LDN193189 (100nM), Day 8-24 FGFb (20ng/ml) + EGF (20ng/ml), Day 24-70 BDNF (20ng/ml), Day 70+ vitamin A was included).

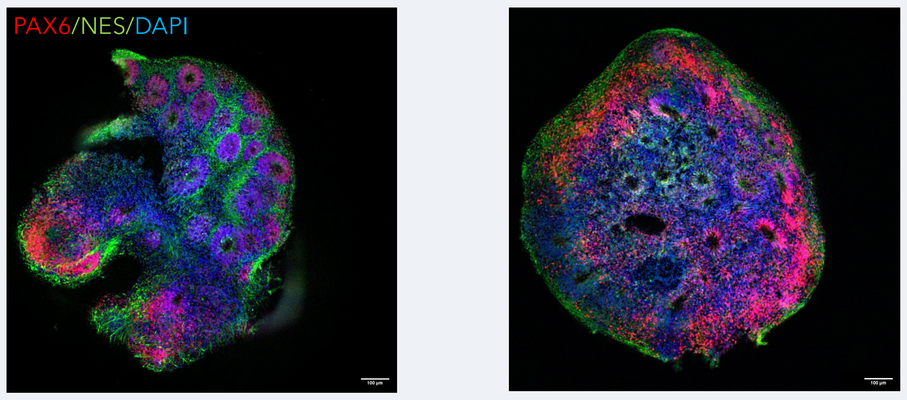
iPSC derived microglia (iMGs) were differentiated using a protocol as previously described in Garcia-Reitboeck et al. (2018) and Xiang et al. (2018), (3,4). In brief, embryoid bodies (EBs) were differentiated in a 96-well ultra-low attachment plate using StemFlex media with 50ng/ml BMP4, 50ng/ml VEGF and 20ng/ml SCF and seeded at 10,000 cells per well. After 4 days, EBs were transferred to T75 flasks and kept in X-Vivo 15 media with 100ng/ml MCSF and 25ng/ml IL3. IMGs were harvested and plated into 6-well plates and fed on DMEM F-12 media with 100ng/ml IL34, 25ng/ml MCSF and 5ng/ml TGFb1 for 2 weeks of maturation.

The development of **Org-iMG co-cultures** is currently being optimised. Initially, we seeded WT microglia at $1x10^5$, $2.5x10^5$ and $1x10^6$ per organoid. Myeloid progenitor cells were added directly to the 6-well plate that the organoids were cultured in. Co-cultures were then harvested 7 days after initial seeding for characterisation. Further optimisation of iMG numbers and co-culture time is ongoing.

Results

Forebrain Organoid Model

We successfully established and characterised a forebrain organoid model of PGRN-associated FTD. Whole mount immunocytochemistry using Sunjin Lab RapiClear 1.52 Solution (RC152002) was performed to confirm successful neuronal differentiation across our lines, (see *Figure 1).*



GRN+/+ bottom plane

GRN-/- mid plane

Figure 1. To confirm successful neuronal differentiation whole mount immunocytochemistry was performed on organoid sections at 30 DIV. DAPI (blue), Nestin (green) and PAX6 (red) were used to confirm the presence of neuronal progenitor cells. Scale bar show 100µm.

Characterising phenotypes in organoid model

We then wanted to assess whether our organoid model displayed any disease relevant phenotypes. We initially examined **TDP-43 expression and cleavage** using western blot. As seen in Figure 2, TDP-43 fragments (35kDa) are trending towards increased in 90DIV and significantly increased 180DIV GRN-/- organoids compared to WT controls. Further TDP-43 phenotypes will be explored using ICC to investigate cytoplasmic mislocalisation.

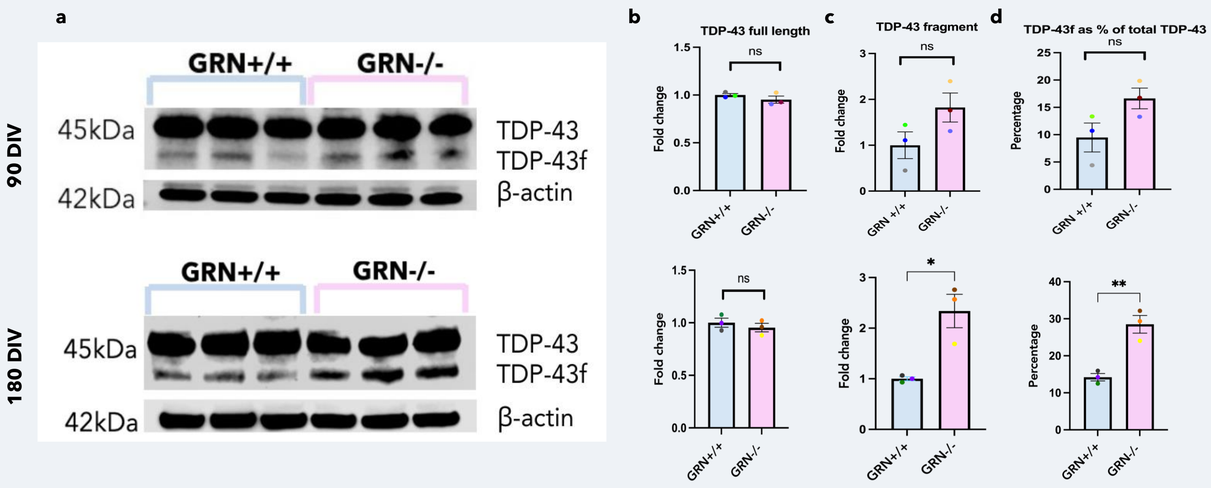


Figure 5. ***a) western blot of GRN+/+ WT and GRN-/- forebrain organoids at 90DIV (top) and 180DIV (bottom), probed for TDP-43 and ß-actin as a loading control. b) quantification of TDP-43 full length (45kDa) expression, normalised to loading control at 90DIV (top) and 180DIV (bottom) c) quantification of TDP-43 fragment expression, normalised to loading control at 90DIV (top) and 180DIV (bottom). d) TDP-43 fragment as a percentage of total TDP-43 (total intensity of full length and fragment band) at 90DIV (top) and 180DIV (bottom). Fold change for figures calculated from average of WT controls (n=3) on each blot. Each data point represents one sample. Unpaired two-tailed t-tests were performed *P<0.05, **P<0.01 Data is displayed as mean ± SEM. (N=3 pooled organoid samples, for each genotype).***

We wanted to assess **lysosome** dysfunction within our organoid model. We performed ICC for LAMP1 to assess whether we could see an increase, as has been shown previously (5). As can be seen in Figure 3, LAMP1 (green) intensity was significantly increased in our GRN -/- organoids compared with controls. This provides preliminary evidence of lysosomal dysfunction in our forebrain organoid model of PGRN-FTD. See Figure 3 for these images and quantification.

Results

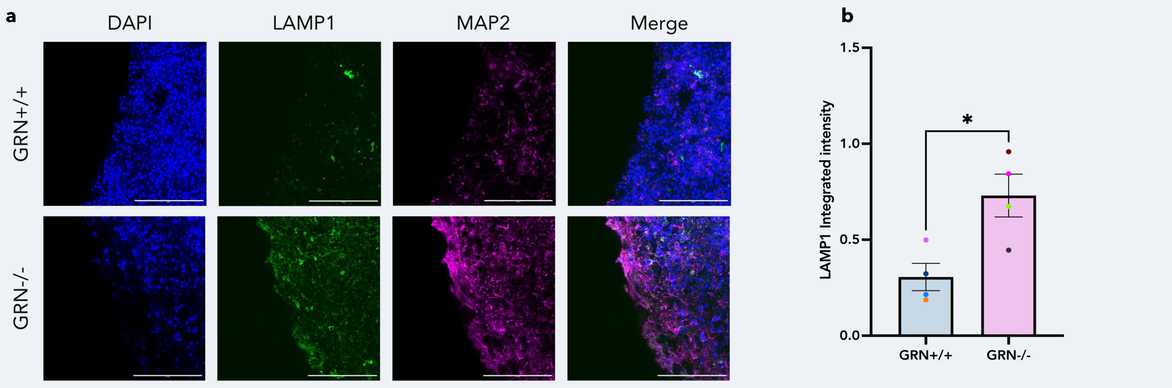


Figure 3. a) ICC to investigate LAMP1 in 180DIV GRN+/+ WT and GRN-/- (R493X homozygous mutation) forebrain organoid sections. Organoids were stained with MAP2 (far red), LAMP1 (green). Merge image shows LAMP1, MAP2 and DAPI composite image. The scale bars show 100µm. (100x magnification). b) shows quantification of LAMP1 integrated intensity. ***Unpaired two-tailed t-tests were performed *P<0.05. Data is displayed as mean ± SEM.***

IPSC derived microglia

As seen in *Figure 4* below, we characterised our iMGs using ICC, staining for Iba1 and TREM2.

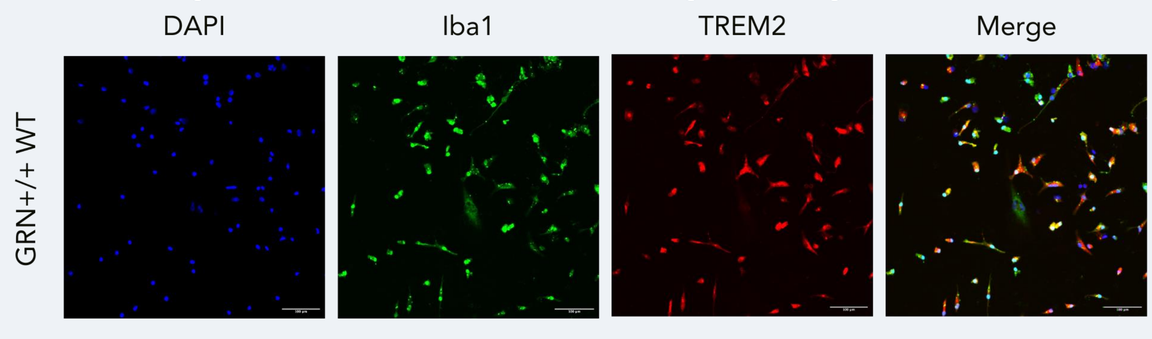


Figure 4. Characterisation of iPSC derived microglia (iMGs) using immunocytochemistry. IMGs were stained with DAPI (blue), IBA1 (green) and TREM2 (red) to confirm microglial like identity. Scale bar show 100µm.

Characterising phenotypes in microglia model

As seen in Figure 5, to measure early lysosomal changes that might be happening in our PGRN deficient microglia model, we conducted ICC for LAMP1 as well as TREM2 for basic microglia characterisation. All cells were TREM2 positive, confirming microglial differentiation. LAMP1 was seen as puncta throughout the cells and quantification of these images demonstrated a trend towards an increase in LAMP1 in GRN KO microglia. This preliminary data highlights potential lysosomal dysfunction as a phenotype in our microglia derived from isogenic GRN KO lines.

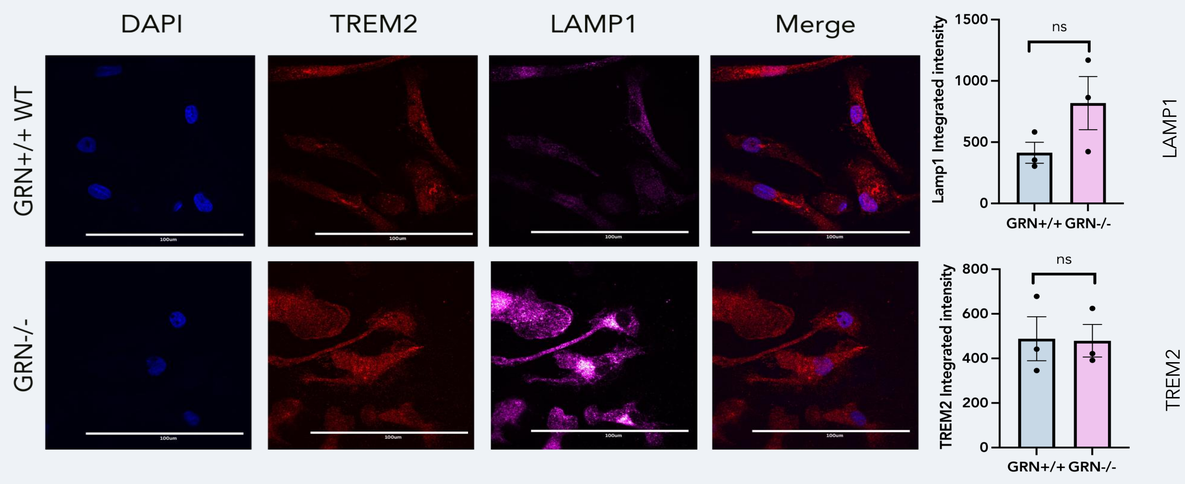


Figure 5. ICC to investigate lysosomal phenotypes in WT and R493X homozygous microglia. Microglia were stained with TREM2 (red) and LAMP1 (far red). Merge image shows TREM2 and DAPI composite image. The scale bars show 100µm. (100x magnification). ***Unpaired two-tailed t-tests were performed *P<0.05. Data is displayed as mean ± SEM.***

Forebrain Organoid – Microglia Co-Culture

We have begun optimising our organoid-microglia co-culture protocol. As can be seen in *Figure 6*, after staining for Iba1 and MAP2 we saw successful integration of the WT myeloid progenitor cells into the organoids. However, further work is required to quantify integration and optimise our protocol.

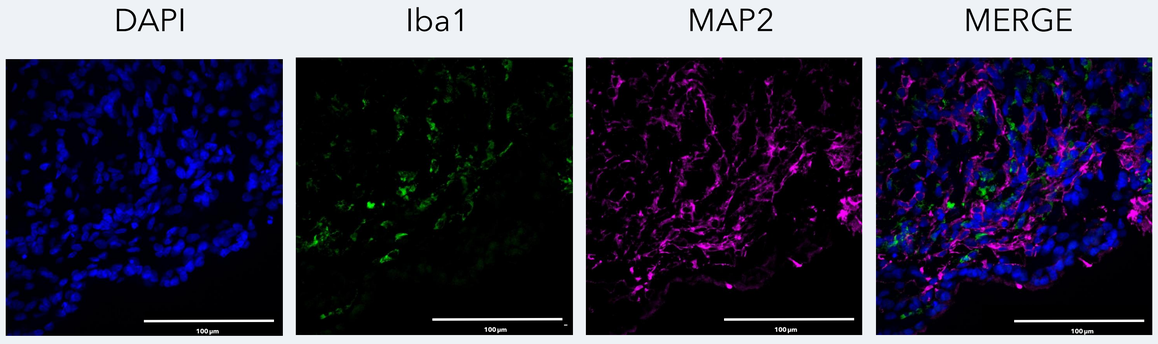


Figure 6. To assess whether iMGs successfully integrated into the organoids, immunocytochemistry was performed on co-culture sections 7 days after adding $1x10^5$ iMGs per organoid to the media. DAPI (blue), Iba1 (green) was used as a marker of microglia and MAP2 (far-red) as a neuronal marker. Scale bar shows 100µm.

Conclusion

In this preliminary study, we established and characterised a forebrain organoid and iPSC derived microglia models of progranulin associated FTD. So far, our preliminary findings suggest increased fragmentation of TDP-43 in GRN-/- organoids and lysosomal dysfunction in both the GRN-/- forebrain organoids and microglia models, in line with previous studies. As part of our second aim, we saw that microglia like cells can successfully integrate into organoids and this will provide the basis of our work going forward. For the next steps, we plan to do a mix-matched approach (e.g. WT microglia with $GRN^{-/-}$ organoids and vice versa) to assess potential non-cell autonomous mechanisms underlying fFTD pathology.

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References: (1) Ramos, D. M., Skarnes, W. C., Singleton, A. B., et al. (2021) Tackling neurodegenerative diseases with genomic engineering: A new stem cell initiative from the NIH. Neuron, 109, 1080– 1083 (2) Bowles, K.R., Silva, M.C., Whitney, K., Bertucci, T., Berlind, J.E., Lai, J.D., Garza, J.C., Boles, N.C., Mahali, S., Strang, K.H. and Marsh, J.A., 2021. ELAVL4, splicing, and glutamatergic dysfunction precede neuron loss in MAPT mutation cerebral organoids. Cell, 184(17), pp.4547-4563. (3) Garcia-Reitboeck, P., Phillips, A., Piers, T.M., Villegas-Llerena, C., Butler, M., Mallach, A., Rodrigues, C., Arber, C.E., Heslegrave, A., Zetterberg, H. and Neumann, H., 2018. Human induced pluripotent stem cell-derived microglia-like cells harboring TREM2 missense mutations show specific deficits in phagocytosis. Cell Reports, 24(9), pp.2300-2311. (4) Xiang, X., Piers, T.M., Wefers, B., Zhu, K., Mallach, A., Brunner, B., Kleinberger, G., Song, W., Colonna, M., Herms, J. and Wurst, W., 2018. The Trem2 R47H Alzheimer's risk variant impairs splicing and reduces Trem2 mRNA and protein in mice but not in humans. Molecular neurodegeneration, 13, pp.1-14. (5) Sung, W., Noh, M.Y., Nahm, M., Kim, Y.S., Ki, C.S., Kim, Y.E., Kim, H.J. and Kim, S.H., 2024. Progranulin haploinsufficiency mediates cytoplasmic TDP-43 aggregation with lysosomal abnormalities in human microglia. Journal of Neuroinflammation, 21(1), pp.1-20.

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