“Innovative Human 3D Neural Network Model for the Efficient Propagation of Human Prions”

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In this project, we aimed to exploit for CJD research a novel and innovative culture neuronal model. We had two main objectives: **(1)** to create an in vitro CJD model more similar to brain environment that will be composed of **neurons, astrocytes, oligodendrocytes, and microglia**. To achieve this task neural stem cells (NSC) generated from human pluripotent stem cells (hPSC) are induced to differentiate into a 3D adult neural network for several weeks (Choi et al., 2014), and mixed with microglial progenitors obtained following differentiation from hPSC **(2)** to validate the model by testing the therapeutic effect of well-known anti-prion agents.

1. CJD Infection of 3D neural networks.

While we were setting up the induction of microglial progenitors from human pluripotent stem cells and since we were able to show in a preliminary experiment that the cell overexpressing PrP could be infected with sCJD and vCJD cases following 3D neuronal differentiation, we first reproduced CJD infection (sCJD and vCJD) in 3D networks (Figure 1). NSC overexpressing PrP were mixed with geltrex matrix and seeded to form a 0.4cm thin layer, at D0, in 3D medium conditions. To improve the infection we will use NSC overexpressing human PrP. Inoculation was performed using sCJD and vCJD isolates after two weeks of culture. 3D networks were stopped, collected and lysed after 42, 52 and 66 days. Western blot analysis of PK digested lysate, performed with Saf mix cocktail of anti-prion antibodies, showed the presence of PrPSc signals that increase during the differentiation process.

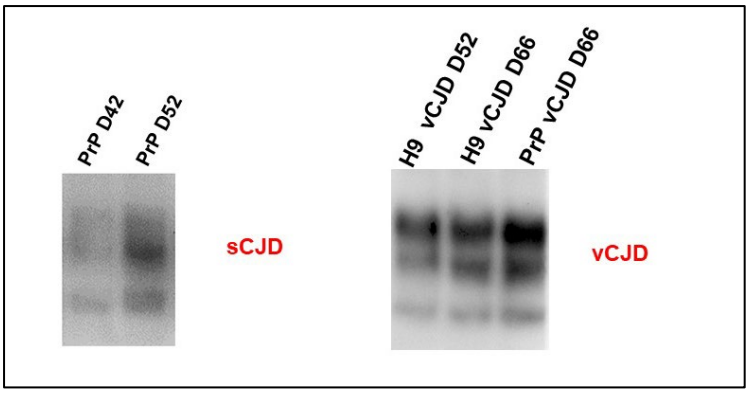

Figure 1: Confirmation of the prion replication in 3D neural networks: 3D neuronal networks from H9NSC (expressing only GFP) were performed and were infected with sCJD and vCJD. Lysates and Western blot of the PK resistant PrP were performed at different time points (42, 52 or 66 days post inoculation). We were able to observe an increase of PrPSc signal during the differentiation process.

2. Validation of 3D models for the study of prion inhibition.

As one of the potential application of 3D models is to obtain a cellular model for anti-prion molecules screening, we checked the effect of some anti-prion molecules on prion infection and propagation. For that purpose we used 3D neural networks that were obtained using NSC overexpressing anti-prion molecules such as the dominant negative mutants of PrP (E219KPrP-DN, E168RPrP-DN) (Crozet et al., 2004) that are known to inhibit prion replication and were shown to promote a 20% increase of the incubation time when used to treat prion infected mice (Toupet et al., 2009). Using these models we observed a decrease of the signal between day 24 and day 66 (Figure 2) showing that there is an inhibition of the prion propagation.

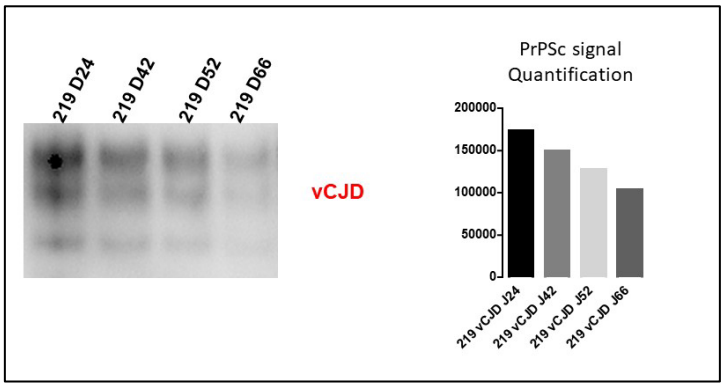

Figure 2: Inhibition of prion replication in 3D neural networks expressing E219KPrP-DN mutant. On the left: western blot using SAF mix cocktail of anti-PrP antibodies on cell lysates treated with PK. On the right: Quantification of the PrPSc signal obtained by WB.

3. *Generation of microglial progenitors from hPSC:*

Microglial cells are resident, tissue-specific macrophages that perform several critical roles in the development and maintenance of the CNS (Hanish et al., 2007). To generate microglial progenitors, we used a robust and reproducible protocol (Figure3) from human pluripotent stem cells (iPSCs) in chemically defined conditions (Douvaras et al., 2017). This protocol, that has been recently described by Douvaras et al., 2017, mimics the embryonic development. We first committed the cells in the myeloid lineage during 26 days to obtain progenitors that could then differentiate into microglial cells. These progenitors were isolated by FACS on the basis of the expression of CX3CR1 and CD14 that are two specific microglial progenitors markers (Figure 3C) before being integrated into 3D neural networks. We also try to allow them to differentiate alone into microglia but we do not find the right conditions to amplify them and differentiate them into microglia.

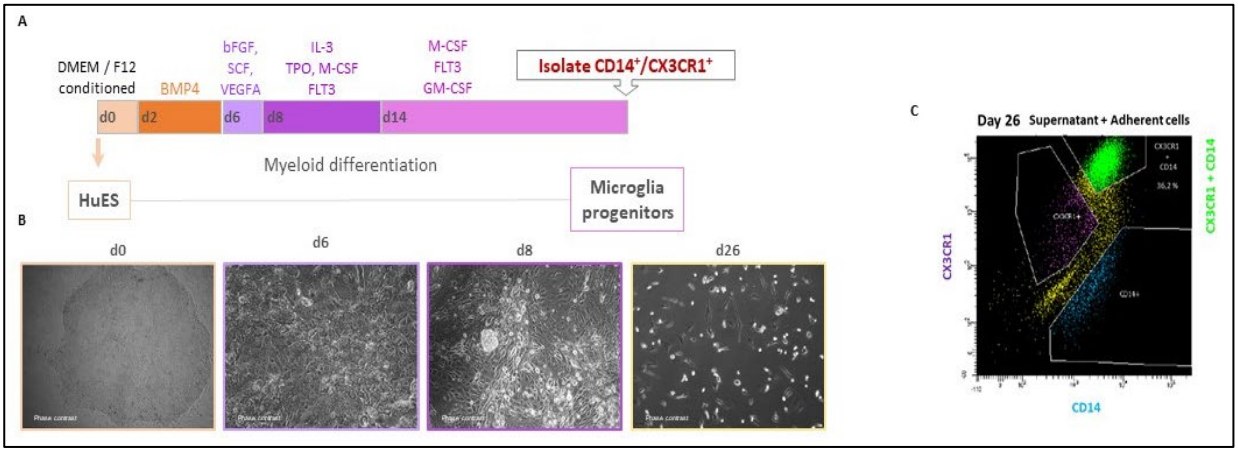

Figure 3: iPSC differentiation into microglia progenitor through myeloid induction
(A) Diagram depicting the major steps of the microglial differentiation protocol with the main growth factors addition
(B) Panel of representative images of cells during microglial differentiation in phase contrast.
(C) FACS profil of cells on day 26 during isolation.

4. *Co-culture of CD14+/CX1CR3+ cells and 3D neural networks.*

For the co-culture system, NSC were first induced to differentiate into a 3D neural network for 6 weeks; microglia progenitors obtained from hPSC were then inserted into the 3D culture in the presence or absence of GM-CSF/IL34 cytokine that are known to improve the microglia differentiation and integration (Haenseler et al., 2017) The co-culture was maintained during 12 more weeks (18 weeks for the whole experiment). After 18 weeks, we fixed the coculture and we characterized the cells by immunofluorescence of βIII-tubulin for neurons, GFAP for astrocytes and Iba1 for microglia. In co-culture containing microglia and 3D neural networks, in neural differentiation medium, we observed the presence of microglia (Figure 4A), neurons and astrocytes (Figure 4B). Curiously when GM-CSF and IL34 were added (Figure 4C), the 3D neural networks disappeared but we got nice Iba-1+ cells suggesting that in addition to the presence of microglia, the conditions were too drastic for the maintenance of the neural network.

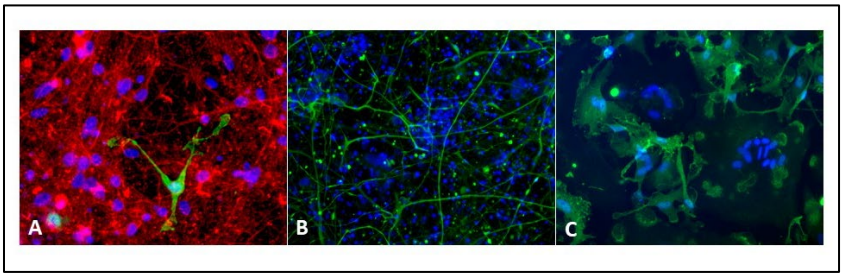

Figure 4: 3D culture containing : (A) βIII positive cells (neurons) in red, Iba1 positive cells (potential microglia) in green, (B) GFAP positive cells (astrocyte) in green. When 3D neural networks are cocultivated with microglia in presence of IL34 and GM-CSF (C), we observe an increase of Iba1 positive cells, but no neurons or astrocytes.

As these experiment were very long to set up and because we were not able to maintain the microglia progenitors in culture to amplify and keep a cell stock, we need to prepare microglia progenitors extemporaneously for each experiments. Regarding these constraints, we had just the time to begin a new 3D neural networks differentiation using NSC cells overexpressing PrP in parallel to a hPSC myeloid induction. 3D networks and microglial progenitors

will be mixed together in few weeks and then infected with CJD to validate the model. The same experiment using NSC cells in which DN-PrP mutant are present are also ongoing.

During this one year program, since we have set up all the tools to achieve our objective, we are confident with is new 3D neural culture system that would cast light on making new CJD cellular models that could comprehensively recapitulate pathogenic cascades of prion pathology in human brain-like environment.

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