Human keratinocytes have two interconvertible modes of proliferation

Amit Roshan1,5, Kasumi Murai2, Joanna Fowler2, Benjamin D. Simons3,4, Varvara Nikolaidou-Neokosmidou1 and Philip H. Jones1,2,6

Single stem cells, including those in human epidermis, have a remarkable ability to reconstitute tissues in vitro, but the cellular mechanisms that enable this are ill-defined. Here we used live imaging to track the outcome of thousands of divisions in clonal cultures of primary human epidermal keratinocytes. Two modes of proliferation were seen. In ‘balanced’ mode, similar proportions of proliferating and differentiating cells were generated, achieving the ‘population asymmetry’ that sustains epidermal homeostasis in vivo. In ‘expanding’ mode, an excess of cycling cells was produced, generating large expanding colonies. Cells in expanding mode switched their behaviour to balanced mode once local confluence was attained. However, when a confluent area was wounded in a scratch assay, cells near the scratch switched back to expanding mode until the defect was closed. We conclude that the ability of a single epidermal stem cell to reconstitute an epithelium is explained by two interconvertible modes of proliferation regulated by confluence.

Recent advances in cell culture have highlighted the capacity of single adult stem cells to generate self-assembling organ-like structures. This property was first demonstrated in cultures of primary human epidermal keratinocytes. A subset of proliferating keratinocytes has the potential to generate large colonies that subsequently fuse to form multilayered sheets, recapitulating the organization of the epidermis. In such cultures, proliferation is confined to the basal cell layer, with differentiating cells stratifying into overlying cell layers when local confluence is attained. This culture system has been widely used to study epidermal stem cells and their regulation. In addition, epithelial sheets generated in vitro are used for autologous transplantation in burns patients. The cultured grafts persist as a histologically normal epidermis for many years.

There is marked heterogeneity in the proliferative potential of cultured keratinocytes, with colony sizes ranging from two to hundreds of cells one week after plating. Subcloning suggests that there are at least three kinds of clonogenic cell. ‘Holoclones’ have extensive cell renewal potential and generate large proliferating colonies, whereas small, differentiated colonies arise from ‘paraclones’ with minimal proliferative potential. A third class of ‘meroclone’ cell, produces colonies with intermediate properties.

These results may reflect a proliferative hierarchy of three or more cell types. However, this seems at odds with the results of in vivo lineage tracing in homeostatic murine epidermis, where only one or two progenitor populations have been identified. We reasoned that live imaging of a large sample of cells to resolve the dynamics of individual cells may reveal the basis of clonal diversity and how single cells can reconstitute epidermal sheets.

RESULTS

We used a high-definition time-lapse microscopy system to image clonal cultures of human neonatal foreskin epidermal keratinocytes (NFSKs). Imaging did not alter the colony size distribution at 7 days (Fig. 1a). Staining revealed both microscopic differentiated colonies and large colonies containing numerous proliferating cells, indistinguishable from non-imaged controls (Fig. 1b). We next tracked 2,208 complete cell cycles over 7 days of culture. Median cycle length, excluding the first division after plating, was 15.7 h (range 4.7–100.2 h, n = 2,127, Fig. 1c). We constructed lineage trees for 81 colonies, with a final size between 2 and 722 cells (Figs 1d,e and 2 and Supplementary Table 1). In the largest colonies it was feasible to track cells over only four sequential rounds of cell division so multiple sets of subclones spanning four cellular generations were tracked within each colony. Keratinocyte division generates cells that go on to either exit the cell cycle and differentiate or divide. More than 99% of divisions occurred within 48 h (Fig. 1c). We therefore classified cells that...
Figure 1 Live imaging of cultured keratinocytes. (a) Size distribution of live imaged (n=81) and non-imaged control (n=1,487) colonies after 7 days culture, in three independent experiments. Box boundaries indicate the 25th and 75th percentiles. Line across box is the median. Whiskers indicate 1st and 99th percentiles. There is no statistically significant difference between the distributions (P=0.15 by Kolmogorov–Smirnov test; NS, not significant). (b) Typical colonies cultured for 6 days, treated with EdU and fixed 24 h later. White, differentiation marker KRT1; yellow, EdU; green, keratinocyte marker KRT14; blue, DAPI. Images are representative of 3 independent experiments. Scale bars, 100 μm. (c) Cycle times of 2,127 live imaged cells from three independent experiments; median 15.7 h; 99% of all divisions occur within 48 h. (d,e) Representative examples of two types of lineage tree, expanding (d), and balanced (e), from three independent experiments. Dividing cells are green, non-dividing cells are magenta and cells observed for <48 h are grey. See Fig. 2a,b and Supplementary Table 1 for complete data set and Supplementary Videos 1 and 2 for example videos. (f,g) Division outcomes in expanding (f, 928 divisions) and balanced colonies (g, 930 divisions), expressed as percentages with 95% confidence intervals. (h) Cell cycle time distributions in balanced and expanding colonies. Box boundaries indicate the 25th and 75th percentiles. Line across box is the median. Whiskers indicate 1st and 99th percentiles. (i) The length of the preceding (maternal) cell cycle for daughter cells with each division outcome. Box boundaries indicate the 25th and 75th percentiles; line across box is the median. Whiskers indicate 1st and 99th percentiles. There is no significant difference between cycle time distributions for any division outcome (P=0.18 by Kruskal–Wallis test, n=1,109 divisions for PP, 338 PD and 330 DD).
did not divide within 48 h as differentiating (D) and those that divided as proliferative (P, Fig. 1d,e). Cells that could not be tracked for 48 h were classified as unknown (U) and excluded from further analysis (n = 288) (Figs 1d,e and 2 and Supplementary Table 1). The validity of these assignments was supported by staining for proliferation and differentiation markers at the end of the experiment (Fig. 1b).

Three outcomes of cell division were observed, symmetric divisions producing two proliferating or two differentiating cells (PP or DD) and asymmetric PD divisions. After classifying division outcomes,
that the tissue is maintained by a single progenitor population that tracing in homeostatic mouse epidermis, where modelling argues maintained. The latter interpretation is consistent with genetic lineage single population in which the outcome of an individual cell division far simpler explanation is that the diversity of trees arises from a distinct cell type specified to undergo a particular series that generate them. It might be that each variety of tree derives colonies may give insight into the proliferative properties of the cells of these colonies was far smaller than the expanding group (median 422, range 108–722 cells). On average, 13 P cells discernible pattern to the occurrence of PD or DD divisions within divisions (Figs 1d,f and 2a and Supplementary Video 1). There was no apparent at each round of cell division, resulting in a wide diversity of colonies `expanding’. In the remaining 70 colonies, PP, PD and DD divisions were apparent at each round of cell division, resulting in a wide diversity of trees (Figs 1e,g; Fig. 2b; Supplementary Video 1). There was no discernible pattern to the occurrence of PD or DD divisions within each colony. These colonies typically contained hundreds of cells by 7 days (median 422, range 108–722 cells). On average, 13 P cells were produced for each D cell, leading us to term this group of colonies ‘expanding’.

The varied nature of the lineage trees in the second group of colonies may give insight into the proliferative properties of the cells that generate them. It might be that each variety of tree derives from a distinct cell type specified to undergo a particular series of PP, PD and DD divisions, but this seems unfeasibly complex. A far simpler explanation is that the diversity of trees arises from a single population in which the outcome of an individual cell division is unpredictable, but the likelihood of PP, PD or DD outcomes is maintained. The latter interpretation is consistent with genetic lineage tracing in homeostatic mouse epidermis, where modelling argues that the tissue is maintained by a single progenitor population that undergoes PP, PD and DD divisions, with the likelihood of PP and DD divisions being equal\textsuperscript{15–18,21,22}. Strikingly, across the 70 trees, the frequency of PP divisions (38%, n = 353) was not significantly different from DD divisions (34%, n = 318; Fig. 1g). We therefore termed these trees ‘balanced’ as, on average, approximately equal proportions of P and D cells are produced per cell division. The observation of such ‘population asymmetry’ in dissociated cells in culture is remarkable and suggests that the balanced likelihood of PP and DD divisions is highly robust\textsuperscript{20,27}.

We next examined whether there was a relationship between cell cycle time and division outcome. There was only a modest difference in median cycle time between balanced (15.8 h, n = 860 divisions) and expanding (15.8 h, n = 917 divisions) colonies (P = 0.06, Mann–Whitney test, Fig. 1h). We found no correlation between the length of the preceding cell cycle and division outcome (median 15.2 h for 1,109 PP divisions, 15.0 h for 338 PD divisions and 15.3 h for 330 DD divisions, P = 0.18, Kruskal–Wallis test, Fig. 1i). This contrasts with retinal cells that have the same three types of division, but the preceding cell cycle time is found to be longer for DD than for PP and PD outcomes\textsuperscript{28}.

We then imaged cells cultured directly from normal homeostatic adult abdominal epidermis (Supplementary Table 2). A total of 1,156 complete cell cycles were observed in 56 colonies (Supplementary Figs 1 and 2a–c and Supplementary Table 1). The median cycle length was 15.0 h (range 5.5–62.3 h), and 99.6% of divisions occurred within 48 h (Supplementary Fig. 2a). The same three division outcomes

---

**Figure 3** Division outcomes of sister cells. (a) Time-lapse images of a typical colony from 81 independent videos in 3 independent experiments (dashed white line), with related cells outlined in dark blue (index cell), yellow (sister), cyan (cousin) and black (second cousin). Scale bar, 100 µm. (b) Mean number of cells separating sisters (n = 345 sister pairs), cousins (456 pairs) and second cousins (390 pairs); error bars indicate 95% confidence intervals; data from 3 independent experiments. (c) Division outcomes (percentage, 95% confidence interval) in sister pairs in balanced (n = 305 divisions) and expanding (n = 415 divisions) colonies. (d,e) Division outcomes in sister, cousin (balanced 413, expanding 689) and niece (balanced 617, expanding 782) cell pairs; data from 3 independent experiments. Legend indicates relationship and outcome, overlaid onto population averages (grey bars) for balanced (d) and expanding (e) lineages. Error bars indicate 95% confidence intervals.

---

**ARTICLES**

© 2016 Macmillan Publishers Limited. All rights reserved
were observed within 6 expanding and 50 balanced type trees (Supplementary Figs 1 and 2a,c). The median sizes of balanced and expanding colonies at 168 h were 4 (range 2–93) and 299 (range 175 ± 876) respectively. We conclude that adult epidermal cells exhibit the same behaviours as NFSKs and that these modes of proliferation do not arise from prolonged cell culture.

Are division outcomes dependent on the behaviour of neighbouring cells and/or the microenvironment? To investigate this we examined pairs of sister NFSKs in which both cells went on to divide. Sister cells remained adjacent to each other throughout their life span in 98% of cases (n = 349), minimizing microenvironmental differences between the cells (Fig. 3a,b). Comparing an index cell to its sister revealed no difference in division outcomes compared with the entire dividing cell population in either expanding or balanced colonies (Fig. 3c). We also analysed division outcomes in spatially separated index ‘niece’ and index ‘cousin’ pairs and again found no significant differences from the population average (Fig. 3b,d,e).

These results argue against extracellular factors as determinants of particular division outcomes, as if this was the case, the outcomes in adjacent cells would be expected to be similar. Furthermore, sister pair

**Figure 4** Transcriptional analysis of colonies. (a) Protocol. Single NFSKs were flow sorted into individual wells of a cell culture plate, cultured for 60 h, feeders removed, and entire 8-cell colonies lysed in situ. Following amplification, cDNA was analysed by array analysis. (b) Hierarchical clustering reveals two groups of colonies, A and B. Heat map shows transcripts with twofold or higher differential expression in 11 colonies (see also Supplementary Tables 4–6). (c) Gene set enrichment analysis plots showing transcripts differentially expressed between groups A and B. Reactome ‘translation’ (c) and ‘metabolism of proteins’ (d) gene sets are shown (nominal P values both <0.001, normalized enrichment scores 2.8 and 2.7 respectively; see also Supplementary Table 7). (e) Mean O-propargyl-puromycin fluorescence per colony after 60 h of culture in situ. 60 h, feeders removed, and entire 8-cell colonies lysed (see also Supplementary Table 7). (f) Appearances of typical colonies from 1 of 3 independent experiments stained for O-propargyl-puromycin (OP-Puro; green), KRT1 (red) and DAPI (blue). Scale bars, 10 μm. (g) RNA and protein knockdown of CBX5 following siRNA transfection. Inset shows a typical quantitative capillary isoelectric focusing immunoassay. Mean of four independent experiments for both protein and RNA. (h) Colony size distribution 7 days after CBX5 knockdown. Typical example of three independent experiments is shown; *P < 0.02 by Kolmogorov–Smirnov test; n = 139 control and 164 CBX5 colonies; details of other experiments are shown in Supplementary Table 9.
Figure 5 Expanding colonies switch towards balance. (a) Typical large colony (12.5 mm² area) after 12 days of culture, treated with EdU for 24 h before fixation. Left panel: phase image overlaid with KRT14 staining in green. Right panel: immunofluorescence image with differentiation marker KRT10 (red), EdU (yellow) and DAPI (blue). Insets show EdU staining in centre and margin of colony. Image is representative of three independent experiments. Scale bars: main panels 0.5 mm, insets 0.1 mm. (b) Still from typical video from three independent experiments showing colony after 9 days of culture. Inner third (by area) is bounded by yellow line; colony edge is indicated by white dashed line. Scale bar, 100 μm. (c,d) Lineage trees from the inner third (c) and outer two-thirds (d) of colonies, derived from live imaging from 168 to 288 h; dividing cells are green; non-dividing cells are magenta. (e) Still from typical video from three independent experiments showing margins of two fusing colonies (dashed lines). Yellow line indicates contact between keratinocytes in adjacent colonies, displacing overlying feeder cells. Scale bar, 100 μm. (f) Lineage trees showing behaviour of cells within 7 cells of the colony edge at the time of fusion, derived from three independent videos of fusing colonies. Time is indicated from the first contact between colonies (5–9 days after plating); dividing cells are green; non-dividing cells are magenta.

analysis excludes lateral inhibition, in which intracellular signalling drives adjacent cells into different fates, as a mechanism for achieving the similar likelihoods of DD and PP divisions observed in the balanced group²⁹,³⁰. Thus, within colonies up to 7 days after plating, division outcomes seem cell autonomous. This observation supports a critical, but previously untested assumption made in the analysis of in vivo lineage tracing data²⁰–²³,³¹,³².

Collectively the results of the live imaging experiments suggest that cells within each colony are in one of two proliferative modes, balanced or expanding. The proliferative mode of the founder cell is inherited by its progeny, so the likelihood of a given outcome is the same for all divisions in the colony. This ‘two mode’ model, based directly on live imaging data, makes testable quantitative predictions that we set out to validate.

First, we examined whether the wide distribution of colony sizes seen in 7 day NFSK cultures could be recapitulated by such a simple model. We performed a computer simulation of 7 days of growth of 30,000 virtual colonies based on the cell cycle time and probabilities of each division outcome observed by live imaging. Virtual founder cells were assigned to balanced or expanding modes in the observed proportions and each division was treated as an independent event. There was no significant difference between the
Figure 6  Cell density and ROCK2 kinase activity regulate the switch from expanding to balanced mode of proliferation. (a) Colony size in cells and area after 7 days of culture in media with (EGF10, black) or without (EGF0, blue) supplemental EGF; data from three independent experiments. (b) Summary of cell dynamics in EGF0 media. From 0 to 96 h, all cells grow in expanding mode, but between 96 and 168 h, cells switch towards balanced mode in the inner two-thirds of colonies, remaining in expanding mode at the colony rim. See also Supplementary Fig. 5c,d and Supplementary Table 1. (c) Size distribution of colonies cultured for 7 days in 10 μM ROCK2 inhibitor Y27632 compared with controls. Typical example of three independent experiments shown; ***P = 0.0007 by Mann–Whitney test, n = 302 control and 293 Y27632-treated colonies. Box boundaries indicate the 25th and 75th percentiles. Line across box is the median. Whiskers indicate 1st and 99th percentiles. (d) Fluorescence micrographs of typical large colonies from three independent experiments cultured for 7 days in standard (EGF10) media with 10 μM Y27632 (1,307 cells) or control (1,207 cells), treated with EdU (red) for the final 24 h of culture. DAPI is blue; white dashed line indicates boundary of stratified cell layer in dimethylsulphoxide (DMSO) colony (no stratification was seen in Y27632-treated colonies). Insets show distribution of EdU-positive cells. Scale bars, 100 μm. (e) Summary of effect of Y27632 on expanding mode colonies; no switch to balanced mode behaviour is seen in the inner third of colonies cultured for up to 212 h after plating. See also Supplementary Fig. 6a and Supplementary Table 1.

To further challenge the model we performed cell tracking on a larger scale. NFSKs were labelled with the fluorescent membrane dye PKH26, plated at clonal density, and the number of cells per colony.
Figure 7 Effects of loss of confluence on mode of proliferation in large colonies. (a–f) Typical stills from large colonies (n = 45 from three independent experiments), 9 days after seeding, scratched with a pipette tip. (a,b) Immediately before (a) and after (b) scratch at t = 0. (c) Typical appearance at 12 h after scratch. Yellow dashed line indicates edge of scratch; MF, migrating front; PZ, proliferative zone; white solid line indicates boundary between MF and PZ. (d) MFs meet at 17 h after scratch. (e,f) Later images at 40 and 118 h after scratch showing completion of repair. Scale bars, 100 μm. (g–i) Lineage trees of proliferating cells in the centres of large colonies 9 days after plating subjected to scratching; g is from migrating front (62 divisions from 27 colonies in 3 independent experiments) and h from proliferative zone pre-scratch closure (346 divisions from 36 colonies in 3 independent experiments). 0 is time in hours since time of scratch in g and h. i shows trees of cell divisions post-scratch closure (352 divisions from 45 colonies in 3 independent experiments; 0 h is time of closure). (j–l) Summary of cell behaviour; green indicates proliferating cells; magenta indicates differentiating, non-dividing cells; numbers are percentages of cells with a given division outcome with 95% confidence intervals in brackets. See also Supplementary Table 1 for full data set.

determined every 24 h (n = 333 colonies in total from 3 independent experiments; Supplementary Fig. 3 and Supplementary Table 3; refs 33,34). To classify colonies into balanced or expanding we drew on the live imaging data. Most balanced colonies contained only differentiated cells by 144 h, but in those that retained proliferating cells, the proportionate increase in cell number between 144 h and
Two interconvertible modes of proliferation underpin epithelial reconstitution in vitro. (a) Keratinocyte proliferation in clonal culture. Small ‘paraclines’ and some ‘meroclines’ with limited subclonal potential are derived from cells in balanced mode division (magenta). Large ‘holoclines’ and the remaining ‘meroclines’ are derived from cells in expanding mode (cyan). Divisions at the centre of the holoclines switch from expanding to balanced divisions with local confluence. Grey cells represent differentiating keratinocytes, and suprabasal cells are omitted for clarity. (b) Fusion of adjacent large colonies triggers a switch from expanding to balanced mode. As additional colonies fuse, a large sheet of keratinocytes results, with the overall epithelium in balance. (c) If the centre of a large colony is wounded, the central keratinocytes in balanced mode switch from balanced to expanding mode to re-epithelialize the defect, and revert to balanced divisions when the gap is closed. (d) Two interconvertible modes of proliferation. The average division outcomes of balanced and expanding mode cells are shown with the likelihood of each type of cell indicated as a percentage. Confluence promotes a switch from expanding to balanced mode, a process that requires ROCK2 activity. Loss of local confluence in a scratch assay results in a switch from balanced to expanding mode.

168 h was less than 50% whereas in expanding mode colonies it was significantly higher ($P < 0.0003$ by Mann–Whitney $U$-test, Supplementary Fig. 4a,b). We therefore used the ratio of colony size at 168/144 h to classify colonies as balanced or expanding (Methods; Supplementary Fig. 4b–d).

This larger data set was then used to further challenge the predictions of the two-mode model. First, for a population of balanced mode colonies, the mean number of cells per colony is predicted to increase linearly, as on average, each division adds one differentiated and one proliferating cell to the colony. This linear growth signature discriminates balanced mode dynamics from other behaviours, for example transit-amplifying cells that differentiate after a fixed number of divisions$^{20}$. Across 304 balanced colonies, mean cells per colony did indeed increase linearly with time ($r^2 = 0.98$), whereas the mean size of expanding mode colonies ($n = 29$) rose exponentially as expected (Supplementary Fig. 4c,f).

A second prediction of the model, based on the computer simulation, was that colonies between 50 and 150 cells at 168 h could arise from either of the two modes of division. Some colonies in expanding mode could, by chance, have a longer than average cycle time for a few early rounds of division, whereas rare colonies in balanced mode may retain some proliferative cells (Supplementary
Fig. 2e). Examination of dye-labelled colonies in this size range indicated that, as predicted, some were balanced whereas others were expanding (Supplementary Fig. 4c,d). This observation may explain why there seem to be three types of clonogenic cell when clonal 7 day cultures are subcloned[7]. The large-colony-forming 'holoclone' and small-colony-forming 'paraclone' cells are in expanding and balanced modes respectively. However, rather than reflecting a third cell type, the intermediate size 'meroclone' colonies may reflect their being a mixture of the largest balanced and the smallest expanding mode colonies[7].

Taken together, the above results lead us to conclude that the proliferative heterogeneity observed in clonal density 7 day human keratinocyte cultures is explicable by just two modes of cell proliferation tuned to either exponential proliferation or the balanced production of proliferating and differentiating cells.

Each mode of proliferation would be expected to produce colonies with a distinct transcriptional profile. We therefore investigated global transcription in colonies containing 8 cells, 60 h after plating (Fig. 4a,b). In expanding lineages, all 8 cells were in cycle, and a computer simulation indicated that balanced 8-cell colonies would be likely to contain 3–4 differentiating cells at this time point. Enzymatic digestion to isolate single cells grossly perturbed transcription, so we opted to analyse 8-cell colonies, selected on cell number alone. Colonies were lysed in situ and transcriptional analysis of the entire colony was performed (Fig. 4a). Hierarchical clustering analysis using all transcripts expressed above background levels revealed two groups of colonies, A and B (Fig. 4b). Differentially expressed transcripts are shown in Supplementary Tables 4 and 5. Group B had significantly higher levels of the $S100$ calcium-binding proteins and transcripts are shown in Supplementary Tables 4 and 5. Group B had significantly higher levels of the $S100$ calcium-binding proteins and downstream signalling proteins were apparent (Supplementary Table 6). Gene set enrichment analysis revealed that group B colonies also had higher levels of messenger RNAs associated with translation, RNA metabolism and biogenesis (Fig. 4c,d and Supplementary Table 7). To validate these results we measured global translation using a metabolic and biogenesis (Fig. 4c,d and Supplementary Table 7).

To validate these results we measured global translation using a fluorescence-based $\text{-propargyl-puromycin}$ assay in colonies cultured in EGF0 and standard media with $10$ ng ml$^{-1}$ EGF (Supplementary Fig. 5b–d and Supplementary Tables 1 and 10). These findings are consistent with increased cell density promoting the transition from expanding towards balanced behaviour in large colonies.

These findings led us to reason that cytoskeletal signalling may play a role in the confluence regulated switch from expanding to balanced mode[31,39]. We tested the effect of treating NFSKs with Y27632, an inhibitor of the actin cytoskeleton, ROCK2 kinase, which is used in establishing primary cultures of adult stem cells from multiple lineages[5,51,52]. ROCK2 inhibition in NFSKs results in decreased terminal differentiation and increased proliferation, prolonging the lifetime of the cells in culture[53,54]. As expected, we found that culture of NFSKs in $10\mu M$ Y27632 for 7 days increased colony size and altered the distribution of proliferating cells within expanding colonies (Fig. 6c,d). Live imaging for 10 days post plating revealed that the switch from expanding mode in large colonies was blocked in the presence of Y27632, but balanced behaviour was unaffected (Fig. 6e and Supplementary Fig. 6 and Supplementary Tables 1 and 11). These observations argue that the switch from expanding to balanced mode requires ROCK2-mediated cytoskeletal signalling.

Finally, we explored whether the confluence-driven transition from expanding to balanced mode was reversible. The central regions of large colonies seeded 9 days previously, in which cells are in balanced mode, were scratched with a pipette tip and the cells close to the resulting 'wound' were tracked by live imaging (Fig. 7a–f). We observed two discrete regions around the scratch, recapitulating keratinocyte behaviour at a wound margin in vivo[31].
Keratinocytes within 4 cell positions of the edge of the scratch formed a ‘migrating front’ from which collective migration of cells closed the defect within 72 h. Cell divisions in the migrating front were rare (62 divisions in total in 27 colonies) and produced an excess of non-dividing cells (Fig. 7g,i). In contrast, there were numerous divisions in the proliferative zone behind the MF (346 divisions in total in 36 colonies) where cells switched from balanced towards expanding mode, reverting to balanced mode when the defect had closed (Fig. 7h,i,k,l). These results indicate that keratinocytes can switch reversibly between the two modes of proliferation.

**DISCUSSION**

We conclude that clonal culture of human keratinocytes reveals two modes of proliferation, balanced and expanding, maintained across cellular generations (Fig. 8a). Once local confluence is attained, expanding mode cells switch to balanced mode, providing a simple and robust mechanism for generating an epithelium from a single cell (Fig. 8b). If confluence is removed by a scratch injury, balanced mode cells local to the defect transiently and reversibly switch to expanding behaviour until the defect is repaired (Fig. 8c). These results argue that rather than there being a hierarchy of stem and progenitor cells in human epidermal cultures, proliferating cells can switch between homeostatic and regenerative behaviour, a finding in keeping with recent lineage tracing studies in mice.

The observation that ROCK2 inhibition prevents the switch from expanding to balanced mode in the confluent centres of large colonies joins with a significant body of evidence linking the fate of keratinocytes to the actin signalling pathway. For example, inhibition of the β1 integrins that connect the extracellular matrix to the actin cytoskeleton substantially increases the number of small, differentiated balanced mode type colonies in clonal culture. Restricting the contact area of keratinocytes with basement membrane also promotes their differentiation through SRF signalling as does inhibition of the actin regulator RAC1 or activation of ROCK2. This study does not reveal whether cells commit to proliferation or differentiation before, during or after cell division or how the mode of proliferation is determined when cells are plated at clonal density. However, the transcriptional analysis of 8–cell colonies gives potential clues to the molecular basis of the modes. Increased translation is a strong signature of balanced mode dynamics, and rapamycin treatment that suppresses translation, promotes the formation of large, expanding type colonies.

The two interconvertible modes of keratinocyte proliferation may be viewed within a wider perspective of behavioural plasticity of adult stem and progenitor cells. Across evolution, tissue maintenance and repair by a hierarchy of cells with invariant fate is rare in comparison with population asymmetry, stochastic fate and behavioural plasticity in response to injury. Switching between modes of proliferation provides a robust cellular mechanism for organ repair and tissue reconstitution by single cells in vitro.

**METHODS**

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.
et al. 2016. Macmillan Publishers Limited. All rights reserved
METHODS

Ethics. Work with human material was carried out in compliance with the UK Human Tissue Act (2004) under a protocol approved by the UK National Research Ethics Service (08/H0306/128). All subjects gave written informed consent for the use of their surplus tissue in research.

Cell authentication. Neonatal foreskin keratinocytes (NFSKs) were obtained directly from ATCC (PCS-200-010). J2-3T3 fibroblasts were a gift from J. Rheinwald (Department of Dermatology, Brigham and Women’s Hospital, USA), not authenticated. All cells were tested for mycoplasma on initial culture and at least 3 months thereafter using a Mycoprobe Mycoplasma Detection Kit (CUL001B, R&D systems) according to the manufacturer’s instructions. No mycoplasma-positive cells were used in this work.

Feeder layer. Keratinocytes were cultured with feeder cells. 3T3 J2 cells were maintained in Dulbecco’s modified Eagle medium (D-MEM; Invitrogen 12320-032) supplemented with 10% donor bovine serum (Invitrogen 16030-074) at 37 °C in an atmosphere of 5% CO2. Cultures were passaged twice per week by 5 min treatment with 1 × 0.25% trypsin-3.4 mM ethylene-diamine-tetra acetic acid (EDTA, diluted from Sigma-Aldrich T4174) to detach cells. Trypsin–EDTA solution was neutralized with culture medium and cells were seeded to new flasks at a dilution of 1 in 6. 3T3 cultures were maintained up to passage 16.

Feeder layers were prepared by incubating 3T3 cells for 3h with 4 μg ml−1 mitomycin C (Sigma-Aldrich M4287), followed by washing with phosphate buffered solution (PBS: 10 mM Na2HPO4, 1.8 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl at pH 7.4). Cells were then detached by trypsinization as above and seeded at densities of 2 × 105 cells into 75 cm2 flasks (~25,000 cells cm−2) before the addition of keratinocytes.

Keratinocyte culture. Keratinocytes were maintained in complete FAD medium (3500 high-glucose D-MEM (Invitrogen 11971-025); D-MEM F12 (Invitrogen 31330-038), supplemented with 5 μg ml−1 insulin (Sigma-Aldrich I5500), 1.8 × 10−4 M adenine (Sigma-Aldrich A3159), 0.5 μg ml−1 hydrocortisone (Calbiochem 386698), 1 × 10−8 M cholera toxin (Sigma-Aldrich C8052), 10 ng ml−1 epidermal growth factor (EGF, PeproTech EC 100-15) and 5% fetal calf serum (FCS, PAA Laboratories A15-041)) with a 3T3 J2 feeder layer.

Cultures were maintained at 37 °C in 5% CO2. Cells were passaged at around 80% confluence and split 1 in 20 following detachment by incubation for 15 min in 1× trypsin–EDTA (~8,000 cells cm−2) at 37 °C. NFSKs were used between passages 3 and 7.

Isolation of adult keratinocytes. Adult keratinocytes were obtained directly from discarded skin from abdominoplasties. Anonymized details of donors are shown in Supplementary Table 2. Excess human skin from abdominoplasty surgery discarded skin from abdominoplasties. Anonymized details of donors are shown in Supplementary Table 2. Excess human skin from abdominoplasty surgery was purchased from hospitals in the UK, with ethical approval of the use of the skin obtained as excess from plastic surgery procedures (see Supplementary Table 2 for donor details). Neonatal foreskin keratinocytes (NFSKs) were obtained from Invitrogen (Invitrogen 11971-025). Neonatal foreskin keratinocytes were used between passages 3 and 7.

For immunostaining macroscopic colonies at 12 days post seeding, individual colonies were detached from culture plates by 5 min incubation in 5 mM EDTA at 37 °C. Colonies were fixed in 4% PFA for 30 min and stored in PBS at 4 °C until further analysis. Results presented are from a minimum of three dishes at each time point in each experiment.

Immunostaining and microscopy. For immunostaining, fixed cell plates were incubated in fish skin gelatin (FSG) buffer, comprising 0.5% Triton X-100 (BDH 28817-295), 0.25% FSG (Sigma-Aldrich G7765), bovine serum albumun (BSA, Calbiochem 126575), and 10% donkey or goat serum (Sigma-Aldrich D9663 or G9023 respectively) depending on secondary antibody species in PBS. Primary antibodies were diluted in FSG and incubated overnight at 4 °C. Following three 5 min washes in PBS secondary antibodies (Invitrogen Molecular Probes) were diluted in FSG, incubated for 3h at room temperature, plates washed three times with PBS and mounted in Vectashield Hardset mounting medium with 4′,6-diamidino-2-phenylindole (DAPI, Vector Labs H-1500).

For scratch assays NFSKs were cultured for 9 days at clonal density. Large colonies were then scratched with a plastic pipette tip creating a defect approximately 750 μm wide across the centre of the colony. Live imaging was then performed in the Incucyte system as described above.

PKH26 dye labelling of keratinocytes. Keratinocytes were labelled with PKH26, a lipid-binding yellow-orange fluorescent dye, with a PKH26 Cell Linker Kit (Sigma-Aldrich PKH26GL), following the manufacturer’s instructions. A suspension containing 2 × 105 keratinocytes was washed in PBS to remove serum and resuspended in 1 ml of diluent C (labeling solution; an iso-osmotic aqueous solution). This cell suspension was added to a PKH26 dye solution to achieve a final dye concentration of 2 μM. The mixed dye/cell suspension was incubated for 5 min at room temperature with periodic agitation. Adding serum and further incubation

DOI: 10.1038/ncb3282

NATURE CELL BIOLOGY © 2016 Macmillan Publishers Limited. All rights reserved
for a minute stopped the labelling reaction. The cell suspension was then centrifuged and washed three times in media.

To track cell growth, cells were then plated at 25 cells/cm² on to a feeder layer and cultured as described above. Colonies were identified by fluorescence imaging and recorded with a Zeiss Axio Observer D1 microscope and the x and y coordinates of each colony recorded with a Vernier graduated stage. Colonies were re-examined every 24 h and the number of cells was counted, until 168 h when dishes were fixed and stained as described above.

To resolve multicellular colonies into balanced or expanding mode, the ratio of the number of cells at 168 h to that at 144 h was calculated. On the basis of the properties of balanced and expanding mode cells we expected that this ratio would be <1.5 in balanced mode colonies owing to the accumulation of differentiated cells and the likelihood of exclusively PP divisions decreasing as the number of progenitors in the colony increases (Fig. 2b and Supplementary Fig. 4a,b). In contrast, the size of expanding mode colonies was expected to rise by more than 50% between 144 h and 168 h, whereas for colonies of more than 150 cells, comprising mostly expanding mode cells in the simulation, the ratio was >1.5 (Supplementary Fig. 4c). In colonies of between 50–150 cells, some colonies had a 168 h:144 h size ratio <1.5, whereas for colonies of more than 150 cells, comprising mostly expanding mode cells in the simulation, the ratio was >1.5 (Supplementary Fig. 4c).

Immunoblotting. NFSKs were seeded at 10³ cells/cm² in media containing 0, 10 or 20 ng/ml supplemental EGF. At the times indicated, cells were scraped into lysis buffer comprising 20 mM Hepes-NaOH pH 7.9, glycerol 10%, NaCl 0.4 M, NP-40 0.5%, EDTA 0.2 mM, dithiothreitol (DTT) 1 mM, and 1 μM Protease and Phosphatase inhibitor (Thermo Fisher 784400) and centrifuged at 18,500g at 4°C for 10 min. Protein concentrations were measured using standard Bradford protein assays (BioRAD QuickSTART Bradford Dye Reagents 500-0202). Lysates were mixed 1:1 with loading buffer (2× concentration of Tris-HCl pH 6.8, 4% SDS, 20% glycerol, bromophenol blue and 0.2% β-mercaptoethanol). Samples were heated at 95°C for 5 min. Six micrograms of each sample was loaded onto a 1.5% or 10% SDS–polyacrylamide gel. Proteins were separated by electrophoresis and transferred onto Immobilon-P membrane (pore size 0.45 μm, Millipore IPVH00101). Membranes were first incubated in blocking buffer (5% dried skimmed milk, PBS, 0.1% Tween-20) at room temperature for 1 h and then with primary antibodies diluted in blocking buffer for 1 h at room temperature or overnight at 4°C on a rocking platform. After rinsing in PBS, 0.1% Tween-20, membranes were placed on a SNAP-id protein detection system (Millipore SNAP2MB1). Washing and incubation with HRP-conjugated secondary antibody were performed on the system according to the manufacturer's instructions. Proteins were detected using Immobilon Western Chemiluminescent HRP substrate (Millipore WBKLS0500) or SuperSignal West Femto Chemiluminescent Substrate (ThermoFisher Scientific 34095) for high sensitivity.

Antibodies. Details of antibodies used are given in Supplementary Table 12.

Transcriptional analysis of 8-cell colonies. A single-cell suspension of NFSK cells was sorted by flow cytometry into half-area 96-well plates (Costar 3969), gating on forward and side scatter to isolate keratinocytes and excluding dead or dying cells by gating out the 7AAD-positive population and then cultured in standard media with feeder cells. After 2.5 days, feeders were removed by pipetting and 8-cell colonies were identified by phase microscopy. Colonies were lysed in TRIzol (Invitrogen) on the plate and RNA was extracted according to the manufacturer's instructions. RNA was precipitated with linear acrylamide (Ambion) and the RNA pellet was dissolved in Direct Lysis buffer from WT-Ovation One-direct Kit (NuGEN), after which cDNA was amplified following the manufacturer's protocol.

Amplified cDNA was hybridized to Affymetrix Human Gene 1.0 ST arrays. Data were analysed as follows. The Bioconductor R xps package was used to create an expression matrix from Affymetrix data by Rostock Multi-array. Each array was normalized. The samples were then filtered on the basis of detection above background. Hierarchical clustering was performed for values with a minimum log fold change of 0.5 and the Linear Models for Microarray Data Bioconductor package used to determine differentially expressed genes.

Functional analyses were carried out using DAVID (http://david.abcc.ncifcrf.gov) and GSEA software (Broad Institute of MIT and Harvard University, http://www.broad.mit.edu/gsea). For GSEA analysis the reaction genome database was selected. Default parameters were maintained except that the permutation type was by gene set with 1,000 permutations. We defined gene sets as being enriched if they had a FDR < 25% and a nominal P value < 0.05.

Measurement of translation by O-propargyl-purumycin assay. Forty-eight hours after seeding at clonal density, NFSKs were cultured with 50 μM O-propargyl-purumycin (Fena Bioscience, NU-931-05) in keratinocyte media for 1 h. Plates were then fixed with 4% paraformaldehyde for 10 min on ice. Colonies were stained following the same protocol described above for immunostaining with EDU using a 1:500 dilution of a Keratin 1 antibody (AF-87, Covance) and an Alexa Fluor Click-IT kit (Invitrogen, C10357) to detect O-propargyl-purumycin. Colonies containing 6–10 cells were imaged using a Zeiss Axio-Observer D1 microscope with Zeiss Axiosview software and mean colony fluorescence was quantified using NIH ImageJ software.

CBXS knockdown. A total of 0.1 x 10⁷ NFSK cells were seeded in a 12-well plate along with a J2 feeder layer. Forty-eight hours post seeding, feeder cells were removed using gentle pipetting and 1 ml fresh CFAD medium was added. For RNA and protein knockdown experiments 80 pmol per well SilencerSelect siRNA directed against CBXS (523885, Life technologies) or Negative control siRNA (Life technologies 4390843) was added to each well with 3 μl Lipofectamine 3000 transfection reagent in 1 ml CFAD as described by the manufacturer (Life Technologies). Cells were left to incubate for 24 h before the medium was changed and a 3T3 feeder cell layer re-seeded. Forty-eight hours post transfection cells were lysed in either RLT buffer (Qiagen) for RNA extraction or RIPA (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, ThermoFisher Scientific 89900) for protein buffers for assays.

RNA was extracted using the RNEasy kit (Qiagen, 74104) and reverse transcribed using the Quantitec kit (Qiagen 205331). qPCR was carried out using a StepOne plus PCR machine (ThermoFisher Scientific) with Taqman probes (Hs01127577_m1 CBXS and Hs00999905_m1 GAPDH) and reagents (Life Technologies).

For quantitative capillary isoelectric immunofluorescence, 4 μl of protein was run on the Wes system (Protein Simple) and hybridized using anti-CBX5 and anti-alpha-tubulin as primary antibodies. Run conditions were as recommended by the manufacturer. Peak areas were determined using Compass software (Protein Simple) and normalised to alpha-tubulin. RNA and protein knockdown experiments were performed in biological quadruplicate.

For assaying colony size post CBXS knockdown, cells were transfected with CBXS or control siRNA at 40 pmol per well and cultured for 7 days before being fixed with 4% PFA and stained and imaged as described above. Colony size assays were performed in biological triplicate.

Reproducibility of experiments. Figure 1b shows typical images from each of 3 independent experiments. Figure 1de shows representative examples from a total of 81 lineage trees from 3 independent experiments; full data set is shown in Fig. 2. Figures 3f and 4f show typical images from 1 of 81 colonies videoed. Figure 4f shows typical colonies from 1 of 3 independent experiments. Figure 5a is representative of colonies in 3 independent experiments. Figure 5b shows representative colonies from 3 independent experiments. Figure 6d shows representative colonies from 3 independent experiments. Figures 7a–t shows a representative colony from 3 independent experiments. Supplementary Fig. 3bo shows representative colonies from 3 independent experiments. Supplementary Fig. 5a shows a representative blot from 3 independent experiments.

Supplementary Videos 1, 2 are representative of 81 videos from 3 independent experiments.

Statistics. Pairwise comparison of normally distributed data with similar variance was by unpaired two-tailed t-test. Colony size distributions were analysed by Kolmogorov-Smirnov or Mann–Whitney unpaired tests. Division outcome data are presented with the 95% confidence interval of the proportion. GraphPad Prism 6 software was used for statistical testing and generating plots. Monte Carlo simulations based on live imaging data were run in Excel using a sequential random number generator.

Accession codes. Microarray data were deposited in ArrayExpress with accession number E-MTAB-3128.

69. Jensen, K. B., Driskell, R. R. & Watt, F. M. Assaying proliferation and differentiation capacity of stem cells using disaggregated adult mouse epidermis. Nat. Protoc. 5, 898–911 (2010).
70. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl Acad. Sci. USA 102, 15545–15550 (2005).
**Supplementary Figure 1** Lineage trees of primary adult human keratinocytes in vitro. Keratinocytes cultured directly from adult epidermis in standard media. Scale indicates time since plating in hours. Magenta indicates cells that did not divide within 48 hours, green cells observed to divide, grey cells those which could not be tracked for 48 hours. a, balanced, b expanding trees. Horizontal brackets in b, marked by *, indicate representative cells tracked within a single expanding colony.
Supplementary Figure 2 Live imaging of primary adult human keratinocytes and validation of two mode model. a-c: Live imaging of keratinocytes cultured directly from adult epidermis. a: Cell cycle time distribution b, c: Division outcomes (% with 95% confidence interval) in balanced, b (547 divisions in 50 colonies), and expanding, c lineages (609 divisions, 6 colonies). See Supplementary Figure 1 a, b for lineage trees. d, e Predictions from a large simulation of keratinocyte growth. Simulated and observed colony size distributions. d: Box plots showing day 7 colony size distributions from a computer simulation assuming two modes of growth with division outcomes shown in Figure 1 (Simulated, n=30,000 colonies) compared with observed sizes from cultured NFSK (Observed, n= 1631 colonies pooled from 3 independent experiments). Box boundaries indicate the 25th and 75th percentiles. Line across box is the median. Whiskers indicate 1st and 99th percentiles. There is no statistically significant difference between the distributions (Kolmogorov-Smirnov test P=0.15) e: Proportions of simulated colonies containing one or more cycling cells after 7 days, red line indicates balanced colonies, red shading balanced colonies with one or more proliferating cells and green shading expanding colonies. Arrow indicates overlap of colonies containing 50-150 cells, which may be from cells proliferating in either mode.
Supplementary Figure 3  Tracking colony expansion with PKH26 labelled cells. a: Protocol. NFSK were labelled with PKH26 and plated at clonal density. Every 24 hours the coordinates of each colony and the number of cells per colony were determined by fluorescence microscopy. At 168 hours dishes were fixed and stained. b,c Images of typical colonies, showing phase contrast images overlaid with PKH26 fluorescence (orange) for time points up to 168 hours with the number of cells/colony and the fixed 168 hour colonies stained for Dapi, blue, pan cytokeratin (green) and EdU (red). Scale bars: 100μm. Images are representative examples of 333 colonies in 3 independent experiments.
**Supplementary Figure 4** Analysis of PKH26 tracking data. 

- **a**: Live imaging data (Fig. 2b) shows that in larger balanced mode colonies the accumulation of differentiating cells (red) resulted in a proportionate increase in cell number between 144 and 168 hours <1.5. Two examples of live imaged colonies which retained proliferating cells at 168 hours are shown, with the ratio of cell number (168:144 hour ratio) at the last two time points in each colony. 
- **b**: 168:144 hour ratio from live imaged colonies (n=8 expanding and 7 balanced colonies from 3 independent experiments, Supplementary Figure 1a,b). Red line indicates ratio <1.5 discriminates between modes. 
- **c**: Box plot of 168:144 hour ratio in PKH26 tracked multicellular colonies (n=333 colonies from 3 independent experiments). Box boundaries indicate the 25th and 75th percentiles. Line across box is the median. Whiskers indicate 5-95 percentiles, red line indicates ratio of 1.5. Colony size is stratified following predication of the simulation (Supplementary Figure 3) that colonies of <50 cells are balanced, 50-150 cells a mixture of balanced and expanding colonies and >150 cells are expanding. 
- **d**: Cells per clone in PKH26 labelled colonies. Each line represents a single colony growth with, inset showing enlarged view of colonies between 50-150 cells. 
- **e**: Mean cells per colony versus time in balanced mode colonies (n=304 colonies). The increase is linear (r2=0.98), dotted lines indicate 95% confidence intervals. 
- **f**: Mean cells per colony versus time in expanding mode colonies (n=29 colonies) showing an exponential increase, dotted lines indicate 95% confidence intervals.

**Analysis**

1. **Live Imaging Data**
   - In larger balanced mode colonies, the accumulation of differentiating cells (red) led to a proportionate increase in cell number between 144 and 168 hours, with a ratio <1.5.
   - Two examples are shown in live imaged colonies, with the ratio of cell number at the last two time points.

2. **Box Plot Analysis**
   - A box plot shows the 168:144 hour ratio for live imaged colonies, stratifying colonies into <50, 50-150, and >150 cells.
   - The median line across the box indicates the ratio of 1.5, with whiskers showing 5-95 percentiles.
   - Colony size is stratified based on prediction from simulation.

3. **Cell Per Clone**
   - A graph shows the mean cells per colony versus time for balanced mode colonies, with linear increase (r2=0.98) and 95% confidence intervals.
   - An enlarged view of colonies between 50-150 cells is shown.

4. **Exponential Increase**
   - A graph shows the mean cells per colony versus time for expanding mode colonies, with an exponential increase and 95% confidence intervals.
**Supplementary Figure 5** Effect of increased cell density on NFSK proliferation. 

**a:** Immunoblot analysis of EGF signalling in NFSK. Lysates were collected at the times indicated after plating keratinocytes in media containing 0, 10 or 20 ng/ml added EGF. Arrowheads indicate EGFR degradation product. Position of size markers (kDa) is as indicated. Blots shown are representative of 3 independent experiments. 

**b:** Cell cycle time distribution of NFSK cultured in media without supplemented EGF (EGF0). Scale indicates time since plating in hours. Magenta indicates cells that did not divide within 48 hours, green cells observed to divide, grey cells those that could not be tracked for 48 hours. c, balanced, d expanding trees. Horizontal brackets marked by * in b indicate representative cells tracked within a single expanding colony. In d large green circles, arrowed, indicate cells lying in outer third (by area) of expanding colony, other green cells lie within inner two thirds of colonies at 96 hours.
Supplementary Figure 6  Effect of Y27632 on NFSK proliferation. **a**, **b**: Lineage trees of NFSK cultured in standard media in the presence of 10μM Y27632. Scale indicates time since plating in hours. Magenta indicates cells that did not divide within 48 hours, green cells observed to divide, grey cells those that could not be tracked for 48 hours. **a**, balanced, **b** expanding trees. Horizontal brackets in **b**, marked by *, indicate representative cells tracked within a single expanding colony.
Supplementary Figure 7 Scans of Western blots Scans of four blots (1 to 4) presented in cropped form in Supplementary Figure 5a.
Supplementary Table Legends

Supplementary Table 1 Division time and outcomes
Excel spreadsheet showing division times and outcomes, each sheet corresponds to the stated lineage tree figure panel.

Supplementary Table 2 Details of donors of adult skin used for primary culture:

Supplementary Table 3 Live tracking of PKH26 labelled keratinocytes
Data is pooled from 3 independent experiments, n =333 colonies.

Supplementary Table 4 Transcripts expressed at higher levels in group A 8-cell colonies (Fold change > log20.5, P<0.01).

Supplementary Table 5 Transcripts expressed at higher levels in group B 8-cell colonies (Fold change > log20.5, P<0.001).

Supplementary Table 6 Expression of transcripts upregulated on keratinocyte differentiation in group A and B colonies

Supplementary Table 7 Functional Annotation of transcripts differentially expressed in 8-cell colonies
Table shows output of DAVID analysis for transcripts listed in Supplementary Table 5.

Supplementary Table 8 Levels of O-propargyl-puromycin fluorescence in colonies 60 hours after plating.
Values shown are mean fluorescence intensity per colony (size range 6-10 cells, median size 8 cells). Colonies containing 1 or more cells staining for KRT1 were scored KRT1+ve, the remainder KRT1-ve.

Supplementary Table 9 Day 7 colony sizes (cells/clone) in Control and CBX5 knockdown NFSK cultures.
Results from 3 independent experiments are shown.

Supplementary Table 10 Cell division outcomes in media without supplemented EGF (EGF0)
Percentages of all divisions in each colony type with 95% confidence interval at the times and locations indicated (inner two thirds (by area) and outer third). Total numbers of divisions imaged were balanced 341 divisions in 45 colonies, expanding 515 divisions in 6 colonies.

Supplementary Table 11 Cell division outcomes in expanding colonies cultured in 10 µM Y27632
Percentages division outcomes of all divisions in balanced and expanding colony types with 95% confidence interval. 3 independent experiments were imaged, with 118, 236 and 222 total divisions per experiment.

Supplementary Table 12 Antibodies used in this study
Antibodies, suppliers and the applications they were used for.

Supplementary Video legends

Supplementary Video 1 Time lapse imaging of typical NFSK expanding type colony
Clock indicates time since plating, yellow dashed line NFSK cells, other cells are mouse 3T3 J2 fibroblast feeder cells, scale bar 100µm.

Supplementary Video 2 Time lapse imaging of typical NFSK balanced type colony
Clock indicates time since plating, yellow dashed line NFSK cells, other cells are mouse 3T3 J2 fibroblast feeder cells, scale bar 100µm.