p16INK4A Influences the Aging Phenotype in the Living Skin Equivalent

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TO THE EDITOR

Senescence is a state wherein cells are metabolically active but are unable to replicate due to the increased expression of cell cycle checkpoints, such as p16INK4A, that prevent passage of cells through the cycle. p16INK4A expression increases significantly in the basal layer of human epidermis and in dermal fibroblasts, making it a reliable biomarker of cellular aging in human skin (Krishnamurthy et al., 2004; Ressler et al., 2006; Waaier et al., 2012). Moreover, p16INK4A has continued to gain strong support as an important component of the aging process (Sharpless, 2004; Campisi and d’Adda di Fagagna, 2007; Baker et al., 2011).

Using in vitro living skin equivalent (LSE) models constructed in-house (Aho et al., 2012) with epidermal keratinocytes (HEKs) from photo-protected sites of different age human donors, and a single neonatal fibroblast donor (Supplementary Information online), we demonstrate that the age of the keratinocyte donor differentially impacts the model’s phenotype. Additionally, we have notably demonstrated that it is possible to develop an atrophic, aged model made from young (30–40 years) donor HEKs and to revert aged (53–66 years) donor HEK LSEs to a younger phenotype, by modulating the level of p16INK4A expression.

Similar to in vivo, we confirmed that the level of p16INK4A in the aged model increases in the basal cell layer and in the lower epidermis, supporting its usefulness as a biomarker for the aging phenotype. HEKs from two young donors formed normal stratified epithelium with a well-organized basal layer, distinct cell layers, and a normal pattern of differentiation (Figure 1a, left). Models using five different aged donor HEKs displayed an unorganized basal cell layer, fewer spinous and granular layers, and impaired differentiation, as reflected by decreased filaggrin immunostaining (Figure 1a, right). In this model, substantially fewer Ki-67 positive–stained cells were seen in the basal layer. This decrease is in agreement with the observations of Gilhar et al. (2004) in skin biopsies; however, others have seen either an increase (Giangreco et al., 2010) or no change (Ressler et al., 2006) in Ki-67 staining with age in vivo. This may be due to variations between donors or due to in vitro to in vivo differences.

We compared p16INK4A expression in LSEs made from young and aged donor cells by immunohistochemistry (IHC) and immunoblot, and the micrographs shown are representative of the donor strains tested. In the young donor LSEs, light staining of p16INK4A was observed throughout the epidermis and at a later time point, staining was specifically localized to the basal layer. In these samples, p16INK4A was barely detectable by immunoblot (Figure 1b and c). Conversely, we saw higher p16INK4A expression in the aged LSEs from an early time point and throughout the experiment. Strong staining was identified within the basal cell layer and in the lower stratum spinosum by IHC, substantiated by increased protein detected by immunoblot. This increase in p16INK4A appears to be inversely correlated with the decrease seen in markers of proliferation and differentiation, as first observed by Ressler et al. (2006).

Next, we altered the expression of p16INK4A in LSEs produced from young donor HEKs by lentiviral expression of p16INK4A, driven by a truncated
keratin 14 (K14) promoter (Di Nunzio et al., 2008) to target delivery to the physiologically relevant basal layer of epidermis. Here, we observed a dramatic difference in phenotype between LSEs overexpressing p16INK4A and the controls (Figure 2a). Cultures with increased p16INK4A expression showed significant atrophy, with a thinner viable epidermis and no apparent stratum corneum, analogous to the LSEs we had previously prepared from aged donor HEKs. We confirmed these findings with another young donor (not shown), and in both models we observed a phenotypic dose-dependent response, where the severity of atrophy was dependent on the level of p16 gene expression in the model (Figure 2b).

In the young donor LSEs overexpressing p16INK4A, dose-dependent downregulation of Ki-67, filaggrin, and caspase-14 was observed as compared to the control samples. Moreover, increased levels of p16INK4A protein were detected by IHC in the p16INK4A-infected LSEs, with strong staining in the basal layer reflecting detection of both endogenous and recombinant protein. This was confirmed by the differential

**Figure 1.** p16INK4A is associated with the aging epidermis. (a) Formalin-fixed, paraffin-embedded (FFPE) sections from young and aged donor keratinocyte living skin equivalent (LSE) models harvested at 7 days post air exposure. Sections were stained with hematoxylin and eosin or immunostained for detection of filaggrin (FLG), Ki-67, and p16INK4A. (b) Expression of p16INK4A protein from monolayer cell lysates (15 μg) harvested at the time of application to the LSE. (c) Representative immunoblot analysis for p16INK4A from young and aged LSE lysates (15 μg) from duplicate cultures harvested at three different time points post air exposure. Bar = 100 μm, applies to all FFPE images.

**Figure 2.** Reversal of phenotype by targeting p16INK4A in the LSE. (a) Young donor keratinocyte living skin equivalents (LSEs) infected with K14 promoter driven either p16INK4A (left) or lacZ control (right) lentivirus, harvested at 11 days post air exposure. Formalin-fixed, paraffin-embedded (FFPE) sections were stained with hematoxylin and eosin (H&E) or immunostained for V5, filaggrin (FLG), Ki-67, or p16INK4A. (b) Immunoblot analysis for caspase-14 (6 μg, left) and for p16INK4A (25 μg, right, bottom). (c) Aged donor keratinocyte LSEs infected with either p16 miR (left) or non-silencing control miR (right) lentivirus, harvested 7 days post air exposure. FFPE sections were stained with H&E or immunostained for filaggrin (FLG), Ki-67, or p16INK4A. (d) LSE lysates (15 μg) were analyzed for p16INK4A and for the differentiation markers loricrin and caspase-14. r-p16, recombinant p16INK4A; e-p16, endogenous p16. Bar = 100 μm.
Properly Selected Skin Cancer Treatments Are Very Effective

TO THE EDITOR

We read with interest Chren et al.’s study on the recurrence rates of nonmelanoma skin cancer (NMSC) treatments and the accompanying commentary by Dr. Stern entitled Cost Effectiveness of Mohs Micrographic Surgery (Chren et al., 2013, Stern, 2013). Dr. Chren described the recurrence rates for NMSCs diagnosed and treated at the San Francisco VA and UCSF dermatology clinics. The biopsying dermatologists selected the skin cancer treatment method (destruction, excision, or Mohs surgery.) The authors concluded that these treatments for NMSC were at

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