Editorial

In vitro eye embryogenesis from induced pluripotent stem cells for ocular surface disease treatment

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In their letter to *Nature*, Hayashi *et al*. describe a new method for the generation of ocular surface epithelium-like cells by incubating human induced pluripotent stem cells (iPS) into four defined concentric zones mimicking the developing eye, which the authors named as self-formed ectodermal autonomous multi-zone (SEAM) (1). Different SEAM zones contained cells with characteristics of the ocular surface ectoderm, the lens, the neural retina and the retinal pigment epithelium. Isolated corneal surface-like cells successfully reinstated a corneal epithelium in a rabbit limbal stem cell deficiency (LSCD) model. The paper offers a new option for patients with LSCD.

Corneal epithelial integrity is essential for visual function. The non-keratinized squamous corneal epithelium is continuously regenerated by the limbal stem cells (LSCs) that reside in the palisades of Vogt of the peripheral cornea. Damage to this region can lead to irreversible LSCD, resulting in impaired regeneration of corneal epithelial cells and keratopathy (2,3).

In cases of unilateral LSCD, autologous LSC transplantation from the healthy to the diseased eye can be performed by limbal autografting, first described by Kenyon and Tseng in 1989 (4). However, while success rates between 45% and 100%, depending on the degree of LSCD and other comorbidities (2,5), have been reported, limitations remain: large limbal biopsies entail a risk of LSCD for the donor eye, as well as minor complications, e.g., discomfort, chronic inflammation, scarring and infection (3). The *ex vivo* expansion of LSCs, established by Pellegrini *et al*. in 1997 (6), showed the risk of donor decompensation could be minimized and has provided a road map for successful stem cell therapy use and approval through regulation (5,6). However, treatment costs are high with the need for clean-room facilities, trained staff as well as GMP-qualified culture reagents (2,7). Finally simple limbal epithelial transplantation (SLET), a one-step surgical procedure combining the placement of healthy limbal tissue fragments on hAM, which is directly anchored on the recipient's cornea, has shown promising results although long-term studies are required (8).

In cases of bilateral LSCD, where autologous LSC transplantation is impeded, other allogenic sources, such as living related or cadaveric donors, are required. However, the long-term success rates are not as good as autologous tissue, and patients will require the use of long-term immunosuppression (2).

Consequently, in recent years, researchers have been focusing on alternative sources of corneal epithelium-like cells. Non-corneal autologous surface epithelia, e.g., conjunctival epithelial cells or cultivated oral mucosal epithelium, have proven to be able to stabilize the corneal surface in the short to mid-time range in clinical trials (9,10).

However, the resulting ocular surface has been shown to vary in its stratification and the number of cell layers, which can lead to a mixed phenotype and suboptimal vision. Further these epithelia do not express anti-angiogenic factors, such as soluble FLT1, TIMP3, and TSP1 and the majority of patients may develop corneal neovascularization in the long-term (10). However, a distinct advantage is the lack of use of oral immunosuppression.

In order to improve on this, alternative stem cell sources have been investigated with the objective of generating cells as closely resembling human corneal epithelial cells as possible:

(I) Pluripotent embryonic SCs (ESCs) are self-renewing and represent a potentially infinite source that can differentiate into virtually any cell type. Human ESCs have been shown to exhibit a corneal epithelial-like phenotype [expressing ΔNp63α (P63) and cytokeratin (K) 3/12] when cultured in limbal fibroblast-conditioned medium (11). However, controversial ethical issues, differentiated cell purity, identity and the risk of teratoma formation have limited the implementation from experimental results towards clinical use (12);

(II) Dental pulp stem cells (DPSCs), express markers in common with LSCs, such as ABCG2, Integrin b1, vimentin, connexin 43 and K3/12 (13). Transplantation of a tissue-engineered cell sheet has been shown to reconstruct rabbit corneas with mild LSCD. However, in extensive LSCD, the reconstructed epithelium consisted of unnatural flattened cells (14). DPSCs also express pro-angiogenic factors increasing the risk of corneal neovascularization (15);

(III) Murine hair follicle bulge-derived stem cells have been chemically induced to a corneal epithelial-like phenotype expressing K12, and have shown 80% repopulation efficiency of the corneal surface in a mechanical mouse LSCD model (16);

(IV) Adult mesenchymal stem cells (MSCs) are proliferative and multipotent stem cells that can differentiate into cells of various lineages. They can be harvested from allogenic sources e.g., umbilical cord linings, but also from autologous sources, such as bone marrow and adipose tissue, hence avoiding ethical issues (17). Human bone marrow and adipose-derived MSCs have been shown to be able to be differentiated into corneal epithelial lineage, improving corneal healing in rat alkali burn models (17,18). However, reports have been variable and feeder cells/conditioned medium and several *in vitro* induction steps impede the implementation of reliable protocols (3,7).

In vitro ocular organogenesis has been described before. Eiraku *et al*. first reported in *Nature* in 2011 the autonomous formation of an optic cup in a three-dimensional culture (Matrigel, Corning Life Sciences, USA) of mouse embryonic stem cell aggregates, that generated stratified neural retinal tissue (19). Nakano *et al*. similarly demonstrated the self-formation of optic cups and stratified neural retina from human ESCs in 2012 (20). The human ESC-derived optic cup was much larger than the mouse ESC-derived one and the neural retina grew into multilayered tissue containing both rods and cones, whereas cone differentiation was rare in mouse ESC culture. Reichman *et al*. demonstrated in 2014 the generation of retinal pigmented epithelial cells and self-forming neural retina-like structures containing retinal progenitor cells from iPS in a floating culture technique (21).

Hayashi *et al*., however, are the first group to specifically grow cells with ocular as well as non-ocular surface ectoderm and lens characteristics within an *in vitro* autonomously grown eye tissue (1). A strong appeal of their approach is that the microenvironment of a SEAM allows for a much more efficient and epigenetically stable differentiation of cells. Further studies are required to show the reproducibility of this technique.

The authors, like Reichman *et al*. before (21), relied on iPS. These stem cells have the benefit of being derived from somatic adult tissue, hence avoiding the controversial ethical issues, and the need for aggressive post-transplantation control for immune-mediated rejections, especially when they can be obtained from autologous sources. However, the widespread utilization of iPS is limited by their potential risks of oncogenic transformation, the problematic epigenetic memory and a low production efficacy. Hayashi *et al*. previously reported the lentiviral generation of iPS from human corneal limbal epithelial cells with a rate as low as 0.0005% (22).

In this publication the authors report favorable efficacies in their SEAM approach with 7.7% of incubated iPS building colonies, of which 67.9% spontaneously separated into four visible concentric zones, developing into all cell types essential for the organogenesis of an entire eye (1).

Ocular surface epithelial cells have been derived from iPS and used for treatment in animal models before. Hayashi *et al*. were the first to reprogram iPS from human

adult dermal fibroblasts and corneal limbal epithelial cells and to induce them to corneal epithelial like character in 2012. However, apart from the aforementioned low reprogramming efficacy, cells were not tested in animal models and DNA methylation analysis revealed epigenetic differences between the iPS-generated and control human corneal epithelium, even though no significant differences in corneal epithelium-related genes such as *K12*, *K3*, and *PAX6* were detected (22). Epigenetic differences to primary corneal limbal cells were confirmed by Sareen *et al*.; limbal-derived iPS had fewer unique methylation changes than fibroblast-derived iPS, suggesting retention of epigenetic memory during reprogramming (23). Cieślar-Pobuda *et al*. found that differentiation of iPS to corneal epithelium-like cells is a slow process (3 weeks) and that pluripotency genes remained activated, implying a risk of malignant transformation (24). Mikhailova *et al*. showed that small molecules added to the medium (TGFβ inhibitor SB-505124, Wnt inhibitor IWP-2 and bFGF) could significantly suppress pluripotency activity and improve differentiation of iPS to corneal epithelium-like cells finding P63 expression in 25–95% of induced cells, depending on the medium composition and time point (25). However, they also described variations in other corneal epithelial cell markers questioning cell purity.

This issue also became apparent in this publication, as cells for corneal epithelial therapy had to be meticulously separated. After media changes and isolation of zone 3 cells by pipetting, Hayashi *et al*. separated corneal epithelial progenitor cells (SSEA-4+/ITGB4+) from the ocular surface epithelial lineage by FACS (14.1%). Conjunctival epithelial cells were obtained as SSEA-4– cells (16.6%) (1,26).

In addition cells expressed different ocular surface tissue characteristics depending on their passage. While P2 cells mainly mimicked conjunctival epithelium [Suppl. Table in (1); PAX6+, K13+, p63+, K12–, HOX– and later also MUC5AC+ and K7+ goblet cells appeared] and non-ocular epithelium characteristics (PAX6–, K13+, p63+, K12–, HOX+), cells from passage 3 expressed cell markers typical of corneal epithelial (PAX6+, K13–, p63+, K12+, HOX–) and limbal cells (PAX6+, K13–, p63+, K12+ low, HOX–). Unfortunately further markers associated with the corneal epithelium (K3, involucrin, connexin 43, ZO1, occludin, CD98, 166 and 340) or limbus (ABCG2 and 5, K15 and 19, vimentin, EGFR, integrin α9 and β1) were not specified.

In the organogenesis approach carried out by the authors, however, ocular surface development proved BMP4/TGFβ dependent, as inhibition of these factors disrupted SEAM formation (1). Induction of transplantable cells was time-consuming (13–18 weeks) and cost intensive, as cells slowly differentiated to mature PAX6+, P63+, K12+ corneal epithelium-like character.

The transplanted sheet reformed a stratified cornea-like epithelium in a surgically induced LSCD model. The authors conclude that they are now in the position to initiate first in human trials. However, further investigations specifying epigenetic differences, e.g., DNA-methylation, teratoma risk, proof of long-term phenotype stability and an ideally simplified and xeno-free GMP differentiation protocol might be necessary before treatment in patients becomes reality.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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