Quantitative proliferation dynamics and random chromosome segregation of hair follicle stem cells

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Regulation of stem cell (SC) proliferation is central to tissue homeostasis, injury repair, and cancer development. Accumulation of replication errors in SCs is limited by either infrequent division and/or by chromosome sorting to retain preferentially the oldest 'immortal' DNA strand. The frequency of SC divisions and the chromosome-sorting phenomenon are difficult to examine accurately with existing methods. To address this question, we developed a strategy to count divisions of hair follicle (HF) SCs over time, and provide the first quantitative proliferation history of a tissue SC during its normal homeostasis. We uncovered an unexpectedly high cellular turnover in the SC compartment in one round of activation. Our study provides quantitative data in support of the long-standing infrequent SC division model, and shows that HF SCs do not retain the older DNA strands or sort their chromosome. This new ability to count divisions *in vivo* has relevance for obtaining basic knowledge of tissue kinetics.

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Introduction

Tissue stem cells (SCs) proliferate, self-renew, and differentiate extensively throughout life, and their cell progeny participate in tissue homeostasis and injury repair (Watt and Hogan, 2000; Fuchs *et al*, 2004). To prevent the accumulation of replication errors by repeated divisions, tissue SCs might be kept quiescent in their resident niches (Cairns, 2002; Potten and Booth, 2002; Ohlstein *et al*, 2004; Lansdorp, 2007). In general, SCs are thought to divide rarely to generate another 'slow-cycling' SC daughter and a rapidly dividing 'transit amplifying' daughter that sustains tissue growth (Bickenbach and Grinnell, 2004; Jensen *et al*, 2004; Sancho *et al*, 2004; Webb *et al*, 2004). The

precise extent of SC proliferation during normal tissue homeostasis is poorly understood, mainly due to lack of quantitative methods to accurately count divisions in unperturbed live tissue. Infrequently dividing or slow-cycling putative SCs are routinely found as label-retaining cells (LRCs) by a repeated 'pulse' with BrdU or ³H-thymidine followed by a 'chase' period (Braun and Watt, 2004; Sun and Lavker, 2004). Label retention depends on the growth kinetics of the tissue. Although insufficient on its own to identify the SCs, it can be used together with more specific markers to obtain meaningful information about SC behaviour. Caution should be exerted when interpreting LRC data, as cells that permanently withdraw from the cell cycle and terminally differentiate soon after labelling can also retain the DNA label.

Hair follicle (HF) SCs and LRCs have been traditionally tightly linked together (Braun and Watt, 2004). HF morphogenesis begins in the embryo and continues until around postnatal day (PD) 17, a stage that initiates the adult developmental phase (Hardy, 1992). Adult follicles undergo repeated cycles of growth (anagen), regression (catagen), and rest (telogen) that are relatively synchronous in mouse skin during the first and second hair cycles (Figure 1A). In catagen, the temporary portion of the HF (bulb) is destroyed by apoptosis, whereas the permanent segment (bulge) survives. In telogen, a pocket of dermal papillae cells comes in contact with the bulge, to which it sends presumptive activating signals to reinstate hair growth from bulge cells (Lavker *et al*, 2003; Cotsarelis, 2006). Infrequently dividing cells reside in the bulge region of the HF, which is now a well-established SC niche (Cotsarelis *et al*, 1990; Taylor *et al*, 2000; Oshima *et al*, 2001). That few bulge cells contribute to hair homeostasis was suggested by LRC tracking experiments (Taylor *et al*, 2000; Tumber *et al*, 2004), by genetic marking of HF cells, long-term lineage tracing *in vivo* (Ghazizadeh and Taichman, 2001; Kopan *et al*, 2002; Morris *et al*, 2004), and by bulge cell transplantation *in vitro* (Oshima *et al*, 2001; Blanpain *et al*, 2004; Morris *et al*, 2004; Claudinot *et al*, 2005). Functional assays demonstrated that a considerable fraction of bulge cells behave as SCs in clonal analyses and long-term transplantations (Claudinot *et al*, 2005). Other studies suggested that bulge LRCs themselves have high proliferative potential *in vitro* (Morris and Potten, 1994; Bickenbach and Chism, 1998; Schoch *et al*, 2004). *In vivo*, LRCs can proliferate in response to tissue growth and injury stimuli (Morris and Potten, 1999; Taylor *et al*, 2000), as well as in response to phorbol ester (Braun *et al*, 2003) and growth hormone (Ohlsson *et al*, 1992). Despite extensive efforts, it remains unclear whether in the bulge, the cells with long-term label retention properties are phenotypically or functionally different from the other cells, and whether these infrequently dividing cells represent the true long-term SC population of the tissue. Interestingly, in the gut, a non-LRC population regenerates the crypts, at least for several rounds of tissue turnover (Barker *et al*, 2007). However, due

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Figure 1 Strategy to detect divisions during hair follicle cycle. **(A)** The hair cycle. After morphogenesis (~PD17), HF enter the adult phase of development characterized by cycles of breakdown (catagen), rest (telogen), and growth (anagen). HFSCs are located in the bulge area. The bulge cells are quiescent during telogen, but proliferate in anagen, when they contribute to differentiated follicle cell lineages and the generation of a new hair shaft (red). APM, arrector pili muscle; DP, dermal papillae; SG, sebaceous gland. **(B)** Top: tetracycline-inducible (tet-off) double transgenic mouse system drives expression of histone H2B-GFP from the epithelial keratin 5 (K5) promoter (pulse). Doxy administration in mouse diet turns off H2B-GFP expression (chase) when proliferating epithelial cells dilute the label between daughter cells by divisions. Bottom: when doxy is added to diet at time points indicated, the proliferation history of bulge cells during the first and second hair cycle can be assessed based on H2B-GFP dilution.

to lack of LRC-specific markers in any tissue, lineage-tracing experiments for these cells have not yet been performed *in vivo*.

Upon initial DNA 'pulse' labelling of cells that are actively dividing, the label can be retained by these LRCs after 'chase' by three possible mechanisms: (1) rare divisions, (2) no divisions, or (3) retaining the oldest, so-called 'immortal DNA strand' by nonrandom chromosome segregation. The last model has been based on a long-standing hypothesis (Cairns, 2006; Lansdorp, 2007; Rando, 2007) supported by data from tissues such as mammary gland (Smith, 2005), muscle (Shinin *et al*, 2006; Conboy *et al*, 2007), nervous system (Karpowicz *et al*, 2005), HF (Morris and Potten, 1999), and intestine (Potten *et al*, 2002), as well as from a few cell culture systems (Merok *et al*, 2002; Armakolas and Klar, 2006). However, this model remains controversial at least in part due to lack of more quantitative methods to address it unambiguously in uninjured tissues, and to an apparent lack of its general applicability among all adult tissue SCs (Kuroki and Murakami, 1989; Kiel *et al*, 2007; Lansdorp, 2007).

In this study, we addressed this question in HF, in the intact unperturbed tissue. We refined the capability of our previously developed strategy to detect cells in skin tissue based on their proliferation history (Tumbar *et al*, 2004). Specifically, we used tetracycline-inducible mice driving histone H2B-GFP to follow cell proliferative status through the dilution of GFP label. Unlike nucleotide labeling, our method does not require active cell proliferation at the 'pulse' stage. The histone H2B-GFP dilutes equally between two daughter cells at division (Kanda *et al*, 1998; Brennand *et al*, 2007), as expected from dynamic interchange of H2A-H2B dimers among nucleosomes (Luger and Hansen, 2005). Here, we used this tool to count divisions of HF bulge cells *in vivo* during adult tissue regeneration, and distinguish among

different possible mechanisms hypothesized for many decades to maintain the SC genome.

Results

H2B-GFP system counts bulge cell divisions during HF homeostasis

Previously, we generated double transgenic tetracycline-inducible mice to express histone H2B-GFP in skin epithelium driven by the keratin 5 (K5) promoter (Figure 1B, top) (Diamond *et al*, 2000; Tumbar *et al*, 2004). H2B-GFP expression is activated upon tetR-VP16 protein binding to the tetracycline response element (TRE) DNA fragment, and can be turned off efficiently by addition of a tetracycline analogue (doxycycline, doxy) to the mouse diet (Kistner *et al*, 1996). Upon H2B-GFP repression in adulthood and a chase period, we found the brightest skin cells in the hair bulge (Tumbar *et al*, 2004). Here, we employ this tet-off system to examine the proliferation history of bulge HFSCs, in live skin, during the first and second normal cycles of hair regeneration (Figure 1B, bottom). We define the HFSC-enriched populations by (a) *in situ* localization in the outer root sheath of the bulge region or (b) CD34 and α 6-integrin (α 6) cell surface expressions in freshly isolated skin cells. CD34 is expressed in all-bulge cells in telogen at the time of our analyses (Supplementary Figure S1A). Although not yet assayed by lineage tracing experiments in intact tissue, the CD34 + α 6 + isolated bulge population has been demonstrated by growth and transplantation *in vitro* assays to contain self-renewing multipotent SCs (Trempeus *et al*, 2003; Blanpain *et al*, 2004).

To examine the efficacy of K5tTA-controlled H2B-GFP labelling of bulge cells in K5tTA/pTRE-H2B-GFP mice at the first telogen (PD21), we used confocal microscopy of frozen skin sections (Figure 2A, left panels) or fluorescence-activated cell sorting (FACS) of isolated skin cells

Figure 2 H2B-GFP system counts cell divisions in bulge cells. (A) Confocal images collected side by side from skin sections of pTRE-H2B-GFP/K5tTA mice at PD21 (no chase; Telogen I); PD49 (4-weeks chase; Telogen II), and PD77 (8-weeks chase; Telogen III). Top panels: note decrease of overall H2B-GFP signal, with brightest signal retained in the bulge area. Bottom panels: TOPRO-3 is a DNA stain. (B) Skin cells stained for surface expression of CD34 and $\alpha 6$ -integrin analysed for fluorescence (FL) by FACS: dot plot of all live PI negative skin cells (left); GFP histograms of double positive CD34 + $\alpha 6$ -integrin + cells at time points indicated (right). PD21 (no chase, blue); PD49 (4-weeks chase, red); and PD77 (8-weeks chase, green). (C) Average frequency for each GFP peak sub-population as defined in (B, right) in CD34 + $\alpha 6$ -integrin + cells with standard deviation among mice at time points indicated (PD49, $N=7$; PD77, $N=6$; PD21, $N=2$). (D) Linear regression analysis of GFP intensity (Int) in bulge sub-populations at PD49 shows decrease of the GFP level by two-fold. (E) FACS analyses of single cells isolated from mice treated on the back skin with TPA (mouse back left of midline) or acetone (mouse back right of midline) for 3 weeks during the doxy chase. (F) Projections through confocal stack images show hair follicles from 3-weeks acetone-treated (left) versus TPA-treated (right) skin section (100 μ m). TOPRO-3 DNA stain is shown in red. Note lack of bright GFP signal in TPA-treated skin. Hair follicles were in telogen in acetone treatment area and in anagen in TPA treatment area of skin, as expected from the known effect of TPA in promoting anagen. (G) Projection through fluorescence image optical stack collected from skin section (40 μ m) shows BrdU incorporation (red) by bulge cells after 1 week of both TPA and BrdU treatment, and 5 weeks of doxy chase. Bu, bulge. (H) Quantification of BrdU + cells in bulge cells shown in (F), represented as three arbitrary classes of GFP brightness, after acetone versus TPA treatment. (I) Normal log of GFP^{Peak1} median intensity (Int) relative to time of chase allows derivation of H2B-GFP degradation rate from the slope. (J) GFP^{Peak1}- (top panels) and GFP^{Peak2} (bottom panels)-sorted cells from PD49 mice treated with BrdU during the entire 4-weeks doxy chase period. Note rare BrdU + cells (red) in GFP^{Peak1} population and nearly 100% BrdU + cells in GFP^{Peak2}. DAPI is DNA staining. (K) Quantification of BrdU + cells in GFP sub-population from mice in (J). Data are shown as average of cells counted from three mice at PD49, with standard deviations. Total number of counted cells is indicated at top of the graph. Designation of GFP cell population as defined in Figure 2B is shown at the bottom with presumed divisions in each population. Scale bars, 50 μ m.

(Figure 2B, blue line). The FACS histogram of CD34 + α 6 + bulge cells at PD21 showed uniform H2B–GFP fluorescence in ~86% of cells (~10⁴ arbitrary fluorescence units, AFUs). The remaining bulge cells showed FACS GFP signals that were either lower or at background levels (<10^{2.1} AFU). These CD34 + α 6 + GFP-negative cells found in unchased mice were >90% positive for undifferentiated keratinocyte markers K5 and K15 (data not shown), and were likely the results of mosaic transgene expression. This assessment was consistent with rare individual or clustered GFP-negative follicles found by fluorescence microscopy of skin sections (data not shown). Thus, our data showed high H2B–GFP signal uniformity in a large fraction of bulge cells before the chase. Furthermore, we examined how rapidly the H2B–GFP mRNA was repressed in CD34 + α 6 + bulge cells after 2 days of chase. We found a substantial reduction of GFP mRNA levels in chased versus unchased cells by RT–PCR analyses (Supplementary Figure S1B). Importantly, this data attested to rapid H2B–GFP mRNA repression in all bulge subfractions analysed.

To examine the proliferation profile of bulge cells upon SC activation during hair regeneration, we performed 4-weeks (PD21–PD49) and 8-weeks (PD21–PD77) doxy ‘chases’ for one or two hair cycles (Figure 1B). As expected, the H2B–GFP signal in confocal images of skin sections decreased over time (Figure 2A), but also displayed markedly different fluorescence levels in CD34 + α 6 + cells within each bulge after chase (Figure 2A, top middle panel; Supplementary Figure S1A). To quantify these H2B–GFP levels, we used FACS of skin cells from mice with telogen follicles throughout the body (as confirmed by microscopy of small skin samples, data not shown). The FACS profiles of CD34 + α 6 + bulge cells showed progressive decrease of H2B–GFP signal at the second (PD49), and third (PD77) telogen after 4- and 8-weeks chase, respectively (Figure 2B). The H2B–GFP signal after chase displayed distinctive peaks of fluorescence in all animals analysed (Figure 2C), with little variability across four back skin regions (Supplementary Figure S1C). We referred to these peaks as GFP^{Peak1–6}, starting from the brightest; the GFP-negative population was GFP^{Peak7}, whereas the unchased population was GFP^{Peak0} (Figure 2B). All sorted sub-populations were highly positive for undifferentiated keratinocyte markers K5, K15, and β 4-integrin (Supplementary Figure S2A–C). Taken together, these data demonstrated distinct and highly reproducible levels of H2B–GFP fluorescence in bulge cells, which decreased as a function of time upon chase.

The H2B–GFP dilution over time is dependent upon division, when the GFP signal is halved between daughter cells at mitosis (Brennan *et al*, 2007). To quantify this effect, we measured the median H2B–GFP signal of GFP^{Peak1–7} bulge cell sub-populations after chase and performed linear regression analyses (Figure 2D). We found a precise two-fold decrease from one sub-population of bulge cells to the next, suggesting that we might be able to count the numbers of cell divisions in bulge cells over time. To further assess the ability to count divisions *in vivo*, we treated skin of double transgenic mice with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which is a potent stimulator of skin keratinocyte and bulge cell proliferation (Morris *et al*, 1985; Braun *et al*, 2003). Simultaneously, we fed mice doxy for 3 weeks starting at PD21. A striking decrease of H2B–GFP levels in bulge cells

was apparent either by confocal microscopy (Figure 2F) or by FACS (Figure 2E; Supplementary Figure S1D). Moreover, 4-weeks chased bulge cells with different H2B–GFP fluorescence incorporated BrdU at higher levels in 1 week TPA-treated skin than in acetone-treated control (Figure 2G and H). These data suggested that TPA induced proliferation of all bulge cells irrespective of their normal frequency of divisions. Even the brightest H2B–GFP cells were capable of dividing upon drug stimulation, as shown by their BrdU incorporation and the shift of GFP signal to lower levels. Moreover, this analysis further demonstrated our ability to detect distinctly increasing numbers of divisions using the H2B–GFP pulse-chase system. Our FACS data suggested that bulge cells divided at least once more during 3 weeks of drug stimulation, whereas most bulge cells divided multiple times more.

To quantify the H2B–GFP protein degradation we analysed CD34 + α 6 + cells by FACS after different chase times. We detected a downshift of all GFP peaks relative to no chase control, suggesting similar degradation in all bulge cells (not shown). The intensity median data of the brightest GFP peak were fit to a first-order decay function to estimate the degradation rate constant, which then was used to calculate a half-life (Belle *et al*, 2006) of $T_{1/2} = \ln 2/k(\text{slope}) \sim 24$ days (Figure 2I). According to these data, the brightest GFP^{Peak1} cells after 4-weeks chase and the GFP^{Peak2} cells after 8-weeks chase never divided during these times, and the loss of H2B–GFP signal in these cells was solely due to protein degradation. To verify this prediction, we continuously labelled mouse skin with BrdU administered in the drinking water during the 4-weeks doxy chase, to mark all the cells that proliferated during the first hair cycle. Next, we examined by microscopy the BrdU staining in each sorted GFP sub-population (Figure 2J and K). At 4-weeks chase, the H2B–GFP^{Peak1} cells were <5% BrdU +, whereas the H2B–GFP^{Peak2} cells were ~90% BrdU +; virtually all the other H2B–GFP^{Peak3/4, 5/6 and 7} fractions displayed 100% BrdU + cells, as expected from dividing cells. Similarly, at 8-weeks chase only few (<10%) of the rare GFP^{Peak2} cells (0.5% of CD34 + α 6 +) incorporated BrdU after long-term labelling (data not shown).

In conclusion, we used the regulated H2B–GFP transgenic system to quantify the proliferation history of HF CD34 + α 6 + bulge cells during normal homeostasis. In one hair cycle, only few bulge cells remained undivided (5.7%), whereas the rest divided a few times: H2B–GFP^{Peak2} (one division: 13.6%), H2B–GFP^{Peak3} (two divisions: 15%), H2B–GFP^{Peak4} (three divisions: 12.9%), H2B–GFP^{Peak5} (four divisions: 12.3%), and H2B–GFP^{Peak6} (five divisions: 8.7%). The H2B–GFP^{Peak7} (31.7%) cells had GFP signal at background level, and divided at least 6 ×. After two hair cycles, the undivided cells were even more infrequent (0.5%). A fraction of bulge cells (28.5%) remained highly infrequently dividing with fewer than four divisions in two consecutive hair cycles, whereas the other bulge cells divided more frequently.

In situ quantitative confocal analysis of H2B–GFP LRCs

The existence of bulge cell sub-populations with distinct number of division was intriguing. How tightly controlled is the number of divisions among equivalent tissue SC niches (bulges) in a single activation cycle? Classical SC niches contain quiescent support or ‘niche’ cells (Doerner, 1998; Fuchs *et al*, 2004; Ohlstein *et al*, 2004), and it was important to find whether similarly, each bulge contained undivided

cells. In skin sections, we quantified the H2B-GFP fluorescence in a volume for cells localized in the outer most layer of the bulge area by Z-sectioning and confocal microscopy (see Materials and methods and Supplementary data). Each bulge analysed at 4-weeks chase displayed cells with a wide range of H2B-GFP fluorescence (Figure 3A and B), and the brighter H2B-GFP cells seemed to cluster preferentially in one area of the bulge. We integrated the H2B-GFP intensity of all relevant Z-sections (Figure 3C) for each of the 246 cells analysed from 10 follicles (two mice). The brightest cells ($N=19$) were

distinct in 3D projections through the stack of each follicle analysed (Figure 3B). The remaining 227 cells could be split into five distinct classes of H2B-GFP fluorescence differing from one another by two-fold, as shown by linear regression (Figure 3D and E). These data showed that we were able to detect distinct divisions *in situ* in individual HFs.

To establish the exact number of divisions for these bulge cells during 4 weeks of chase, we compared their H2B-GFP intensity with that of bulge cells before the chase. We determined the average of H2B-GFP signal in a volume of

Figure 3 H2B-GFP system counts cell divisions in individual bulges *in situ*. (A) Confocal optical Z-stack is shown as tiled images. Numbers indicate actual optical slice and arrows point to a bright H2B-GFP cell throughout the stack. (B) Stack in (A) is shown as maximal projection through the slices on XY, ZY, and XZ planes. Arrow points to the same cells as in (A). (C) Total intensity after background subtraction (Int) in each optical Z-section for cell indicated by arrows in (A, B) used to obtain total 3D intensity. (D) Average 3D intensity (Int) of bulge cells measured at PD21 (no chase, black) and six sub-populations with average intensities decreasing by two-fold at PD49 (4-weeks chase, grey) is shown with standard errors of the mean (s.e.m.) bars. (E) Linear regression analysis to verify the ranking of bulge cells in classes differing by two-fold in GFP intensity. The slope measured experimentally did not differ significantly from a -1 slope, as predicted by two-fold dilution ($P=0.97$, Student's *t*-test). (F) Cross comparison of average intensity of cells from each bulge (no chase) is shown with error bars (s.e.m.). Accurate definition of nuclei was difficult in unchased follicles, which contained dense clusters of bright cells. This resulted in higher H2B-GFP signal variation than expected from previous FACS analyses. Eight out of ten follicles showed no significant differences in intensity ($P=0.3147$, Wilcoxon test). HFs 5 and 6 were significantly different from the eight follicles ($P=0.001$, Wilcoxon test). (G) Distribution of the six GFP populations defined in (D) with distinct divisions ($0 \times -5 \times$) detected at PD49 (4 weeks of chase) among 10 HFs (hf01–hf10). Average intensities per HF for each of the six GFP populations detected at PD49 (4 weeks of chase) are shown with error bars (s.e.m.). Wilcoxon test showed no significant difference in the GFP signal among each population in all HFs ($P=0.1997$), with the exception of one population ($1 \times$ division) in two HFs (hf01 & hf06; $P=0.06$). HFs 5 and 10 marked with asterisks were partially truncated by our skin and/or optical sectioning, and were also missing the undivided ($0 \times$) cell population.

69 PD21 unchased bulge cells (10 HF, two mice) (Figure 3D, black bar) and documented the variability among hair bulges (Figure 3F). When we adjusted this average for H2B-GFP degradation, we computed a value ($\sim 5 \times 10^6$) that was comparable to that measured experimentally ($\sim 6 \times 10^6$) in the brightest cells after 4-weeks chase. This suggested that the bright cells never divided during the chase (Figure 3D, compare back and first grey bar). Finally, we examined the distribution of the six categories of H2B-GFP cells, which divided $0-5 \times$, in 10 HF, analysed (Figure 3G). We found that with very few exceptions, potentially attributable to the experimental limitation of our measurements, each bulge contained cells of all division stages.

In conclusion, our confocal analyses and quantification of H2B-GFP fluorescence in a volume allowed us to establish *in situ* localization of bulge sub-populations with defined division stages. We determined that virtually all follicle bulges analysed contained cells that presumably divided $1 \times$, $2 \times$, $3 \times$, and $>3 \times$ within one hair cycle, whereas undivided cells were rare and limited to 1–2 cells per bulge. These data documented a high degree of uniformity in

distribution of cell divisions among bulges during one cycle of SC activation and quiescence.

Bulge BrdU LRC proliferation history and labelled chromosome segregation

The retention of nucleotide label by putative SC populations has been attributed to infrequent divisions or to a specialized mechanism to preserve the original 'immortal DNA strands' through asymmetric segregation of chromosomes at mitosis (Cairns, 2006; Lansdorp, 2007; Rando, 2007). Our data showed that a majority of bulge cells divide infrequently during one hair cycle, but a fraction of them divided five times or more. To test if bulge cells segregated randomly the older, BrdU-labelled DNA strands, we asked whether BrdU decreased by two-fold in bulge cells that divided multiple times as shown by the H2B-GFP signal. We obtained bulge BrdU LRCs by a well-established pulse-chase scheme (Cotsarelis *et al*, 1990; Taylor *et al*, 2000), in which we labelled skin of pTRE-H2B-GFP/K5tTA double transgenic mice with BrdU during morphogenesis and chased until adulthood. Simultaneously, we turned off the H2B-GFP

Figure 4 Bulge cells dilute the BrdU label in divisions counted by H2B-GFP system. (A) Diagram showing BrdU and doxy simultaneous pulse-chase scheme. Arrows represent six BrdU subcutaneous injections at PD3–PD5. Mice were killed at PD21 (2-weeks chase) and PD47 (6-weeks chase). (B) Mice not treated with BrdU were used to estimate the frequency of GFP sub-populations (as defined in Figure 2C) among CD34 + / α6 + bulge cells (PD21, $N = 2$; PD47, $N = 3$) and α6 + cells (PD6, $N = 2$), with standard deviation bars. (C) Sub-population of bulge cells from mice in (A) sorted on slides and immunofluorescence stained showed BrdU + cells in all sorted bulge cell fractions. (D) Frequencies of BrdU + cells in each bulge cell sub-population are shown as average among mice with standard deviation bars ($N = 2$ mice at PD21 and 3 mice at PD47). (E) Total intensity of BrdU signal per cell normalized between two experiments is plotted as average for each GFP sub-population at PD21. Note decrease of BrdU signal from one population to another. (F) Same as (E) for mice killed at PD47. (G) Average BrdU intensity measured in GFP^{Peak1–7}-sorted bulge sub-population from (E, F) is plotted as log₂ and shows linear regression fit supporting two-fold dilution of BrdU with divisions.

expression by feeding the mice doxy (Figure 4A). First, we documented the FACS histograms in bulge cells after 2- and 6-weeks chase in the presence and absence of BrdU labelling (Figure 4B; Supplementary Figure S3). Despite some BrdU-induced toxicity, we obtained sufficient CD34+/ α 6+ bulge GFP sub-population, and quantified by microscopy their BrdU immunofluorescence signal (Figure 4C–F). Similar to the decrease in GFP levels, the BrdU signal also decreased by two-fold in the bulge sub-populations (Figure 4E–G). This suggested that the older, BrdU-labelled strands, segregated equally between bulge cell daughters at division.

Our data thus far did not support the chromosome-sorting model but some questions still remained. Although in general

6 weeks of chase is considered sufficient to enrich in putative SCs (Potten and Booth, 2002; Potten, 2004), it was possible that at the time points analysed, SCs had not yet switched from presumed symmetric to asymmetric chromosome segregation. Therefore, we investigated the division pattern of more long-term BrdU LRCs. Because the length of the chase was limited to 6 weeks due to H2B–GFP dilution below detectable levels by divisions, we shifted the doxy chases from morphogenesis and first hair cycle over to the first and second hair cycles (Figure 5A). First, we performed BrdU immunostaining of skin sections at PD47 and PD77 (Figure 5B). The BrdU signal decreased in bulge cells over time, and the bright BrdU and H2B–GFP cells colocalized in

Figure 5 Long-term BrdU LRCs divide infrequently and dilute the label over time. (A) Diagram showing BrdU and doxy pulse-chase scheme. Small arrows represent six subcutaneous BrdU injections at time points indicated. (B) Skin sections from mice in (A) were stained for BrdU with fluorescently labelled antibodies. Scale bar, 50 μ m. Blue is DNA DAPI stain. (C) Quantification of BrdU signal in fluorescence images of skin sections at time points indicated: PD6 is no chase; PD21, 2-weeks BrdU chase; PD77, 10-weeks BrdU chase. Images of BrdU-stained follicles were acquired with the same exposures and the brightest cells in each follicle were surveyed for level of BrdU signal using the IP Lab Imaging software. Note progressive decrease of label over time. (D) Skin cells from mice in (A) stained for CD34 and α 6-integrin surface expression, sorted in GFP bulge sub-populations on microscopy slides and immunostained for BrdU. Percent BrdU+ cells in each GFP^{Peak1-7} was determined relative to negative control (secondary antibody or no BrdU-labelled cells); PD47, N = 3 mice; PD77, N = 4 mice. Total number of cells counted is indicated at top in appropriate colour. (E) Data obtained in (D) are transformed to illustrate the frequency of BrdU LRCs with defined numbers of presumed divisions among total bulge LRCs. (F) A random pool of sorted cells from (D) was measured for total BrdU signal in single wide-field fluorescence images (N = 4 mice at PD77 (black)). For comparison of BrdU levels at PD21, the H2B–GFP cells corresponding to one division during morphogenesis (2 weeks of BrdU and doxy chase: PD6–PD21) were also sorted and stained for BrdU (white dots, N = 2 mice). The BrdU signal is plotted as log₂. Presumed divisions indicated by H2B–GFP intensity at bottom. Under each column dot pattern in black represents cells from the four mice analysed. White circles underline 2 of 850 cells (found in 1 of 4 mice) that did not follow the decreasing trend of BrdU signal with increasing number of divisions. Number of cells analysed is shown at top. NC1 (negative control 1) are all live cells from mice not injected with BrdU. NC2 (negative control 2) are bulge cells from PD77 mice stained with secondary antibody only.

Figure 6 Labelled chromosomal foci were diluted in nuclei of dividing cells. CD34+/ α 6+ bulge cells with distinct H2B–GFP levels and presumed divisions were sorted as before from PD77 mice treated with BrdU and doxy as shown in Figure 5A. Cells were spotted on slides, fixed, and stained for BrdU. Each panel shows fluorescence images of a single nucleus as sum projections through 3D optical Z-stacks. Examples are of the brightest BrdU+ cells found in individual sorted bulge sub-population. Numbers in corner indicate presumed divisions during two consecutive hair cycles. Note only 1–2 BrdU nuclear foci representative of distinct chromosome labelling in nuclei from cells with >4 presumed divisions. Scale bar, 5 μ m.

fluorescence images of skin sections. Moreover, we found a progressive decrease in BrdU signal when we compared HF in skin section images after 0, 2 and 10 weeks of chase (Figure 5C). Taken together, these data suggested that the BrdU LRCs divided infrequently and diluted the label over time.

To precisely quantify the frequency of BrdU LRC divisions during one or two hair cycles, we sorted on slides individual CD34+/ α 6+ cells with distinct H2B–GFP levels (GFP^{Peak1–7}) from mice treated as shown in Figure 5A. Next, we immunostained the cells for BrdU and counted the BrdU+ cells in immunofluorescence images (Figure 5D). The total number of BrdU LRCs detected at PD47 decreased substantially by PD77, consistent with our assessment of skin tissue, suggesting that a majority of the 6-weeks BrdU LRCs might divide multiple times during one hair cycle and dilute the label at division. At each stage analysed, the BrdU LRCs were enriched only in GFP cells with predicted 0–2 divisions, whereas cells that divided 3 times or more contained rare or no such cells (Figure 5D and E). Finally, for a random pool of the PD77-sorted cells (10-weeks BrdU chase), we measured the total BrdU signal per cell in fluorescence images and found overall decreasing BrdU levels with increasing number of divisions (Figure 5F). Only 2 out of 850 total cells ($N=4$ mice) fell out of this pattern (white circles in Figure 5F). The brightest BrdU LRCs were found in the undivided cells. The BrdU signal showed high variability in each GFP sub-population, suggesting that these cells divided several times during morphogenesis. These data demonstrated that cells with high BrdU label retention divided very rarely during adult hair cycling, contradicting the premise of the immortal strand hypothesis. Cells that divided multiple times lost detectable BrdU label.

Our data thus far suggest that most if not all chromosomes are randomly segregated in dividing bulge SCs. As in some instances only few chromosomes might be selectively retained (Armakolas and Klar, 2006), it was possible that our analyses were not sensitive enough to detect such a subtle effect. To address this possibility, we analysed the high-

resolution nuclear pattern of BrdU label retaining bulge cells that divided increasing number of times, at the end of the second hair cycle. Individual chromosome territories are known to appear as bright BrdU-labelled nuclear foci after multiple cell divisions (Cremer and Cremer, 2001). If all 40 mouse chromosomes are randomly segregated, GFP-negative cells with 5 or more divisions, would display 1–2 if any BrdU+ foci, whereas the undivided cells might display multiple BrdU+ foci. In contrast, if we assume several chromosomes are nonrandomly segregated to retain the older DNA strands in one daughter cell, we should find several BrdU-labelled foci in some cells even after multiple divisions. High-resolution 3D imaging of BrdU-stained cells with distinct divisions revealed rare BrdU+ chromosomal foci in cells with multiple divisions, and bright multiple BrdU-labelled foci in cells with few or no divisions (Figure 6). In particular, we carefully analysed a large number of GFP-negative bulge cells and found only 13 of 2199 (0.5%) BrdU+ cells displaying 1–2 BrdU-labelled foci (Figure 6). Thus, we conclude that bulge cells with multiple divisions did not retain together several BrdU-labelled chromosomes, and instead segregated them randomly at division.

Discussion

Proliferation dynamics and chromosome segregation in HFSCs

Regulation of cell proliferation is thought to be essential for accomplishing SC function in tissue regeneration and in preventing disease (Watt and Hogan, 2000; Fuchs *et al*, 2004). For decades LRCs have been attributed SC properties, and the mechanisms responsible for DNA label retention have been controversial (Lansdorp, 2007; Rando, 2007). These mechanisms have been considered highly relevant for the SC genome maintenance. Here, we addressed this question by examining three distinct possibilities for the label retention phenomenon in the HF: (1) no division; (2) infrequent division; and (3) asymmetric segregation of

chromosomes. To accomplish this goal, we adapted a previously developed strategy (Tumbar *et al*, 2004) to be able here to quantitatively measure divisions *in vivo*, based on H2B–GFP dilution over time and systematically examine the proliferative properties of cells in the HFSC pool, the bulge.

No divisions of bulge cells? As bulge cells were thought to be highly quiescent, one possibility examined here was that the bulge contained some cells that never divided in adulthood. Classical SC systems such as those of *Drosophila* germ line or plant root apical meristem include quiescent ‘niche’ cells that contact the SCs and maintain their potential (Doerner, 1998; Fuchs *et al*, 2004; Ohlstein *et al*, 2004). Suggestively, in a previous systematic genomic screen we found that the most quiescent bulge cells produced extracellular factors that contributed to the general makeup of the SC environment, qualifying them as candidates for niche cells (Fuchs *et al*, 2004; Tumbar *et al*, 2004). Because BrdU pulse chase depends upon active cell proliferation at the labelling stage, this question could not be addressed before with the existing tools. Using the H2B–GFP labelling under the epithelial K5 promoter, we accounted for all bulge cells irrespective of their initial proliferative status. Indeed, in our analyses we find bright H2B–GFP bulge cells that were not detected by the superimposed BrdU pulse chase. We were able to obtain quantitative data clearly showing that each bulge contains only 1–2 undivided cells after one complete hair cycle. Significantly, these quiescent cells were not maintained after a subsequent hair cycle, when the number of undivided cells dropped by 10-fold (as estimated from the sorted bulge population after one and two hair cycles). Moreover, our data showed that all bulge cells were capable of proliferation when stimulated. Taken together, our data ruled out the putative existence of a long-lived permanently quiescent bulge cell, potentially specialized in niche maintenance. Thus, it remains to be established if all bulge cells work in concert to create and maintain the niche environment.

Infrequent divisions of bulge cells? A second possibility for the bulge SC proliferation was infrequent division, to preserve the SC genomic integrity during life. This characteristic has been generally attributed to many tissue SCs, although it has been unclear how frequent their total divisions would be throughout life. An estimate of ~100 divisions for a somatic SCs’ life has been previously proposed (Lansdorp, 1997). To address this question, we refined our H2B–GFP pulse-chase strategy to a quantitative level and counted cell divisions in the SC pool during hair cycles. Hair cycles encompass SC proliferation periods, marked by periods of rest or quiescence, during normal tissue homeostasis. We showed that a majority of bulge-retained cells divided 1–5 times (average of ~3 times) in one hair cycle. (Cells that left the bulge either died or repeatedly divided, as the H2B–GFP label was retained in epithelial skin at high levels only in the bulge.) Mouse hair cycles take ~3–4 weeks to complete and in older mice are interspersed by prolonged periods of rest (Paus *et al*, 1999), allowing us to infer fewer than 20 hair cycles in ~2 years. By a simple calculation, our data show that bulge cells would undergo on average fewer than 100 divisions during the entire life of a mouse. Given a low estimated rate of mutation of 10^{-6} per gene per replication (Drake, 1999), this number of total divisions is clearly insufficient to result in

significant error accumulation. Thus, our data numerically proves the original model for tissue SC genome preservation. This finding is also in agreement with the observation that active maintenance of telomere length is not required in the first few generations of telomerase knockout mice (Blasco, 2005).

Asymmetric chromosome segregation of bulge SCs? The modest number of total SC divisions estimated here, is at odds with the third possibility described above, that chromosomes are sorted according to the age of their DNA strands, to prevent accumulation of errors during repeated replications (Cairns, 2006; Lansdorp, 2007; Rando, 2007). In fact, we found that the cells detectable as BrdU LRCs over one or two complete hair cycles divided only 0–3 times. Thus, the long-term BrdU retention must simply be the result of rare bulge cell divisions. Moreover, we quantitatively showed here that cells with increasing number of divisions progressively diluted the DNA nucleotide label by two-fold, down to background levels. Only very rare cells with multiple divisions displayed weak label retention, which was limited to 1–2 BrdU-labelled chromosomal foci. Thus, our data refute the chromosome-sorting model in the HFSC pool, and instead show that chromosomes are randomly segregated with respect to the earlier labelled DNA strands.

The chromosome-sorting model is the subject of a recent debate, as it has received increasing support from studies in many injured tissues or *in vitro*, in cultured cells, to the point of becoming the rule rather than the exception among adult SC types (Cairns, 2006; Lansdorp, 2007; Rando, 2007). The epidermal and bulge cells were known to dilute the label over time (Kuroki and Murakami, 1989; Braun *et al*, 2003), but it could be argued that drug treatment used in those studies perturbed the normal patterns of SC divisions. Conversely, it can be argued that results supporting chromosome sorting might be artifacts of injury, *in vitro* assays, or lack of quantitative methodology (Lansdorp, 2007). Only the haematopoietic SCs appeared to dilute the BrdU label over time in the absence of drug treatment or injury of the tissue (Kiel *et al*, 2007). Therefore, a logical follow-up question was whether blood SCs would be unique among tissue SC types. Here, we showed that a second well-characterized adult tissue SC system, besides blood, utilizes random chromosome segregation at division, in the absence of any perturbation by injury or drug treatment. Moreover, we provide a quantitative approach to accurately evaluate this question *in vivo*, in tissues less active than blood in which the BrdU is retained for long periods of time, without the need to identify extremely rare mitotic cells or grow the cells in culture.

Significance of quantitative division measurements: future lessons of tissue kinetics

The quantitative proliferation history of bulge SCs documented here contradicts the generally adopted model that portrays the bulge as an inactive ‘storage’ niche (Ohlstein *et al*, 2004). Our data indicated a much more extensive turnover rate of bulge cells during one activation cycle than previously recognized, an aspect of bulge cell behaviour that needs to be considered in future models. The extent of bulge cellular output has been essentially unknown (Cotsarelis, 2006), although it has been estimated that only up to four bulge-derived SCs would contribute to the formation of the hair

bulb *in vivo* (Kopan *et al*, 2002). Additional contribution must be made to the outer layer of the follicle (Legue and Nicolas, 2005). Previously, the only significant mitotic activity has been detected at the beginning of anagen, when only some bulge SCs were thought to divide and contribute to the new follicle (Cotsarelis, 2006). In contrast to the general expectations, we found that virtually *all* bulge cells divided during normal hair cycle and a majority of these cells divided repeatedly (3–5 times). The bulge is made of roughly 100–200 cells that by an average of three divisions would generate 600–1600 cells. This would lead to three to eight-fold bulge size increase in a single hair cycle in the absence of extensive compensation by cell loss. This loss could be accomplished by substantial direct bulge progeny contribution to tissue growth and/or by a significant rate of bulge cell death; any of these events do not agree with our current view of this cell compartment thought of as permanent and highly static.

Finally, our quantitative platform is amenable to mathematical modelling of cell population dynamics in a manner similar to that previously used with membrane dye (CFSE) staining of cultured cells (Bernard *et al*, 2003). This type of analyses could allow informed prediction about tissue SC behaviour *in vivo*, and provide guidelines for further experimental testing. Cell and tissue kinetics parameters can be derived from fitting a mathematical model that describes bulge cell behaviour to proliferation history data. We tested simple exponential models of cell division in which the bulge is made of a single, homogenous cell population with either a fixed or time-dependent division rate. Interestingly, and unlike Clayton *et al* (2007) who analysed the proliferative compartment of inter-follicular epidermis, such simple models did not fit our data (R Tumber and D Shalloway, personal communication). This suggested that the bulge population might be heterogeneous and/or has cell generation-dependent kinetics (Leon *et al*, 2004). The simplest model that fits both the 4- and 8-weeks chase data assumes that the bulge is composed of two equal-sized sub-populations with different rates of cell division but the same rate of cell loss. The data can be well fit whether export from the bulge occurs synchronously or asynchronously with division. These mathematical models describing bulge cell kinetics will be discussed in detail elsewhere. We are currently addressing experimentally the SC activity and the molecular makeup of potentially distinct bulge cell populations with different division rates and levels of H2B–GFP retention.

In conclusion, we characterized the cell dynamics in the bulge compartment of HF, and ruled out the chromosome-sorting model as a major mechanism of label retention and genome integrity preservation. Analyses of cell behaviour *in vivo* using our quantitative cell division counts reveal previously inaccessible information about cell population dynamics and tissue kinetics during development and homeostasis.

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Materials and methods

Mice

Animal work was carried out according to the Cornell IACCUC guidelines. We crossed hemizygous pTRE–H2B–GFP (Tumber *et al*, 2004) (*CD1*) and K5tTA (*FVB1*) mice (Diamond *et al*, 2000) and identified GFP-expressing animals with an ultraviolet-based portable lamp (BLS Ltd). Mice were fed with 1 g doxy/kg mouse chow (Bio-serv), and killed at PD21, 49, and 77.

Immunofluorescence and wide-field microscopy

Procedures and primary antibodies are described in more detail in Supplementary data and elsewhere (Osorio *et al*, 2008).

Confocal microscopy

Frozen tissue sections (100 µm) were fixed for 1 h in 4% paraformaldehyde and stained overnight with TOPRO3 (1:2000 dilution; Invitrogen). Confocal Z-sections were collected using a Leica microscope.

BrdU and TPA treatment

BrdU was injected subcutaneously to a final amount of 50 µg/g of body weight starting at postnatal PD3–PD5 at 12-h intervals. We checked the efficiency of BrdU labelling at PD6 by immunostaining as described elsewhere (Tumber, 2006). To preserve the nuclear structure for BrdU staining, we sorted cells in Eppendorf tubes with 5% BCS in PBS, and gently spotted them on slides. We fixed the cells for 30 min in 4% freshly made paraformaldehyde in PBS. For administration of BrdU in the drinking water, we used 0.8 mg/ml. For TPA treatment, the right side of the mouse back skin was treated with 200 µl acetone, and the left side was treated with 0.1 nmol/µl TPA in acetone 3 × per week.

FACS analysis

We used SPHERO Rainbow particles, 3.0–3.4 µm (Spherotech), to calibrate the FACS machine before each experiment. All experiments were performed on a FACS Aria (BD Biosciences) in the Cornell University Flow Cytometry facility. Skin cells were isolated from fresh tissue using trypsin digestion and stained with biotin-labelled CD34 antibody (eBioscience) and phycoerythrin-labelled α6-integrin (CD49f) antibody (BD Pharmingen). Secondary antibodies used were Streptavidin-APC (BD Pharmingen), as previously described (Blanpain *et al*, 2004). Live cells were those not stained by propidium iodide (PI; Sigma).

RT-PCR

RNA extraction from sorted cells and RT-PCR was carried out as described elsewhere (Tumber 2006), except for the first strand cDNA synthesis we used the 'Superscript III First Strand Synthesis System (Invitrogen) according to the manufacturer's instruction.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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