Efficient directed differentiation of human induced pluripotent stem cells (hIPSCs) towards photoreceptor progenitors using small molecules.

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Development of a small molecule protocol for the differentiation of human pluripotent stem cells (hPSCs) to photoreceptors

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METHODOLOGY

Photoreceptor (PhR) degeneration and death is the ultimate cause of permanent vision loss,common to all retinal degenerative disease (RDD). Millions of people worldwide are affected, with current therapies lacking long-term efficacy. Human pluripotent stem cells (hPSCs) provide a limitless source cell that can be used for differentiation of all retinal cell types. They offer potential for use in transplantation for a host of RDDs. However, the development of protocols to provide efficient differentiation of hPSCs to PhRs in sufficient number of clinical grade cells, remains a challenge. This 66-day pilot study used PGP1, a CRISPR/Cas9 transgenic reporter hiPSC line, to develop a protocol for directed differentiation of hiPSC to PhRs using small molecules. Preliminary results across five timepoints demonstrate the presence of cells expressing early PhR markers, along with transcription factors at relevant time points. Live cell images at D28 indicate activation of the mCherry-tagged *RCVRN* gene. Further immunocytochemistry supports the high expression of NR2F2 at D30, and of CRX, PRDM1 and RCVRN by D46. This is followed by expression of OPSIN-M and ARRESTIN at D66. Flow cytometry data at D45 supports activation of cerulean-tagged VSX2. This initial study demonstrates the potential to generate homogeneous populations of PhRs without the need for cell selection. This protocol can be refined to give a Good Manufacturing Practice (GMP) protocol for pre-clinical study.

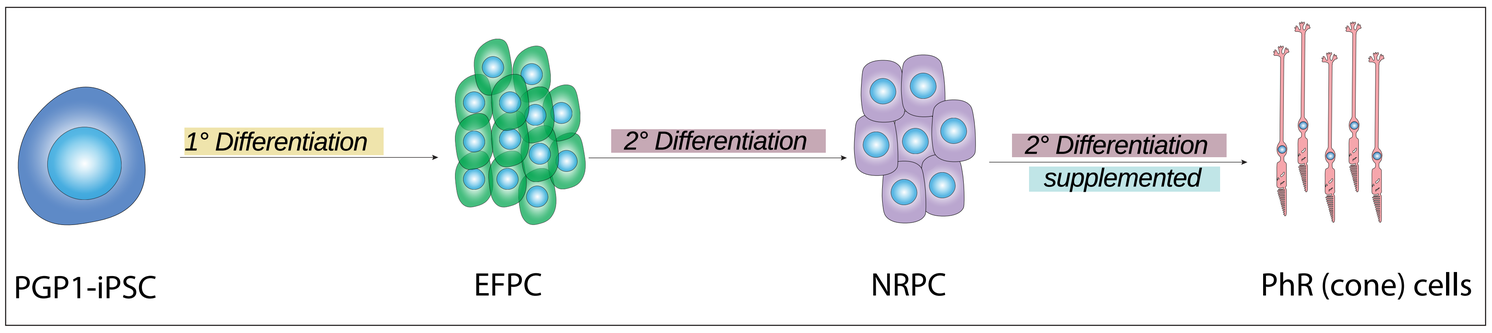

Figure 1| A schematic representation of PGP1-hIPSC differentiation towards eye-field progenitor cells (EFPCs) and onward towards the neural retina progenitor cells (NRPCs) and finally cone photoreceptors (PhRs).

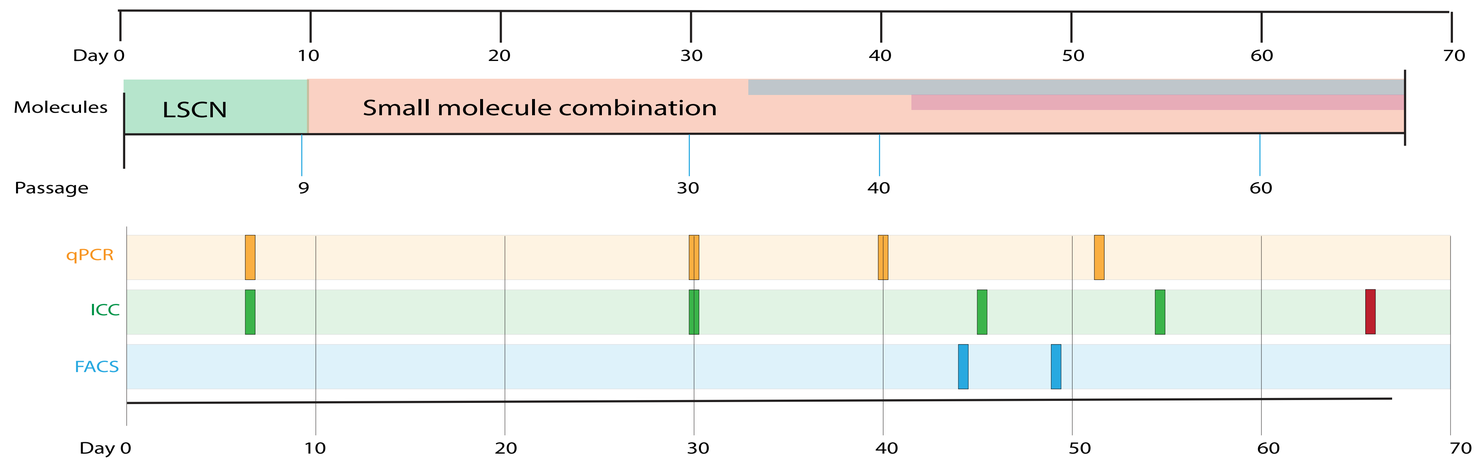

Figure 2 | Experimental timeline of conditions, passages and analyses conducted over time of development *in vitro.*

hiPSCs were plated on reduced growth factor Matrigel (BDAA354230; Bio-strategy) and directly induced into primary differentiation following addition of medium containing LDN193189 (L: Sigma), CKI (C: Sigma) and SB431542 (S: Merk) and Nicotinamide (N: 72340 Merk) over nine days. These molecules in combination facilitated the inhibition of the Wnt and TgF-β pathways, respectively. At D9, cells were analysed for expression of eye-field markers. Cultures were then exposed to secondary differentiation medium to induce differentiation towards neural retina. Medium was supplemented with small molecules to promote development and survival of PhR progenitors. A time course of four passages was determined on the basis of morphological change and the density of cell colonies. Efficiency of fate lineage and determination was monitored using live microscopy, since the PGP1-CRISPR/Cas9 reporter line revealed either cerulean/VSX reporter or mCherry/RCVRN reporter fluorescence. Further characterization of cells using ICC, FACS and qPCR to detect stage-specific gene expression, revealed developmental stages of iPSC, EFPCs, NRPCs, PhR precursors and finally PhR cones.

Figure 3| Morphological characterisation of hIPSC-PhR differentiation

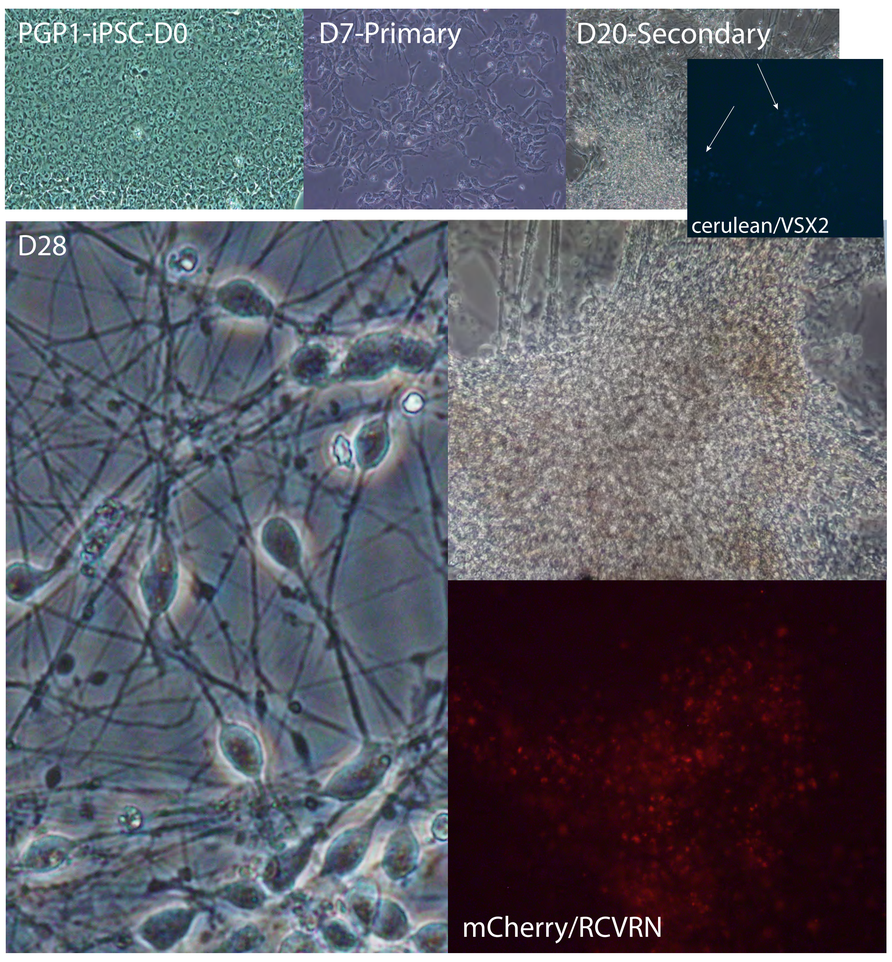

Figure 3 | Morphological changes showing the activation of cerulean tagged *VSX2* and mCherry tagged RCVRN at points throughout secondary differentiation.

Figure 4| ICC characterisation of hIPSC-PhR

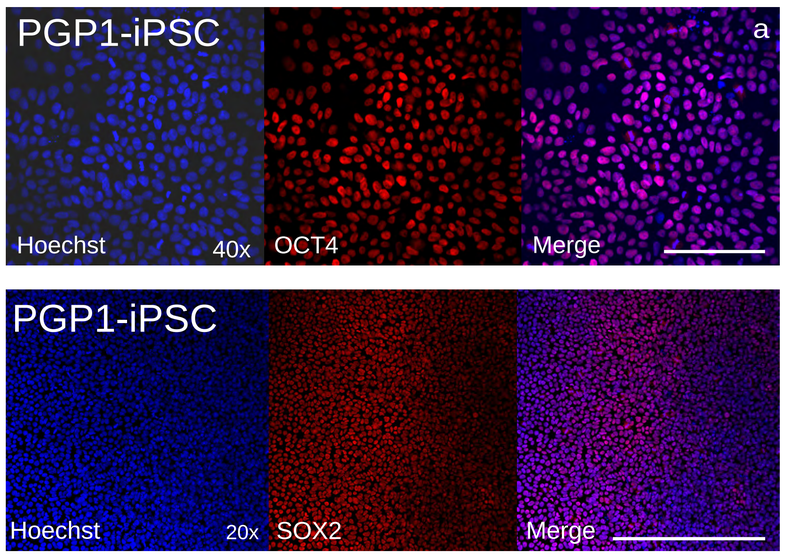

Figure 4| ICC characterisation of hIPSC-PhR

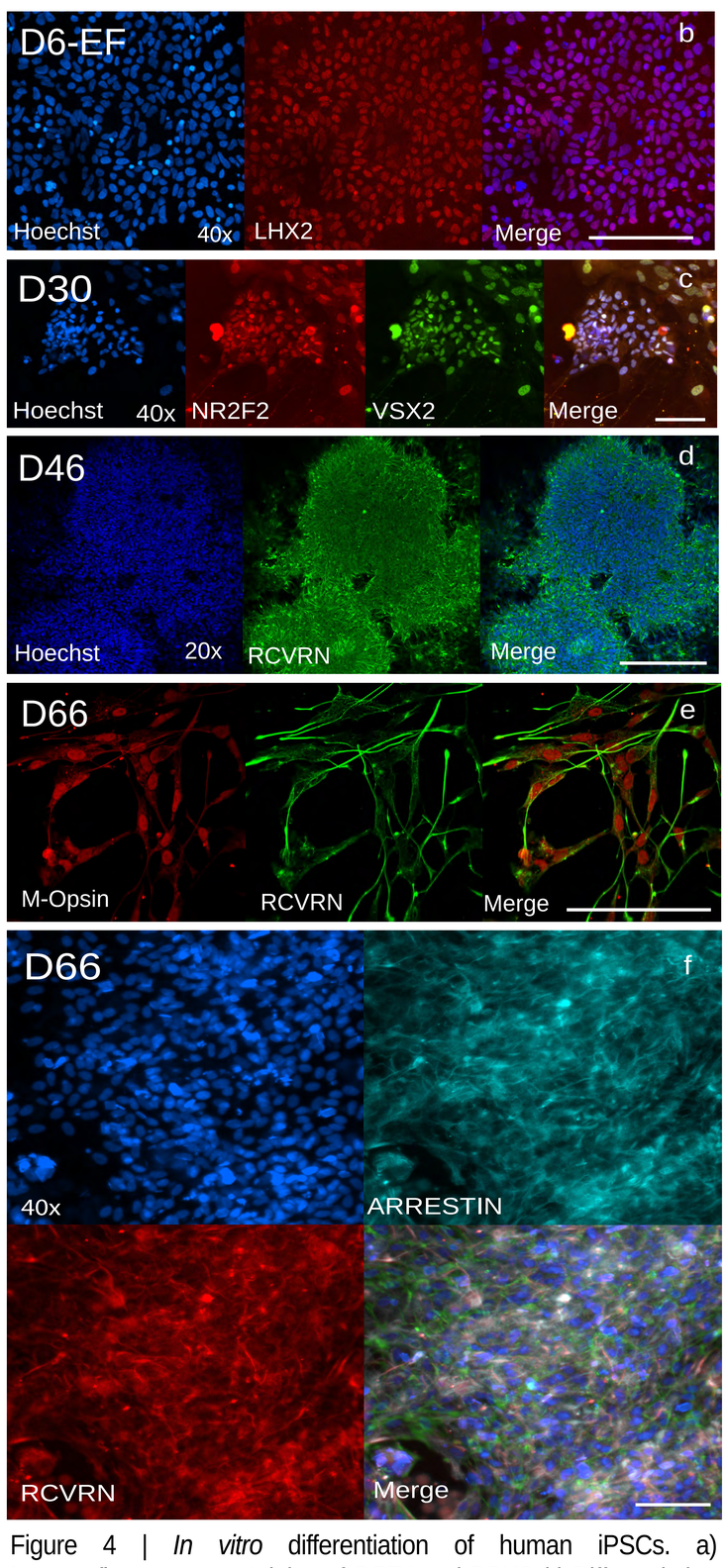

Figure 4 | *In vitro* differentiation of human iPSCs. a) Immunofluorescence staining of OCT4 and SOX2. b) Differentiation towards EFPCs by expression of LHX2. c) D30 expression - NR2F2, VSX2. d) D46 expression - RCVRN e) D66 expression - M-OPSIN, RCVRN. f) D66 expression - Arrestin, RCVRN.

Figure 5| Cerulean/VSX positive cells

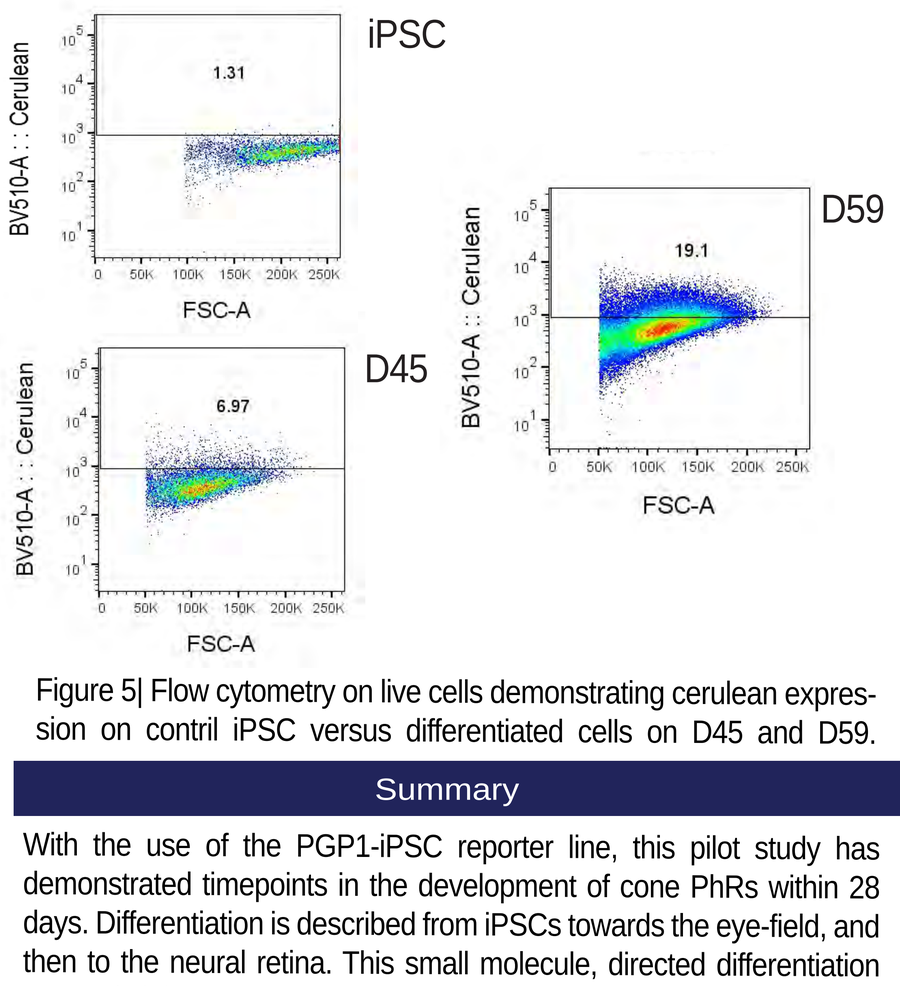

Figure 5| Flow cytometry on live cells demonstrating cerulean expression on contril iPSC versus differentiated cells on D45 and D59.

Summary

With the use of the PGP1-iPSC reporter line, this pilot study has demonstrated timepoints in the development of cone PhRs within 28 days. Differentiation is described from iPSCs towards the eye-field, and then to the neural retina. This small molecule, directed differentiation protocol yields a near homogeneous population of PhRs and their precursors without need for cell sorting or enrichment. The protocol can be used to study RDDs *in vitro* and may also be expanded and investigated for clinical application.

Further/Ongoing Research

1.To repeat and optimise the protocol across different hESC and iPSC lines with extended timepoint analysis.
2.Extensive stage-specific characterisation of each line using RNA sequencing.
3.Retinal implantation of cells to test functionality in a disease model.

Acknowledgments

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