Development of a 3D living skin equivalent to explore the interplay between senescence and the skin ageing phenotype

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Introduction

- Replicative senescence is a stable cell cycle arrest driven by p16 and/or p21 pathways¹.
- Senescent cells accumulate in ageing tissue causing loss of regenerative potential.
- Clearance of senescent cells in mice slows progression of age-associated phenotypes^{2,3}.

Figure 1 – Replicative senescence is driven by the p16 and/or p21 pathways. Continuous replication causes cells to enter senescence at the Hayflick limit as a stress response to critically short telomeres. Lowe *et al.* (2015)⁴ established a protocol to reverse deeply senescent (DS) cells to an early proliferating phenotype (EP) using siRNA knockdown, providing an opportunity to explore the impact of senescence in ageing and disease.

Figure 2 – The skin ageing phenotype. Skin is an ideal tissue to investigate ageing as it exhibits a clear intrinsic ageing phenotype and can be modelled using 3D organotypic methods to create a 'living skin equivalent' (LSE).

Aims

How does senescence influence the skin ageing phenotype?

How can the senescence pathway be manipulated to reverse the stable senescent phenotype?

Can the skin ageing phenotype in an LSE be alleviated using senescence rejuvenation?

Identification of novel markers of senescence and/or ageing.

Figure 3 – Schematic of project aims.

Methods

Figure 4 – Skin can be modelled *in vitro* by creating a 3D LSE. Fibroblasts are suspended in a 3D dermal matrix; keratinocytes are then seeded on the LSE surface and the entire LSE is brought to the air/liquid interface to initiate epidermal stratification. By incorporating either EP or DS adult human dermal fibroblasts (HDFs) in the dermis, young and aged models of skin can be created.

Results

Figure 5 – Serial passaging leads to a DS phenotype in adult HDFs. HDFs show an increase in SA-β-gal when brought to DS (B), compared to EP cells (A) which can be quantified (C) using Hoechst[®] to identify cells. Morphological changes include enlarged nuclei, and a large, flattened shape. Images show SA-β-gal (greyscale) and Hoechst (blue). SB=50μm, n=1.

Figure 6 – Senescent cells can be identified using a panel of markers including CDK inhibitors. DS HDFs exhibit phenotypic changes such as an increased expression of the CDK inhibitors p16 and p21 (A and B). Expression of SA-markers can be quantified, e.g. there is an increase in both p16 and p21 positive HDFs in DS compared to EP (C and D). SB=50μm, n=1.

Figure 7 – Senescence can be induced in HDFs using UVB irradiation. EP HDFs were induced into premature senescence using UVB irradiation. Optimisation of UVB dose showed 12mJ/cm² induced premature senescence (UV-PS) (A), including a morphological change compared to untreated HDFs (B). UV-PS HDFs exhibit a significant increase in the percentage of p16 (C) and p21 (D) positive cells, as previously shown in DS HDFs. UV-PS and DS HDFs both show an increase in DNA damage foci compared to EP HDFs (E-G), however UV-PS exhibit a different DNA damage profile than DS HDFs (H). Further work has demonstrated the UV-PS phenotype is transient in HDFs. SB=50μm, n=2. **p<0.01, ***p<0.001, ****p<0.0001.

Figure 8 – LSEs reflect the dermal and epidermal structure of normal human skin. LSEs using N/TERTs with HDFs (A) show stratification of the epidermis reflecting the structure of human skin (C). LSEs using primary human keratinocytes (PHKs) (B) show increased stratification and cornified layer compared to N/TERTs (A). SB=100μm, n=3.

Figure 9 – LSEs express skin structural markers in a similar pattern to human skin. LSEs using PHKs with HDFs (A) show suprabasal expression of involucrin and dermal expression of vimentin as seen in human skin (B). Keratin 14 is expressed throughout the epidermis in LSEs compared to basal expression in skin. SB=100μm, n=1.

Figure 10 – HDF^{EGFP}s show an altered morphology in 3D dermal gels compared to 2D culture. EP HDFs transduced with EGFP were grown in both 2D and 3D culture conditions and stained with Hoechst[®]. 3D dermal gels were constructed in transwells and transferred to chamber slides for imaging (A). HDFs in 3D gels (C) appear to have an increased number of projections and fibrous morphology compared to HDFs grown in 2D (B). SB=50μm, n=1.

Future Work

- Analyse the phenotypic differences between aged LSE using DS and UV-PS HDFs and EP HDFs.
- Create HDF^{RFP} in order to distinguish between EP and DS HDFs within a mixed population, in both 2D and 3D.
- Optimise senescence rejuvenation protocol using siRNA knockdown of CDK inhibitors.

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