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3D culture of human pluripotent stem cells in RGD-alginate hydrogel improves retinal tissue development

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ABSTRACT

No treatments exist to effectively treat many retinal diseases. Retinal pigmented epithelium (RPE) and neural retina can be generated from human embryonic stem cells/induced pluripotent stem cells (hESCs/hiPSCs). The efficacy of current protocols is, however, limited. It was hypothesised that generation of laminated neural retina and/or RPE from hiPSCs/hESCs could be enhanced by three dimensional (3D) culture in hydrogels. hiPSC- and hESC-derived embryoid bodies (EBs) were encapsulated in 0.5% RGD-alginate; 1% RGD-alginate; hyaluronic acid (HA) or HA/gelatin hydrogels and maintained until day 45. Compared with controls (no gel), 0.5% RGD-alginate increased: the percentage of EBs with pigmented RPE foci; the percentage EBs with optic vesicles (OVs) and pigmented RPE simultaneously; the area covered by RPE; frequency of RPE cells (CRALBP+); expression of RPE markers (*TYR* and *RPE65*) and the retinal ganglion cell marker, *MATH5*. Furthermore, 0.5% RGD-alginate hydrogel encapsulation did not adversely affect the expression of other neural retina markers (*PROX1, CRX, RCVRN, AP2α* or *VSX2*) as determined by qRT-PCR, or the percentage of *VSX2* positive cells as determined by flow cytometry. 1% RGD-alginate increased the percentage of EBs with OVs and/or RPE, but did not significantly influence any other measures of retinal differentiation. HA-based hydrogels had no significant effect on retinal tissue development. The results indicated that derivation of retinal tissue from hESCs/hiPSCs can be enhanced by culture in 0.5% RGD-alginate hydrogel. This RGD-alginate scaffold may be useful for derivation, transport and transplantation of neural retina and RPE, and may also enhance formation of other pigmented, neural or epithelial tissue.

Statement of Significance

The burden of retinal disease is ever growing with the increasing age of the world-wide population. Transplantation of retinal tissue derived from human pluripotent stem cells (PSCs) is considered a promising treatment. However, derivation of retinal tissue from PSCs using defined media is a lengthy process and often variable between different cell lines.
This study indicated that alginate hydrogels enhanced retinal tissue development from PSCs, whereas hyaluronic acid-based hydrogels did not. This is the first study to show that 3D culture with a biomaterial scaffold can improve retinal tissue derivation from PSCs.
These findings indicate potential for the clinical application of alginate hydrogels for the derivation and subsequent transplantation retinal tissue. This work may also have implications for the derivation of other pigmented, neural or epithelial tissue.

1. Introduction

In 2010, it was estimated that globally 32.4 million people were classified blind, and 191 million were visually impaired [1]. Diseases affecting the retina account for approximately 26% of

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blindness globally and 70% of blindness in the UK [2]. The burden of retinal disease is ever growing with the increasing age of the world-wide population [3,4]. Both neural retina and the supportive retinal pigmented epithelium (RPE) fail to regenerate in humans, therefore diseases that cause retinal cell loss, such as age-related macular degeneration (AMD), retinitis pigmentosa (RP) and other hereditary retinal dystrophies including glaucoma and vascular retinopathies, typically result in permanent visual impairment [5].

The transplantation of retinal tissue and other cell types has been explored to treat retinal disease. A clinical trial involving 10 patients either with AMD or RP showed that visual acuity was improved in 70% of patients by the transplantation of human foetal neural retina together with RPE into the subretinal space, without the use of immunosuppression [6]. Conversely, the transplantation of adult retinal cells has proved unsuccessful [7,8]. There is, however, limited availability of foetal tissue for transplantation, and the ethical issues associated with this approach mean that it is unlikely to be a feasible treatment option for a large number of patients.

In 2007, it was shown that human induced pluripotent stem cells (hiPSCs) can be generated from patients' dermal fibroblasts [9]. hiPSCs, like embryonic stem cells (hESCs) can be differentiated into both laminated neural retina and RPE [10–14]. Transplantation of retinal cells derived from hESCs and hiPSCs is considered to be a promising treatment for patients with macular degeneration and inherited retinal disease. Shirai et al. [15] were able to show that hESC can be coaxed to differentiate to laminated retinae which upon transplantation into the subretinal space of rat and primate models of retinal degeneration differentiated into a range of retinal cell types, developed a well-organised outer and inner nuclear layer and formed synaptic connections with the host retina. Patients with advanced retinal degeneration may require transplantation of RPE, photoreceptors, and/or other retinal cells, hence the generation of hESC/hiPSC-derived laminated retina presents a significant step towards the design of human clinical trials. To be able to achieve this, safe, robust and efficient differentiation methods that comply with good-manufacturing practice need to be devised. Preliminary results from stage I/II clinical trials have shown that RPE cells generated from hESCs can successfully be transplanted [16] into the subretinal space without causing adverse events in patients with AMD or Stargardt's disease, however clinical trials on transplantation of neural retinal sheets from hESC or hiPSC have not yet been performed. This is due in part to the length of current differentiation protocols (up to 250 days) [17].

Generation of retinal tissue is useful not only for transplantation purposes but also for the study of retinal diseases *in vitro*. The normal retina consists of multiple layers of neural tissue, which are in direct contact with and supported by the RPE. Both tissues are required for visual function. Several retinal diseases affect the neural retina and the RPE, yet the way in which each of these tissue types are affected, is not well understood [18]. Therefore the development of suitable protocols which result in the generation of neural retina in conjunction with RPE may be useful for studying retinal disease [19].

It is increasingly being recognised that the extracellular matrix (ECM) is important for the correct development and function of the retina both *in vivo* and *in vitro* [20–25], and changes in the ECM are associated with age-related degenerative changes in the retina including AMD [26]. Mutations affecting several components of the retinal ECM have been identified in patients with retinal disease [27–33]. Several animal models have also demonstrated how mutations in other ECM components can affect retinal ontogenesis and are associated with age-related degenerative changes [34–38]. It was hypothesised that recreation of the retinal microenvironment during hESC and hiPSC differentiation may provide the critical micro-environmental cues that are needed for their efficient differentiation to fully laminated neural retina with RPE.

The retinal ECM and Bruch's membrane (BrM), are enriched in proteoglycans [39,40]. A number of studies have shown that the major component of the retina is hyaluronic acid (HA), a large non-sulphated polysaccharide which binds a number of secreted proteins including other ECMs, such as link proteins and proteoglycans [24,40–42]. Recent work has suggested that HA-based hydrogels can drive neural [43] and retinal differentiation under 3D conditions and can also be used to deliver cells into the retina [44–48] as well as other areas of the CNS [49,50]. Similarly, RGD-alginate hydrogels appear to be promising scaffolds and have been successfully used to transplant primary foetal retinal tissue in rats [51] and to promote neural differentiation of mouse ESCs [52]. Alginate has been shown to maintain good viability of encapsulated primary and adult human RPE cell lines [53,54]. Furthermore, encapsulation in 1% alginate hydrogels has been shown to enhance the pigmented RPE phenotype of both human and porcine primary adult RPE and the expression of typical RPE markers such as RPE65 and tyrosinase [53,55]. Additionally, both alginate and HA are used in ophthalmic products, including those used intraocularly [56,57], and are well tolerated in the eye.

The addition of insulin-like growth factor 1 (IGF-1) to serum-free media has previously been shown to enhance formation of hESC-derived 3D laminated neural retina containing functional photoreceptors with membrane capabilities amenable to phototransduction [13]. Here it was investigated whether the 3D culture of hESC- and hiPSC-derived tissue, in the same defined media with IGF-1, within HA, HA/gelatin, 0.5% RGD-alginate or 1% RGD-alginate could enhance retinal differentiation compared with suspension culture in media alone. The resulting effects of hydrogel encapsulation on both RPE and neural retina formation were assessed.

2. Methods and Materials

Unless otherwise specified, all reagents were purchased from Sigma Aldrich (Dorset, UK).

2.1. Cell culture and generation of embryoid bodies (EBs)

The experimental procedure is summarised in Fig. 1. The H9 hESC line (Wicell Inc.) and SB-AD3 hiPSC line (derived and fully characterised) were cultured on growth factor reduced Matrigel-coated 6-well plates in mTeSR1 media (Stem Cell Technologies, Cambridge, UK) supplemented with penicillin-streptomycin (P/S, 1% v/v). EBs were generated by dissociating cells at 90% confluence with Accutase (Thermo Fisher Scientific) and seeding 9000 cells into each well of a 96-well lipidure-coated U-bottom plate (Amsbio, MA, USA) in 100 µl of mTeSR1 with 10 µM ROCK inhibitor (Y27632, Tocris, Bristol, UK). On day 3, media was changed to differentiation media (DMEM/F12 with 1% P/S, Thermo Fisher Scientific 20% KOSR (Thermo Fisher Scientific, Glasgow, UK), IGF-1 (5 ng/ml, R and D systems, Minneapolis, USA) and B27, Thermo Fisher Scientific). Media was changed every 3 days thereafter, with serum reduced to 15% at day 5, then 10% at day 9, before finally culturing the cells in serum-free media from day 37 onwards, supplemented with 1% P/S, 10 ng/ml IGF-1,B27 and N2 (Thermo Fisher Scientific, Glasgow, UK). EBs were maintained in 96-well plates until day 12 when they were transferred to ultra-low-attachment plates (Corning) and either kept suspension in media (controls) or encapsulated in hydrogel (0.5% RGD-alginate, 1% RGD-alginate, HA or HA/gelatin – as specified below) and cultured until day 45. Control EBs were then cultured in 3D suspension in media only throughout the duration of the experiment (45 days) as previously described [13]. Scans, photos and samples of EBs were taken at day 30 and 45 for analysis via immunohistochemistry (IHC), qRT-PCR and flow cytometry.