Long term high glucose exposure induces premature ageing in retinal endothelial cells

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Introduction

- Diabetic retinopathy (DR) is a frequent microvascular complication in diabetic patients and leads to visual impairment and blindness.
- Diabetes has been associated with accelerated ageing.
- Cellular senescence is an irreversible state of cell cycle arrest, characterized by multiple phenotypic changes and impaired cell functionality.
- Features of cellular senescence have been described in diabetic retinal vasculature.

Aim

- To investigate the ageing programme in cells known as senescence by exposing human retinal microvascular endothelial cells (HRMECs) to diabetic-like conditions and characterising their cellular ageing process.

Results

Figure 1. Accelerated cellular senescence in human retinal microvascular endothelial cells cultured under high glucose conditions

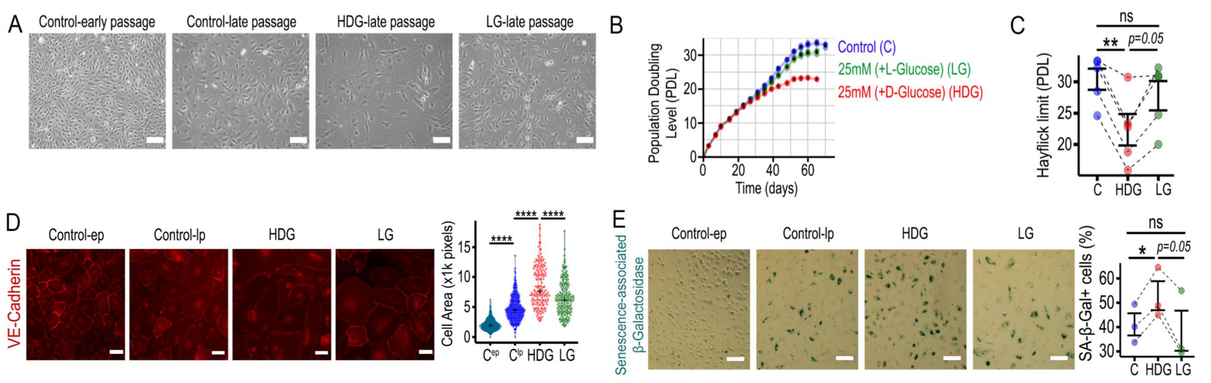

Figure 1. (A) Phase-contrast 10X microscopy images of HRMECs at early passage (P4-P5), late passage (P11-P16) and late passage HRMECs treated with high glucose conditions (HDG or LG). **(B)** Growth curves for control (5 mM D-glucose), osmotic control (25 mM with L-glucose) and high glucose (25 mM D-glucose) HRMECs. **(C)** Graph comparing population doubling level (PDL) at the Hayflick limit, for statistical comparison. **(D)** 20X images of VE-cadherin immunostaining in HRMECs after 4 weeks in culture to evaluate cell surface area. **(E)** 10X phase-contrast images of senescence-associated β-Galactosidase staining in HRMECs after 4 weeks in culture to identify and quantify senescent endothelial cells. *p < 0.05, **p < 0.01, ****p < 0.0001, ns: not significant. PDL, Population doubling level; C, control (5 mM D-Glucose); LG, 25 mM (+L-glucose); HDG, 25 mM (+D-glucose); ep, early passage; lp, late passage. *Scale bar: 150 µm.*

Figure 2. Endothelial function is impaired in HRMECs cultured with 25mM D-glucose for 4 weeks

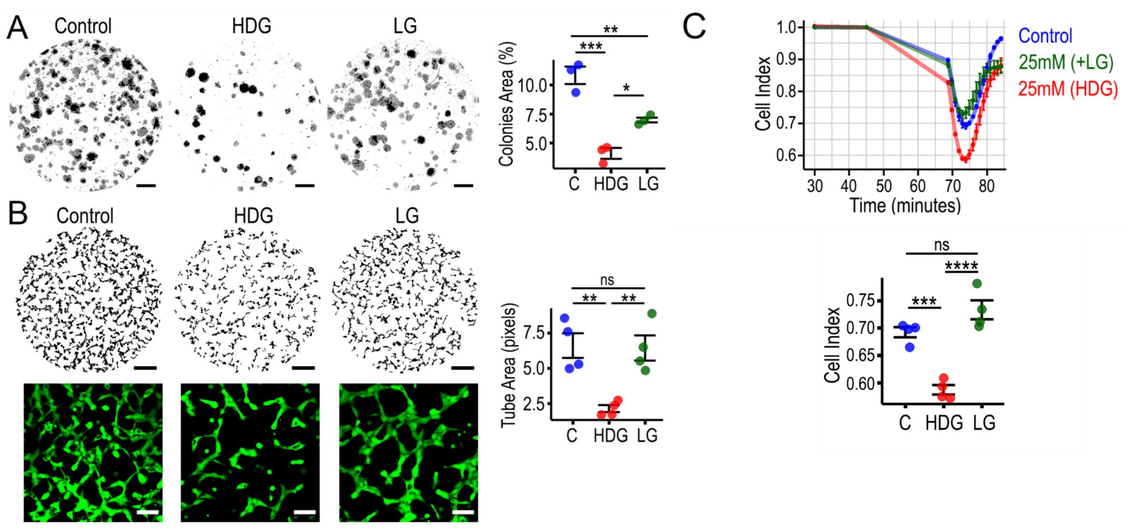

Figure 2. (A) Representative images of clonogenic assay and quantification with statistical comparison. 10X images were stitched together and the whole well of a 6-well plate is shown. *Scale bar: 4 mm*. **(B)** 3D- tube formation assay and quantification with statistical comparison. 10X images were stitched together and the whole well of a 96-well plate is shown. *Scale bar: 500 µm*. For 20X images, cells were stained with calcein in green. *Scale bar: 100 µm*. **(C)** Cell index traces from xCELLigence system to evaluate barrier function. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: not significant.
C, control; LG, 25 mM (+L-glucose); HDG, 25 mM (+D-glucose).

Figure 3. Cell metabolic profiling identified diminished glycolytic capacity in HRMECs cultured under high glucose conditions

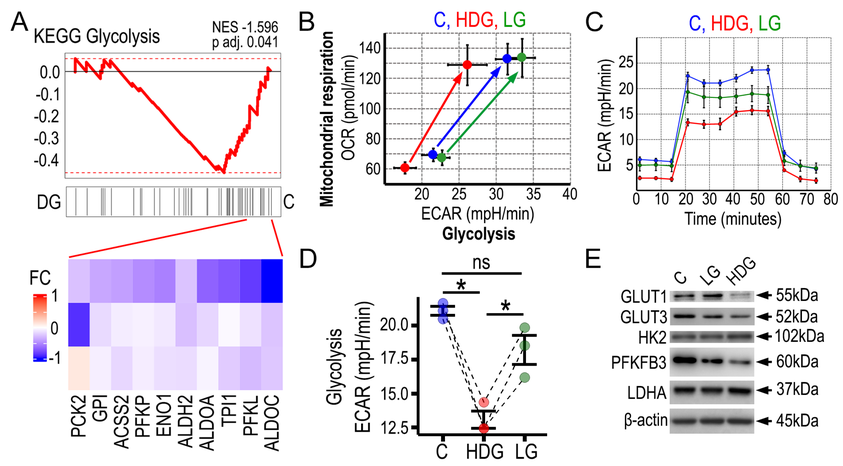

Figure 3. (A) Gene signature from KEGG Glycolysis was used for the gene set enrichment analysis (GSEA). Transcriptome data from bulk RNA sequencing of three biological replicates of HRMECs cultured with 25 mM D-glucose vs. the 5 mM control at 4 weeks of culture. Heatmaps below depict the leading edges for transcripts responsible for major differences. **(B)** Energy phenotype plot from Seahorse XFe96 analyzer depicting assessment of glycolysis as extracellular acidification rate (ECAR) and mitochondrial respiration as oxygen consumption rate (OCR) at baseline and stressed phenotypes. **(C)** Glycolysis stress assay to characterize glycolysis in HRMECs under different culture conditions. Injections in the assay were glucose at 20 min, oligomycin at 40 min, and 2-DG at 60 min. **(D)** Statistical comparison of basal glycolysis measures using Seahorse Glycolysis stress assay in three biological replicates. **(E)** Western blot analysis to compare expression of glycolysis-related proteins. β-actin was used as the loading control. *p < 0.05, ns: not significant. NES: Normalized Enrichment Score. p adj: adjusted p value. C, control in blue; LG in green, 25 mM (+L-glucose); HDG in red, 25 mM (+D-glucose).

Figure 4. Diabetic mice (db/db) retinas exhibit accumulation of senescent endothelial cells

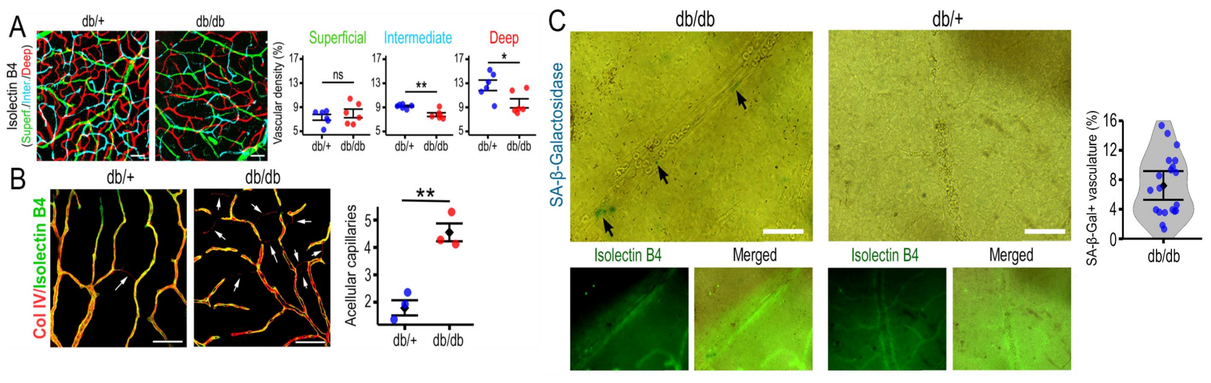

Figure 4. (A) Retinal tissue from 6–9-months mice, comparing db/+ and db/db, stained with Isolectin B4 to identify vasculature, and pseudocolored based on confocal sections to distinguish three retinal plexuses; superficial shown in green, intermediate in cyan, and deep in red. Vascular density was assessed as percentage of total area. **(B)** Retinas were stained with antibody against Collagen IV (Red) and with Isolectin B4 (Green) to identify acellular capillaries and compared their frequency in non-diabetic to diabetic. **(C)** Retinas were stained with SA-β-Galactosidase to identify senescent cells by the blue staining. Counterstaining with Isolectin B4 in green to show senescent cells within the retinal vasculature. **p < 0.01, *p < 0.05, ns: not significant. *Scale bars: 50 µm.*

Figure 5. Diabetic mice (Akimba) retinas exhibit accumulation of senescent endothelial cells

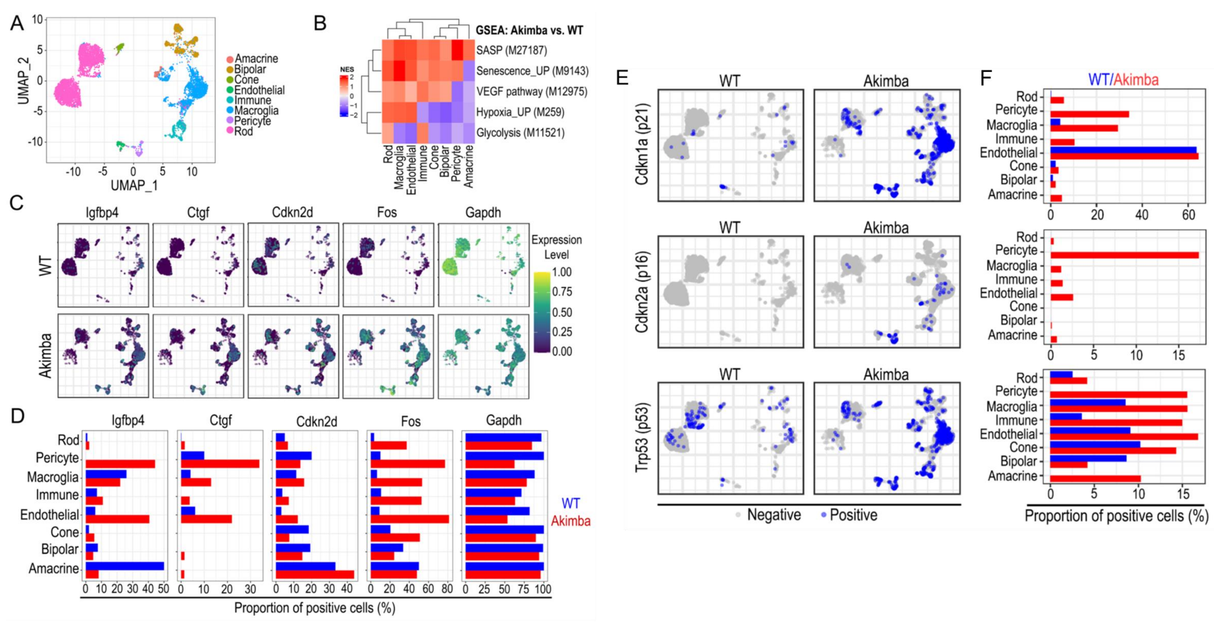

Figure 5. (A) Clustering of retinal cells to distinguish cell types. **(B)** Gene set enrichment analysis comparing diabetic Akimba retinas vs. wild type controls per cell type. **(C)** Expression level for genes from SASP, senescence, and glycolysis signatures to highlight differences at transcript level. **(D)** Bar plots quantifying proportion of positive cells for transcripts. **(E)** Expression level for senescence-associated genes, shown as positive in blue or negative in grey. **(F)** Bar plots quantifying proportion of positive cells for transcripts.

Conclusion

- Long term exposure to high glucose as an accelerated the senescence program in HRMECs, with impaired endothelial function and glycolysis
- We identified accumulation of senescent cells in two mouse models of diabetic retinopathy

- Some unanswered research questions from our findings:

1) The role of aging-associated inflammatory molecules as a driver of retinopathy complications needs further characterisation
2) Cellular aging is related to increased metabolism, but in a diabetic milieu we observed decreased metabolism
3) The identification of a senescence signature in relation to DR will enable the development of therapeutic strategies to delay or reverse cellular senescence in the ageing retina

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