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Research paper

Characterisation of cell cycle arrest and terminal differentiation in a maximally proliferative human epithelial tissue: Lessons from the human hair follicle matrix

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ABSTRACT

Human hair follicle (HF) growth and hair shaft formation require terminal differentiation-associated cell cycle arrest of highly proliferative matrix keratinocytes. However, the regulation of this complex event remains unknown. CIP/KIP family member proteins ($p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$) regulate cell cycle progression/arrest, endoreplication, differentiation and apoptosis. Since they have not yet been adequately characterized in the human HF, we asked whether and where CIP/KIP proteins localise in the human hair matrix and pre-cortex in relation to cell cycle activity and HF-specific epithelial cell differentiation that is marked by keratin 85 (K85) protein expression. K85 expression coincided with loss or reduction in cell cycle activity markers, including *in situ* DNA synthesis (EdU incorporation), Ki-67, phospho-histone H3 and cyclins A and B1, affirming a post-mitotic state of pre-cortical HF keratinocytes. Expression of CIP/KIP proteins was found abundantly within the proliferative hair matrix, concomitant with a role in cell cycle checkpoint control. $p21^{CIP1}$, $p27^{KIP1}$ and cyclin E persisted within post-mitotic keratinocytes of the pre-cortex, whereas $p57^{KIP2}$ protein decreased but became nuclear. These data imply a supportive role for CIP/KIP proteins in maintaining proliferative arrest, differentiation and anti-apoptotic pathways, promoting continuous hair bulb growth and hair shaft formation in anagen VI. Moreover, post-mitotic hair matrix regions contained cells with enlarged nuclei, and DNA *in situ* hybridisation showed cells that were >2N in the pre-cortex. This suggests that CIP/KIP proteins might counterbalance cyclin E to control further rounds of DNA replication in a cell population that has a propensity to become tetraploid. These data shed new light on the *in situ*-biography of human hair matrix keratinocytes on their path of active cell cycling, arrest and terminal differentiation, and showcase the human HF as an excellent, clinically relevant model system for cell cycle physiology research of human epithelial cells within their natural tissue habitat.

1. Introduction

It is still largely unclear how somatic epithelial cell populations self-organise and regulate rapid, prolonged and tightly orchestrated cell cycling and terminal differentiation, without being frequently marred by abnormalities in tissue homeostasis (*e.g.* aberrant cell proliferation). Human scalp hair follicles (HFs) constitute a particularly interesting case in point. These are characterized by a very sustained, years-long phase of massive proliferation and growth (anagen VI) during which rapidly proliferating hair matrix keratinocytes (KCs) continuously must undergo cell cycle arrest as they shift from a state of active proliferation to post-mitotic terminal differentiation in order to uninterruptedly generate a hair shaft over many years (Paus and Cotsarelis, 1999; Xu et al., 2003; Oh et al., 2015; Bernard, 2017). As a complex semi-autonomous mini-organ, this makes the human HF an exemplary model system to study diverse facets of human biology *in vivo* and *ex vivo* (Westgate et al., 1993; Schneider et al., 2009; Gáspár et al., 2010; Kloepper

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et al., 2010; Ramot et al., 2011; Samuelov et al., 2012; Al-Nuaimi et al., 2013; Ernst et al., 2013; Hawkshaw et al., 2015; Langan et al., 2015; Oh et al., 2015), including the dynamics of cell cycling (Purba et al., 2016).

Studying the regulation of cell cycle and terminal differentiation dynamics within the human HF is also of significance in order to help develop treatment strategies to manipulate HF keratinocytes under physiological and pathological conditions (*e.g.*, androgenetic alopecia, alopecia areata, scarring or chemotherapy-induced alopecia) (Van Scott and Ekel, 1958; Weinstein and Mooney, 1980; Harries and Paus, 2010; Kloepper et al., 2010; Gilhar et al., 2012; Harries et al., 2013; Paus et al., 2013; Nieves and Garza, 2014; Langan et al., 2015; Purba et al., 2015, 2016; Dimitrov et al., 2016; Bernard, 2017).

The phenotypical and functional processes of hair shaft formation in human anagen HFs are well understood. For example, the field has greatly benefited from studies that have characterized the expression of specific differentiation-associated hair keratins (Langbein and Schweizer, 2005; Moll et al., 2008; Ramot et al., 2009; Rogers and Koike, 2009; Yamamoto et al., 2009; Ramot and Zlotogorski, 2015), or the epithelial-mesenchymal signalling cross talk between the matrix and the dermal papilla that promotes hair keratinocyte differentiation (Jahoda and Reynolds, 1996; Schneider et al., 2009; Enshell-Seijffers et al., 2010; Lee and Tumbar, 2012; Sennett and Rendl, 2012; Higgins et al., 2013; Rishikaysh et al., 2014; Rezza et al., 2016). However, the cell cycle dynamics of the human HF have as yet been defined only rather incompletely (Bull et al., 2001; Xu et al., 2003; Botchkareva et al., 2007; Purba et al., 2016), and the field is largely dependent on very general analyses of cell proliferation within the HF epithelium and its changes during experimental manipulation (*e.g.*, Samuelov et al., 2012; Al-Nuaimi et al., 2013; Ernst et al., 2013; Hawkshaw et al., 2015; Purba et al., 2016). Importantly, a well-defined model of hair formation from a cell cycle activity/arrest vantage point is still missing.

To fill this gap we have asked where proteins $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$, which belong to the CIP/KIP family of proteins, are expressed in the human hair matrix in scalp HFs *in vivo* and *ex vivo*. The study of CIP/KIP family member proteins is of interest as they are prominently implicated in cell cycle regulation as inhibitors of cyclin dependent kinases (CDKs), and as key regulators of other processes such as differentiation, apoptosis and endoreplication (Hiromura et al., 1999; Lloyd et al., 1999; Zhang et al., 1999; Deschênes et al., 2001; Drexler and Pebler, 2003; Denicourt and Dowdy, 2004; Nguyen et al., 2006; Egozi et al., 2007; Abbas and Dutta, 2009; Pateras et al., 2009; Ullah et al., 2008, 2009a; Fox and Duronio, 2013; Edgar et al., 2014).

Therefore, their expression patterns were assessed in freshly microdissected anagen VI scalp HFs. Furthermore, we probed the expression patterns of CIP/KIP proteins relative to cell cycle activity and arrest in matrix keratinocytes as they graduate from proliferation towards pre-cortical terminal differentiation, assisted by a range of *in situ* makers and techniques (including EdU incorporation during short-term HF organ culture as a marker of DNA synthesis (Langan et al., 2015; Purba et al., 2016)). Expression of the hair shaft keratin, keratin 85 (K85), was employed as a marker for hair matrix keratinocyte commitment to HF-specific terminal differentiation (Moll et al., 2008).

2. Methods

2.1. Tissue preparation

Occipital scalp tissue, from the Crown Cosma Clinic, Manchester, UK, was donated by patients under informed consent. The study was performed under local ethical approval from the University of Manchester. Tissue was handled in accordance to the Human Tissue Act (2004). HFs were microdissected from scalp skin (Langan et al., 2015), embedded in optimal cutting temperature and frozen in liquid nitrogen, and stored at −80 °C. For DNA fluorescence *in situ* hybridisation (DNA FISH), HFs were fixed overnight in 4% paraformaldehyde at 4 °C. Subsequently, HFs were incubated in 10%, 20% and 30% sucrose (in phosphate buffered saline (PBS)) for 30 min, 30 min and 1 h respectively at room temperature (RT) prior to embedding and freezing. 7 μm frozen tissue sections were prepared onto Superfrost™ Plus slides (Thermo Fisher Scientific, Waltham MA). Throughout the study, experiments were conducted in samples from ≥3 patient samples (minimum ≥2 anagen hair follicles per patient, and ≥2 sample sections per stain).

For the morphological evaluation of nuclear size and *in situ* hybridisation (ISH) staining, normal human scalp skin specimens were obtained from the archives of the Pathology Department of Murcia University General Hospital. Tissue had been formalin fixed, paraffin embedded and Haematoxylin & Eosin stained (H&E). After evaluation of the H&E sections, slides from the margins of surgically removed tumours (two cases), and from a cadaver (one autopsy case) were selected. Paraffin blocks from each case that showed normal anagen HFs were retrieved from the archives.

2.2. Immunofluorescence staining and microscopy

Phospho (Serine 10) Histone H3 (pH3) (1:50, rabbit #ab5176 Abcam, Cambridge, UK), 'pS780' – Phospho (Serine 780) pRb (1:50, rabbit #9307 Cell Signalling Technology, Beverly, MA), Keratin 85 (1:300, guinea pig, Lutz Langbein, Heidelberg), Ki-67 (1:20, mouse #M724001, Dako, Denmark), $p27^{KIP1}$ (1:100, Y236, rabbit #ab32034, Abcam, validated in knockout HAP1 cell line), $p57^{KIP2}$ (1:100, rabbit #ab4058, Abcam). Single and double immunofluorescence staining for these markers was conducted using an immunofluorescence microscopy protocol as described previously (Purba et al., 2015), unless otherwise indicated below.

Cyclin A (1:50, H-432, rabbit #Sc-751, Santa Cruz), cyclin B1 (1:50, GNS1, mouse #Sc-245, Santa Cruz), cyclin E (1:200, C-19, rabbit #Sc-198, Santa Cruz), cyclin E1 (1:100, HE12, mouse #ab3927, Abcam) and $p21^{CIP1}$ (1:50, CP74, mouse #P1484, Sigma, knockout validated in HCT116 cells (Ferrandiz et al., 2009)) immunofluorescence staining protocol was modified following fixation with ice-cold Acetone (−20 °C) fixation with the addition of the following steps: 1) 10 min permeabilization at RT *via* treatment of tissue sections with PBS + 0.5% Triton X100, followed by washing with PBS for 3 × 5 min per wash (implied hereafter, after each subsequent step). 2) Blocking, using 10% normal goat serum in PBS for 45 min at RT before proceeding to overnight primary antibody incubation (Purba et al., 2016). Secondary Alexa Fluor antibodies (Thermo Fisher Scientific) were utilised at a 1:200 dilution as appropriate (goat anti-mouse 488/594, goat-anti rabbit 488/594 and goat anti-guinea pig 488) (#A11001, #A11005, #A11008, #A11037, #A11073). All experiments were conducted in parallel with appropriate negative controls (*e.g.* exclusion of primary antibody). Judgement of staining specificity was aided by reference to comparable staining patterns detailed in prior studies in the human hair follicle, human skin or in other human tissues. *E.g.*, cyclins, pH3 and 'pS780' staining in human skin (Zanet et al., 2010), $p21^{CIP1}$ in human skin and hair follicle, (Pontén et al., 1995) $p27^{KIP1}$ in dental pulp (Klinz et al., 2013) and $p57^{KIP2}$ in placenta (Fukunaga, 2004; Merchant et al., 2005).

Immunofluorescence stains were imaged using fluorescence microscopy (Keyence BZ-8000, Osaka, Japan). Single channel fluorescent images were analysed in ImageJ software (NIH), whereby marker expression/signal within a defined region of interest (*e.g.* matrix or pre-cortex matrix) was analysed (*i.e.* fluorescence intensity by cell or region of interest). Values were graphed and statistically analysed using GraphPad Prism (versions 6-7) (Graph-