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A Primer for Studying Cell Cycle Dynamics of the Human Hair Follicle

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Abstract
The cell cycle is of major importance to human hair follicle (HF) biology. Not only is continuously active cell cycling required to facilitate healthy hair growth in anagen VI HFs, but perturbations in the cell cycle are likely to be of significance in HF pathology (i.e. in scarring, non-scarring, chemotherapy induced and androgenic alopecias). However cell cycle dynamics of the human hair follicle (HF) are poorly understood in contrast to what is known in mouse. The aims of this method review are two fold; principally to address this gap we present a primer to introduce histological techniques to study the cell cycle in the human HF whilst concurrently encouraging the exploitation of the human HF as a powerful and clinically relevant tool to investigate mammalian cell cycle biology in situ. To achieve this we describe methods to study general ‘proliferation’ (nuclei count, Ki-67 expression), apoptosis (terminal deoxynucleotidyl transferase dUTP nick end labelling, cleaved caspase 3), mitosis (phospho- histone H3, ‘pS780’), DNA synthesis (5-ethyl-2’-deoxyuridine) and cell cycle regulation (Cyclins) in the human HF, whilst also providing specific examples of dual labelling to further provide
instructive cell cycle analyses or to investigate the cell cycle behaviours of specific HF keratinocyte sub-populations (i.e. Keratin 15+ stem / progenitor cells).

Introduction

The cell cycle dynamics of murine hair follicles (HFs) during HF development and cycling have become progressively better defined over the past two decades (1–5, s1–s4) and have recently been further illuminated by the introduction of instructive in vivo-imaging techniques (6,7). In contrast, the cell cycle dynamics of the human HF are much less well-understood, as they have only been investigated partially in vivo (8,9) or within organ-cultured anagen VI scalp HFs undergoing the early stages of HF regression (catagen) (e.g. (10-14, s5)).

This has left an important gap on the human hair research map that needs to be filled-in. The current Methods Review introduces newcomers on how to best address this and to simultaneously aims to promotes the exploitation of the human HF as an excellent, clinically relevant model system for the study of human and mammalian cell cycle dynamics in vivo, ex vivo, and in situ.

Why human HF cell cycle dynamics are biologically and clinically important

Human HF organ-culture or xenotransplantation (9-11,15,16, s6) permits the in situ manipulation of a complex human (mini-)organ under clinically relevant conditions. By utilising this accessible human tissue and cell interaction system, one can study the function of distinct keratinocyte populations of human adult epithelial progenitor cells (17,18), their differentiated keratinocyte (KC) progeny and how they can interact within the HF microenvironment (i.e. hair matrix-KCs, outer root sheath-KCs, inner root sheath-KCs, companion layer KCS as well as KCS of the hair shaft cortex, medulla and cuticle) (19). As such, the human HF offers a unique model for characterising human epithelial cell cycle characteristics within an intact epithelial-mesenchymal interaction system. If studied under in vivo or xenotransplantation conditions, these human epithelial cell cycle dynamics can be explored in the presence of neural, vascular, nutritional/metabolic, and endocrine inputs.

Characterising and manipulating cell cycle dynamics in the human HF is important for a number of reasons, beyond the fact that cell cycling is important for healthy human hair growth and disruption of normal cell cycling may lead to human HF pathology. For example in conditions such as telogen effluvium, HF miniaturisation during pattern balding (20,21), chemotherapy-induced alopecia (22-
hirsutism, hypertrichosis \((s7)\) and pathological changes in human epithelial stem cell cycling during radiation-induced alopecia \((25)\) / cicatricial alopecia \((26)\).

Cell cycle regulation is an elementary component of human HF physiology, an understanding of which provides essential background information when manipulating human HF growth in culture. Specifically, this background information is needed when assessing and analysing changes in proliferation dynamics in response to novel candidate hair growth promoters (e.g. \((27-31)\)) or inhibitors (e.g. \((32,33,s8)\) as well as HF responses to oxidative damage \((34)\), chemotherapy \((22-24)\) or to UV or ionizing irradiation \((25,35)\) However, our incomplete understanding of human HF cell cycle dynamics impedes the interpretation of the HF proliferation data obtained in such studies.

Due to the need for novel hair growth promoting and inhibitory agents to treat common human hair growth disorders, a comprehensive, professional and standardized method of analysis to investigate intrafollicular cell cycle dynamics is required. This will facilitate quantitative appraisal of novel hair growth agents in micro-dissected HFs or skin biopsies from trials/volunteers from different ethnicities, age groups or genders and will make the results better-comparable between different laboratories. For these reasons, the current primer attempts to provide the field with some basic methodological guidance on how best to study human HF cell cycle dynamics in situ and ex vivo.

**Proliferation dynamics in the human hair follicle: Background**

The majority of proliferating HF keratinocytes (HF-KCs) are found in the hair matrix (HM) of fully growing HFs in the anagen VI stage of the hair cycle \((9)\). Most cell proliferation in the anagen VI HM occurs below Auber’s line, an arbitrary line that passes through the widest diameter of the HF’s mesenchymal “command centre”, the follicular dermal papilla (DP) \((36)(Fig. 1a,S1)\). HF-KCs of the inner root sheath (IRS) are already terminally differentiated and thus do not proliferate anymore, while the HF-KCs of the pre cortical HM are programmed to undergo terminal differentiation towards the trichocytes or hair shaft cuticle cells of the hair shaft (HS) \((19,37,38)(Fig.S1)\). The KCs of the upwards-moving IRS are sloughed-off at the level of the insertion of the sebaceous gland duct, whereas trichocytes and HS cuticle cells are eventually lost during hair shaft shedding (exogen) \((19,39,40,s9)\).
Proliferating KCs can also be found in an intermittent pattern along the HF’s outer root sheath (ORS) as detected by Ki-67 (10,17,41) (Fig.1a,ii, Table 1). However, in unwounded HFs, epithelial HF stem cells in the bulge region of the ORS are quiescent during most of the hair cycle, yet remain in a highly undifferentiated state (17,42) (Fig.S2).

What exactly happens distal to Auber’s line in terms of HF-KC cell cycle dynamics has not yet been comprehensively investigated. Moreover, where (and how) exactly in the ORS HF-KCs exit the cell cycle and become differentiated is also unclear. While the final verdict on this is still out, at least some post-mitotic KCs in the precortical HM that go on to terminally differentiate into the trichocytes of hair shaft cortex and medulla may undergo endoreplication (i.e. continued DNA synthesis (43-45,s10,s11), just as terminally differentiating epidermal KCs do (46).

With the notable exception of epithelial HF stem cells (17,42), the human ORS homogeneously and constitutively expresses the hyperproliferation-associated keratins 6 and 16 (47-49,s12). Yet, truly ORS-specific differentiation-associated keratins are unknown. Also, it is not fully understood where exactly and into which direction human ORS-KCs are moving upon proliferation in the ORS basal layer and to which epithelial structures (if any) they contribute. Moreover, how they are lost and replaced during the many years that a human (scalp) HF can remain in anagen VI is not understood. Furthermore, while moderate, but continuous cell proliferation occurs outside of the bulge stem cell compartment in the ORS (s5,17), little KC apoptosis is detectable in a healthy human ORS in situ, despite the fact that the tissue volume of the ORS stays fairly constant throughout the duration of anagen VI (9).

Thus, the cell cycle dynamics, differentiation and migration patterns, and the mechanisms of tissue homeostasis that underlie human ORS physiology have remained enigmatic. Given that the ORS serves as the major source of locally generated growth factors, cytokines, and (neuro-)hormones that regulate HS production, HF cycling, pigmentation and HF immunology (19) and might even constitute the “power house” of human HF energy metabolism (50), the role of the ORS in the human HF must be better understood. As the cell cycle dynamics of these KCs are likely to impact greatly on these factors in human ORS, a standardised methodology to study this is required.

Moreover, while it is now understood that, regarding cell cycle activity, even telogen HFs are far less “quiescent” than long assumed (at least in the mouse) (4,51), the cell cycle dynamics of human telogen HFs remain to be systematically characterized (9). Thus, our basic understanding of human HF cell cycle dynamics stands in stark contrast to our knowledge of the human HF’s expression of differentiation-associated proteins, most prominently HF-associated keratins (48,49,52-54). The
current Methods Review hopes to encourage other colleagues in the field to help close these important gaps in our understanding of human HF biology by applying the methods recommended below.

**How this primer is organised and should be used**

Newcomers to the field often struggle with picking the best-suited and most instructive cell cycle markers, with selecting well-established (immuno-) staining protocols that provide robust and reproducible results, and struggle with how to evaluate the corresponding results. The current primer strives to facilitate cell cycle analysis in the human HF in situ and ex vivo, i.e. on tissue sections of human HFs within scalp skin (9) or after HF organ culture (10,11). For the use of cell sorting techniques (FACS etc.) as an important complementary cell cycle analysis method, the reader is pointed to excellent available literature (e.g. (20,21,55). This method is not covered here as it requires tissue disruption and cell isolation and studies cell cycle dynamics of isolated cell populations outside of their natural tissue habitat.

Since anagen VI HFs contain the largest total number of proliferating cells during the human hair cycle (9), and can be readily microdissected from human scalp skin and organ-cultured even by newcomers (10,11), this Methods Review purposely restricts itself to discussing human anagen VI scalp HFs. However, the markers outlined here can also be applied to all other hair cycle stages, ideally in conjunction with the use of a recently published guide for the recognition of different stages of the human hair cycle (9). On this basis, we review how and when to employ different markers, which can be used individually and in combination, to investigate human HF cell cycle dynamics (Table 1).

**Techniques and read out parameters**

**Nuclei count**

We recommend to begin with counting the number of nuclei within a given reference zone of the HF, as this background information provides an indirect means to determine HF growth and allows one to predict an increase/decrease in the number of completed mitotic events. This is particularly important when comparing control and test groups of organ-cultured or xenotransplanted human HFs, and is essential for determining the percentage of cells that are positive for a given cell cycle
marker (Table 1). One can use nuclei count to also quantify DP fibroblast emigration (56,57) (for further details see supplementary text S1).

**Ki-67: a marker of cell cycling through G1-S-G2-M**

The detection of Ki-67 using immunohistochemistry (IHC)/ immunofluorescence (IF) techniques has long been the standard target antigen for detecting cycling cells in tissue sections (58) (Fig.1a). However, only a fully quantitative approach (i.e. quantitative immunohistomorphometry) of Ki-67+ (Fig.S3) cells in a defined HF compartment, analysed in as many different HFs and individuals (minimum 3) generates instructive, robust, and reproducible data (10). This allows one to estimate the degree of interindividual and interfollicular variation in the HF’s Ki-67 immunoreactivity, either constitutively or in response to a given experimental manipulation or different clinical phenomena (this basic rule also applies to all other cell cycle dynamics read-out parameters covered here).

It is often ignored that, unless analysed by determining specific intracellular localisation patterns of Ki-67 by confocal microscopy (59), generally one cannot reliably identify distinct phases of the cell cycle by Ki-67 imaging on tissue sections as this antigen labels all cycling cells from G1-S-G2-M (58,60), albeit with varying intensity. This has important consequences for data interpretation, as Ki-67+ cells could be disproportionately residing within a specific cell cycle stage (i.e. in G2 as opposed to S-phase). Moreover, cells could be post-mitotic but still actively engaging in S-phase-associated DNA synthesis (endoreplication) (43-45,s10,s11). Thus, Ki-67 analysis alone is insufficient when one wishes to characterize intrafollicular cell cycle dynamics, and additional markers need to be assessed (Table 1).

**Comparing the balance of proliferating and apoptotic cells**

It is generally useful to assess the balance of proliferating and apoptotic cells in any analysed tissue, as this provides the most instructive insights into how an experimental manipulation or clinical condition has an impact on overall tissue homeostasis. In human HFs, Ki-67 immunofluorescence microscopy (Table 1) is routinely used in combination with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) e.g. as a read out in human HF organ culture experiments (10,11), to quantitatively assess ‘proliferation’ versus ‘apoptosis’ (13,61,62) (Fig.S4). As in the sebaceous gland (6375), besides counting the number of nuclei as basic background information (see above), double quantitative immunohistomorphometry for Ki-67 and TUNEL serves as the backbone and starting point of any HF cell cycle dynamics analysis. (For limitations of the TUNEL methods, see supplementary text S2, Table S1,s13,64-65)
Moreover, Ki-67/TUNEL double immunohistomorphometry is an invaluable basic tool for gauging whether a given test agent has impacted on the intrafollicular balance of proliferating and apoptotic cells in defined HF sub-populations and tissue compartments, e.g. ORS versus hair matrix (examples: \(13,22,29,61,66-68,s14\))

**Anti-phospho Histone H3 (Serine 10) (pH3) and anti-phospho retinoblastoma protein (Serine 780) (pS780) antibodies: M-Phase**

Antibodies raised against phospho Histone H3 \((69,83)\) allow one to specifically detect mitotic events in defined HF regions (Fig.1b) (Table 1). We recommend that mitotic events are identified and quantitated by counting only the number of strongly immunoreactive cells in a well-defined reference region of the HF (arrows), e.g. within the matrix epithelium, where mitotic cells are typically found as isolated pH3+ cells (Fig1.b).

Alternatively, antibodies directed towards the phosphorylated form of retinoblastoma protein (pRb), specifically at serine 780 (‘pS780’) can detect mitotic cells \((70,71,s15)\) (Fig 1c). The designation ‘pS780’ for this antibody has been recommended as specificity for the detection of phosphorylated pRb at serine 780 alone is not indicated, although this antibody is still a reliable identifier of a mitotic epitope \((70,84)\). Moreover the phosphorylation of pRB is a major step in cell cycle progression at G1, rather than in M-phase \((71,s15)\) (supplementary textS3). Therefore, we recommend the use of a pS780 antibody for M-phase detection.

**Cyclins**

As key elements of the core cell cycle machinery, cyclins not only drive cell cycling as such, but integrate cellular proliferation with differentiation \((72,)\). Even though they are likely to be key regulatory components within the developmental programs that drive the hair cycle-associated remodelling of human HF epithelium, cyclins have not before been comprehensively studied in the human HF, perhaps with the exception of cyclin D1 \((12)\). Cyclins bind with cyclin-dependant kinases (CDKs) to engage in regulation of the cell cycle through their phosphorylation activity on specific proteins that are implicated in driving the transitions through G1- to M-phase \((72-74, s16)\) (supplementary text S4) (Table 1)(Fig.S6).
In terminal human anagen VI scalp HFs, abundant cyclin A immunofluorescence is present in the matrix, particularly below Auber’s line, where measuring the mean fluorescent intensity in this region permits one to gauge putative cell cycle activity in S/G2 (Fig.S7a) (71-75,s15-17). Similarly, cyclin B1 can be measured below Auber’s line to assess the overall activity of this cyclin in HM cells as a driving force through M-phase (Fig S7.b) (71-75,s15-17).

Cyclin E is also expressed in the HF matrix and can be investigated as an indicator of cells at the G1/S transition (71-76,s15-17)(FigS7.c) (Table 1, supplementary text S5). Additionally Cyclin D1, required to initially drive the cell cycle in G1 (71-75,s15-17), can be investigated as it has been shown to be expressed in the ORS, but is reportedly absent from the human anagen HM) (12).

Because of the diffuse expression pattern of cyclins within the HF epithelium (and with immunoreactivity in mesenchyme (Fig.S7)), their multi-functionality and dynamic intracellular expression throughout the cell cycle (72,77), quantifying the number of cells that specifically express individual proteins of the cyclin family by IF or IHC can be very challenging. Therefore, alongside mean regional fluorescent intensity as described above, study of the intracellular localisation of cyclins and their expression patterns by high resolution and confocal microscopy is also recommended. Moreover, for optimal instructiveness, cyclin IF/IHC needs to be combined with other cell cycle markers such as Ki-67 (see above) or EdU incorporation (see below).

EdU incorporation as S-phase marker

In organ-cultured human HFs (9) or human HF xenotransplants onto immunocompromised mice (9), the S-phase in intra- and perifollicular cells can be sensitively and accurately assessed by studying the incorporation of BrdU or the less toxic thymidine analogue, 5-ethyl-2’-deoxyuridine (EdU). This is typically administered 4-6 hrs before the end of HF organ culture or xenotransplant harvesting, and is incorporated into actively synthesised DNA. By IF microscopy, incorporation can be visualised on tissue sections via a fluorescent azide click-it reaction (78) (Fig.2a-b) (for details, see supplementary text S6).

Double immunofluorescence visualization methods for instructive cell cycle analyses

Tissue sections treated with EdU can be stained with other cell cycle-related IF protocols (supplemental flowchart S1) before proceeding to the ‘Click-It ® (Thermoscientific, Paisley UK) cocktail reaction’ (Supplementary text S6). This double labelling method allows the specific
simultaneous analysis of cells in S-phase (i.e. EdU+ cells) and in M-phase (i.e. pH3+ cells; (Fig.2c) or pS780+ cells). When Ki-67 is used in combination with EdU, the ratio of proliferating cells that actively engage in DNA synthesis (S-phase) versus those that are in either G1/G2/M can be assessed, (e.g. by investigating the ratio of Ki-67+EdU+ versus Ki67+EdU- cells) (Fig2.d).

Additional double-immunostaining techniques are useful to investigate cell cycle behaviour in specific HF cell subpopulations of experimental interest, such as keratin 15+ epithelial progenitor cells (18)(Fig.S8), gp100+ melanocytes (79) (Fig.59), CD90+ fibroblasts (80), endothelial cells (CD31) (81-88), macrophages (CD68)(82)(Fig.S10), mast cells (c-kit, tryptase) (83) and any other markers of functional interest in hair research. Combined with Ki-67/TUNEL double immunohistomorphometry (10,1114,15), these double immunostaining methods greatly enhance the instructiveness of HF cell cycle dynamics analyses.

Caveats & pitfalls

When engaging in these analyses and interpreting HF cell cycle data, one should be aware of several important limitations, pitfalls and caveats (Table 2).

Hair growth and HF-KC proliferation are surprisingly well-sustained in organ culture of microdissected amputated anagen VI HFs (10,1114,15), which makes it a useful model to study interventions that affect HF cell cycle dynamics. However, the loss of molecular or mechanical signalling cues from the tissue microenvironment will undoubtedly affect the underlying biology and cell cycle behaviour, e.g. the disruption of systemic, neural, or microenvironmental signals that promote quiescence in a given HF stem cell niche or of constitutive signals that maintain active cell cycling. The loss of signals derived from major skin structures e.g. the (arrector pili muscle, sebaceous gland and the eccrine gland, which may form a functionally important integral component of the pilosebaceous unit (84), epidermis/dermis, adipose tissue, vasculature and skin nerves) must be expected to alter intrafollicular cell cycle dynamics compared to the in vivo situation. Moreover the stress imposed by microdissection, the withdrawal conditions provided by a serum-free culture medium, and the relative hyperoxic standard incubation conditions (11) of HF organ culture must also be all considered and acknowledged. Alternatively, one can employ hair-bearing full-thickness human skin organ culture, in which many perifollicular skin structures are still intact (85102) or human HF xenotransplants (9,16) (supplementary text S6)
**Future perspectives**

The straightforward *in situ*-techniques described here, whose application requires only basic understanding of human HF biology (19,37), provide invaluable readout parameters in determining how experimental interventions or defined pathologies affect the cell cycle behaviour of the cell populations in the human hair follicle, from the infundibulum to the matrix. By delineating quantitative methodologies that facilitate an accurate appraisal of both normal HF biology and how test compounds can influence hair growth, we provide a primer which will hopefully enable the identification of efficacious hair growth promoters/inhibitors for the treatment of hair growth disorders. Obviously, the same techniques described here can be applied to the study of human HF pathology (26,86,87) and HF-associated tumors, where they are used much less frequently.

Additionally, application of the methods delineated here will help greatly to better define the as yet enigmatic cell cycle dynamics of the human HF. Moreover, the cycling human HF begs to be exploited as an excellent, autonomous mini-organ that can serve as a clinically relevant model system for understanding regulation of the mammalian cell cycle machinery within a complex tissue interaction context *as such in situ*, e.g. by knocking down key cell cycle related proteins (using simple siRNA techniques, e.g. (13,67,83). Finally, the methods outlined here should be complemented with laser capture microdissection-based cell cycle gene profiling e.g. by microarray or next generation sequencing of defined compartments within the HF epithelium. Moreover, stable fluorescent protein expression is required to trace human HF cells during their passage through the cell cycle *in situ*, and dissecting the epigenetic regulation of human HF cell cycle dynamics as well as the role non-coding RNA species remain to be systematically explored. While these methods have already been used with great benefit to the study of murine HF biology (88-94) they await to be fully translated to human HF cell cycle dynamics research in health and disease as well as under conditions of wounding and repair.

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Conflict of Interest

None Declared

Figure/Table legends (main text)

Figure 1
Cell cycling marker Ki67, and mitotic markers pH3 and ‘pS780’ in the human HF
To assess HF cell cycle dynamics, human scalp HFs can be stained with markers for proliferation and measured in various standardised reference areas. (a) Cells positive for proliferation marker Ki-67 in the HF epithelium can be assessed by Immunomorphometry (i.e. no. of positive cells, (Figure S7) regional fluorescent intensity) in a designated reference zone such as below Auber’s line (dash through the dermal papilla). (a.ii) Similarly Ki-67 labelling in non-quiescent ORS compartments can also be investigated. (b-c.ii) Mitosis associated markers pS780 and pH3 can be examined in the matrix. We recommend quantification of the number of positive cells in a designated reference zone (i.e. sub-bulge basal/suprabasal ORS or below Auber’s line etc.) beyond an arbitrary signal threshold). Scale bars are 20 µm. M - matrix, DP - dermal papilla, ORS – outer root sheath, CTS - connective tissue sheath, HS – hair shaft.

Figure 2
EdU incorporation/multiplexing in the human HF
(a-b) Labelling of EdU incorporation highlights S-phase cells in proliferative compartments of the HF including the matrix and proliferative portions of the ORS. Analysis of the number of cells positive for DNA replication can be analysed (e.g. following experimental intervention versus untreated control) in a designated reference area of the HF. (c-d.ii) Multiplexing allows simultaneous analyses of different phases of the cell cycle on HF tissue sections, such as EdU+ cells (s-phase) with pH3+ cells (M-phase). Note the limited double-positivity for these markers as they label distinct cell cycle phases (arrow). (d) Double labelling of EdU with Ki67 (G1-S-G2-M) can also be conducted, this allows visual identification of sub-set of Ki67+ cells not actively replicating DNA (KI-67+ EdU-, arrows). (a,b c,d) - 20µm scale bars, (b.ii & d.ii) - 10 µm scale bars.

Table 1
Markers for investigating cell cycle dynamics in Human HFs
An array of markers to systematically investigate HF cell-cycle dynamics can be utilised.
Recommended core-read outs are underlined. Please see references for alternative methodological
details, where markers have been utilised in other research contexts. *H3570 (Life Technologies) or AR17992 (DAKO). Please see supplementary ‘flowchart S1’ for experimental protocols.

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## Figures & Tables

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<th>Read outs</th>
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Figure 1

(a) Ki-67 and (a.ii) Ki-67 images showing DP and M regions.

(b) pH3 and (b.ii) pH3 images highlighting DP and M regions.

(c) pS780 and (c.ii) pS780 images with M region indicated.

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Figure 2

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