# Title: Co-differentiation of corneal keratocyte-like cells along with endothelial cells from human pluripotent stem cells - from heterogeneity towards enriched cell populations.

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## Abstract

Corneal keratocytes (CK) and endothelial cells (CEnC) maintain corneal stromal transparency. Damage to these layer causes scarring, clouding, and blindness. Although corneal transplantation is effective, donor shortages limit its availability. Human pluripotent stem cells (hPSC) offer a promising alternative for generating corneal cell types. While hPSC-derived epithelial cells and CEnC are well-studied, progress in hPSC-derived CK has lagged due to reliance on animal-derived materials, hindering clinical translation. This study investigates the differentiation of hPSC to CK using defined culture conditions to enrich both CK and CEnC.

hPSC cultured on LN-521 coating were differentiated using small molecules inhibitors of TGFβ and GSK-3. Additionally, retinoic acid (RA) was used at different concentrations with (mEn protocol) or without (En protocol) FGF, to derive hPSC-CK. Characterization was performed using qPCR and immunofluorescence (IF) with quantification on days(D) 8/10/13. To enrich hPSC-CK, D10 cells were differentiated on collagen-1 coated plates with keratocyte differentiation medium. Similarly, hPSC-CEnC were enriched through metabolic starvation using serum-free medium or by extending the media change durations.

The differentiation yielded a heterogenous culture containing both CEnC and CK. By D6-8, hPSC-CEnC monolayer was formed, and further differentiation produced PAX6-rich cells surrounded by lumican-secreting hPSC-CK. By D13, 'En' protocol-derived hPSC-CK showed significant fold-changes in keratocan (16.17±1.35), lumican (147.35±98.7), and PAX6 (984.8±288.49) with notable expression of CEnC (CD166:38.84%; Na-K:78.38%) and CK (lumican:20.45%; decorin:52.24%) markers. Enriched hPSC-CK were lum+/deco+/pax6-/cd166- and hPSC-CEnC, CD166+/ZO-1+. In conclusion, CK can be derived and enriched from hPSC with xeno-free, defined culture conditions along with CEnC using a single protocol.