



Human keratinocytes are efficiently immortalized by a Rho kinase inhibitor

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Primary human keratinocytes are useful for studying the pathogenesis of many different diseases of the cutaneous and mucosal epithelia. In addition, they can form organotypic tissue equivalents in culture that can be used as epidermal autografts for wound repair as well as for the delivery of gene therapy. However, primary keratinocytes have a finite lifespan in culture that limits their proliferative capacity and clinical use. Here, we report that treatment of primary keratinocytes (originating from 3 different anatomical sites) with Y-27632, a Rho kinase inhibitor, greatly increased their proliferative capacity and resulted in efficient immortalization without detectable cell crisis. More importantly, the immortalized cells displayed characteristics typical of primary keratinocytes; they had a normal karyotype and an intact DNA damage response and were able to differentiate into a stratified epithelium. This is the first example to our knowledge of a defined chemical compound mediating efficient cell immortalization, and this finding could have wide-ranging and profound investigational and medical applications.

Introduction

Somatic cells have a limited lifespan, gradually slow in growth, and stop dividing, a process known as cellular senescence. This process is thought to limit the vulnerability of aging cells to disease. Human keratinocytes are invaluable for the study of skin biology and the pathogenesis of skin-related diseases, but their short lifespan in culture is a limitation. Different conditions have been developed to optimize the culture of keratinocytes; for example, the presence of fibroblast feeder cells increases the proliferative capacity of primary keratinocytes from approximately 20 to 40–60 population doublings (1, 2). Spontaneously immortalized keratinocyte lines, for example HaCaT (3) and NIKS (4), have been used for skin-related research. However, these cell lines have genetic abnormalities, such as mutations in p53 (5) or isochromosomes (4).

Continuous replication of primary human cells is blocked by 2 separate events: mortality stage 1 (M1; replicative senescence) and mortality stage 2 (M2; crisis). At M1, signaling by shortened telomeres results in activation of the p53 and pRB pathways. M2 represents a critical period of genomic instability, with extremely eroded telomeres, resulting in chromosomal fusions and translocations. In retinal pigment epithelial cells and foreskin fibroblasts (which have decreased expression of components of the p16INK4A/pRB pathway), telomerase expression is sufficient to bypass both M1 and M2 and stabilize and elongate chromosome ends (6). However, telomerase expression is not sufficient for immortalization of keratinocytes, and p16INK4A function must also be disrupted (7, 8).

Keratinocytes expressing the E6 and E7 proteins encoded by high-risk human papillomavirus (HPV) types bypass both M1 and M2 blocks and become immortal (reviewed in ref. 9). E7 inactivates and degrades the pRB retinoblastoma tumor suppressor protein to induce G₁/S phase progression of the cell cycle. This process increases the levels of p16INK4A, but inactivation of the pRB pathway renders it ineffectual (7). The E6 protein degrades p53, which is

activated in response to the usurpation of the pRB pathway. E6 also induces expression of telomerase and allows the cells to proliferate beyond senescence and evade crisis. Thus, the oncoproteins of the high-risk HPV types target the p16INK4A and telomerase pathways that are important for keratinocyte immortalization.

Rho GTPases are a subfamily of the RAS superfamily of proteins that play essential roles in cell adhesion, cytokinesis, and cell migration (reviewed in ref. 10). Rho kinase (ROCK) is a downstream effector of the Rho pathway. Mammalian cells encode 2 Rho kinases, ROCK1 and ROCK2, which phosphorylate a wide range of substrates on serine or threonine residues. These substrates are involved in processes such as stress fiber formation, actin stabilization, the development of focal adhesions, and apoptosis (reviewed in ref. 11). ROCK inhibitors, such as Y-27632 and fasudil, have been found to have diverse and profound effects on cell behavior and have great therapeutic promise in many areas of disease (12).

It has recently been shown that inhibition of ROCK greatly increases the cloning efficiency of human embryonic stem cells (13) and human keratinocytes (14). In the current study, we demonstrate that treatment with Y-27632 greatly increased long-term proliferation of primary human keratinocytes and, unexpectedly, enabled them to efficiently bypass senescence and become immortal without detectable cell crisis. This is the first example to our knowledge of a defined chemical compound mediating efficient cell immortalization, and we anticipate that the resulting immortalized keratinocytes will be invaluable for the research of many aspects of keratinocyte biology and may have substantial therapeutic and diagnostic potential.

Results

Y-27632 immortalizes primary human keratinocytes. Rho kinase inhibition has been reported to affect keratinocyte proliferation and differentiation (14, 15). To further explore the effect of Rho kinase inhibition on the long-term growth of keratinocytes, human neonatal foreskin keratinocytes and adult vaginal and ectocervical keratinocytes were cultured in the presence or absence of 10 μ M

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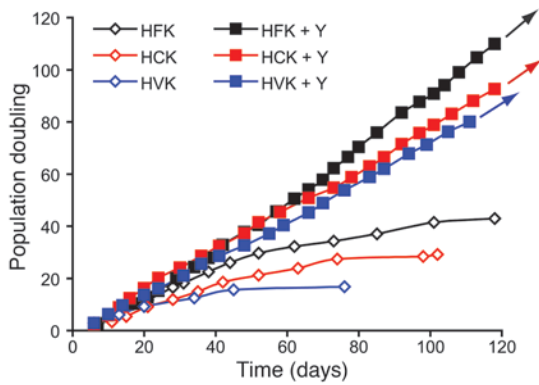


Figure 1
Y-27632 stabilizes the growth rate of primary keratinocytes. Growth rate of human keratinocytes from foreskin (HFK strain c; black), ectocervix (HCK; red), and vaginal tissue (HVK; blue) cultured in the presence (filled squares) or absence (open diamonds) of 10 μ M Y-27634 (Y). The arrows indicate cells lines that continued to divide indefinitely. The growth rate is measured as population doubling per day.

Y-27632, a well-characterized inhibitor of the Rho-associated kinase, ROCK (16). As shown in Figure 1, in the absence of Y-27632, the growth rate of all 3 keratinocyte types slowed with time, and senescence was observed at approximately population doubling 20–40, depending on the specific cell type. However, in the presence of Y-27632, a dramatic increase in cellular proliferation of all 3 types of keratinocytes was observed within days and continued indefinitely. Y-27632–treated cells had a steady growth rate, as indicated by the constant slope of population doubling versus time. All 3 keratinocyte types efficiently bypassed senescence with no observed decline in growth rate. As shown in Table 1, efficient keratinocyte immortalization was observed at least 8 times with 3 different donor pools of foreskin keratinocytes (strains a, b, and c) and twice each with ectocervical and vaginal keratinocytes. Foreskin keratinocytes have been cultured for up to 150 passages for a period of 500 days in media supplemented with Y-27632 and can be considered immortal (see Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI42297DS1). Rare spontaneously immortalized cells would occasionally grow out from quiescent cells that were close to senescence in the absence of Y-27632. However, this only occurred after a long lag period, suggesting that individual cells had picked up rare mutations, allowing them to escape senescence. In contrast, Y-27632–treated cells grew steadily at all times.

Genetic analysis was carried out on the immortalized human foreskin keratinocyte (HFK), using strain a at passage 94, to ensure that it was identical to the original donor cells. Short tandem repeat analysis, a method used to distinguish individuals based on the highly polymorphic nature of certain regions of chromosomes, showed that the immortalized cells were genetically indistinguishable from the original keratinocytes, eliminating the possibility of contamination by an immortalized cell line (Supplemental Table 1).

Immortalization by Y-27632 is dependent on coculture with fibroblasts. Culturing keratinocytes in the presence of fibroblast feeder cells increases the lifespan of keratinocytes (17, 18) and could contribute to the observed immortalization by Y-27632. Therefore, we analyzed the effect of Y-27632 on foreskin keratinocytes cultured on plastic in 2 different types of serum-free medium, in the absence

of fibroblast feeder cells. Y-27632 treatment resulted in somewhat increased proliferation, but this was not as pronounced as that in the presence of feeders. Furthermore, in repeated experiments, these cells did not efficiently bypass senescence (Supplemental Figure 2). Therefore, coculture with feeder fibroblasts is required in concert with Y-27632 treatment to immortalize keratinocytes.

Morphology of Y-27632–immortalized keratinocytes resembles that of early passage, basal-like keratinocytes. At early passages, primary keratinocytes were actively dividing and were small, cuboidal, and homogeneous in shape (Figure 2). When cultured with fibroblasts, they grew in tightly packed colonies and resembled basal keratinocytes. As they approached senescence, their morphology changed, and they became flat and heterogeneous, with an enlarged cytoplasmic volume. The morphology of all 3 types of Y-27632 immortalized keratinocytes was similar to that of early passage, actively dividing cells.

The karyotype of Y-27632–immortalized cells is normal. Immortalization of primary human keratinocytes is rare, and the resulting cells have genetic changes and abnormal karyotypes. To evaluate whether similar genetic alterations occurred during Y-27632–mediated cell immortalization, we analyzed the karyotype for the foreskin keratinocyte lines strain a, which had been cultured in the presence of Y-27632 for 95 passages. The karyotype of the immortalized cells was identical to that of the donor cells, with no apparent abnormalities (Supplemental Figure 3).

Telomerase is upregulated in Y-27632–immortalized cells. Telomerase reverse transcriptase (TERT) is the active subunit of telomerase, which maintains the telomere caps throughout the multiple cell divisions of development. TERT expression is turned off in most somatic cells, and so the telomere ends become progressively shorter over multiple cell divisions. Replicative senescence is triggered as these protective ends shorten (19). To overcome this constraint, most tumor-derived or immortal cell lines have reactivated TERT expression to maintain the telomere ends. Quantitative RT-PCR analysis showed that the level of TERT mRNA increased with passage of foreskin keratinocytes in the presence of Y-27632

Table 1
Life span of keratinocytes cultured with Y-27632

Keratinocyte strain	Experiment	+Y-27632 ^A	–Y-27632 ^B
HFK	a	1	PD195
		2	PD193
		3	PD177
	b	4	PD183
		5	PD145
		1	PD237
c	1	PD150	
	2	PD105	
HCK	1	PD93	
	2	PD67	
HVK	1	PD80	
	2	PD66	

Senescence was defined as a growth rate (population doubling [PD]/day) of less than or equal to 0.2, within the time period of 1 month. ^ACells were cultured to the population doubling shown and were considered to be immortal. ^BCells were determined to be senescent at the population doubling shown. The *P* values for testing whether numbers of populations doublings in the presence and absence of Y-27632 were equal, at day 60 and at day 80, were *P* < 0.0001 and *P* < 0.0001, respectively.

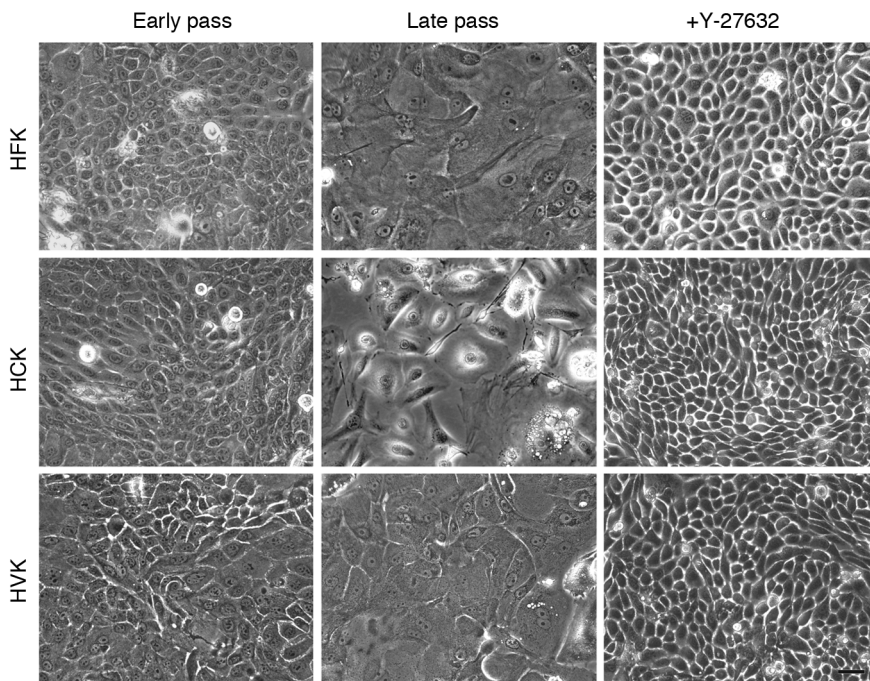


Figure 2

The morphology of Y-27632-immortalized cells resembles that of early pass primary keratinocytes. Images of human foreskin, ectocervical, and vaginal keratinocytes at P1 are shown in the left column. Images of keratinocytes near senescence (HFK P15, HCK P9, and HVK P5) are shown in the center column. Images of keratinocytes immortalized by 10 μ M Y-27632 (HFK P100, HCK P29, and HVK P26) are shown in the right column. Scale bar: 10 μ m.

(see Figure 3A). As a comparison, *TERT* mRNA levels were also determined in keratinocytes immortalized with HPV18. The high-risk HPV E6 protein directly upregulates *TERT* transcription as part of the immortalization process (20). By passage 34 in Y-27632, *TERT* mRNA levels were comparable to those in HPV18-immortalized keratinocytes. A similar induction of *TERT* mRNA was observed in vaginal and cervical keratinocytes immortalized by Y-27632 as well as in another strain of foreskin keratinocytes (Supplemental Figure 4A).

The lengths of telomeres shorten, but are stabilized, in keratinocytes immortalized by Y-27632. In HPV immortalized cells, telomere ends erode despite telomerase induction, but the shortened length becomes stable. We observed a similar phenomenon in Y-27632-immortalized cells. The relative length of telomeres was measured using a quantitative PCR assay. Despite increased levels of telomerase expression, the length of the telomeres in cells cultured with Y-27632 became progressively shorter with passage (see Figure 3B). However, the length became stable from passage 50 to 120 and was similar to the length of telomeres in HPV18-immortalized cells.

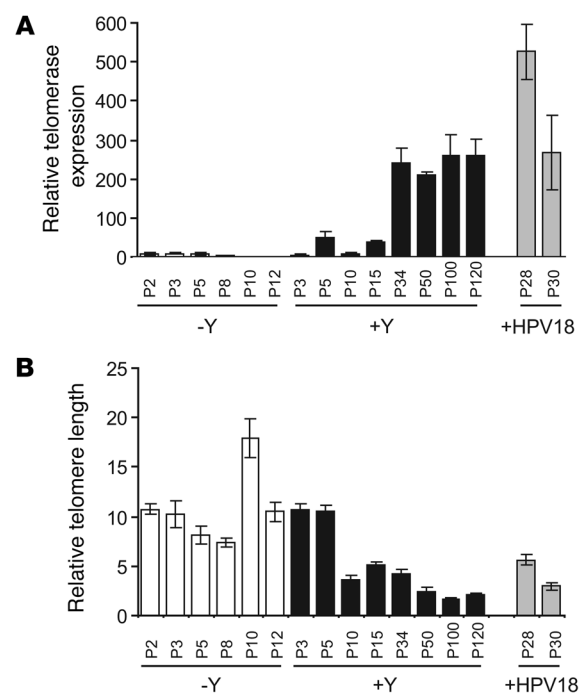
p16INK4A is expressed in Y-27632-immortalized cells. Telomerase expression is not sufficient for immortalization of human keratinocytes, and the pRB/p16INK4A pathway must also be inactivated (8). We examined *p16INK4A* mRNA and protein levels in keratinocytes during long-term culture with Y-27632. p16INK4A pro-

tein expression (see Figure 4A) and *p16INK4A* mRNA expression (Supplemental Figure 4B) were still observed, albeit at a low level, after long-term culture with Y-27632. However, at this point, we do not know whether the observed p16INK4A is functional. In contrast, the level of p16INK4A in HPV-immortalized cells is very high but nonfunctional, because of inactivation of the pRB pathway (7). Y-27632 treatment had no effect on the p16INK4A levels in these HPV-immortalized cells.

MYC is upregulated in Y-27632-immortalized cells. The MYC protein binds to the E-boxes of the *TERT* promoter to induce transcription

Figure 3

Telomerase expression increases over time, and the length of telomere ends stabilizes after culture with Y-27632. (A) Relative levels of *TERT* mRNA from HFK strain a, cultured in the absence or presence of 10 μ M Y-27632 at the pass indicated, as quantitated by real-time PCR. Each bar represents the mean of replicated samples \pm SD. (B) Relative length of telomeres in HFK strain a, cultured in the absence or presence of 10 μ M Y-27632 at the pass indicated, as quantitated by real-time PCR. Each bar represents the mean of replicated samples \pm SD.



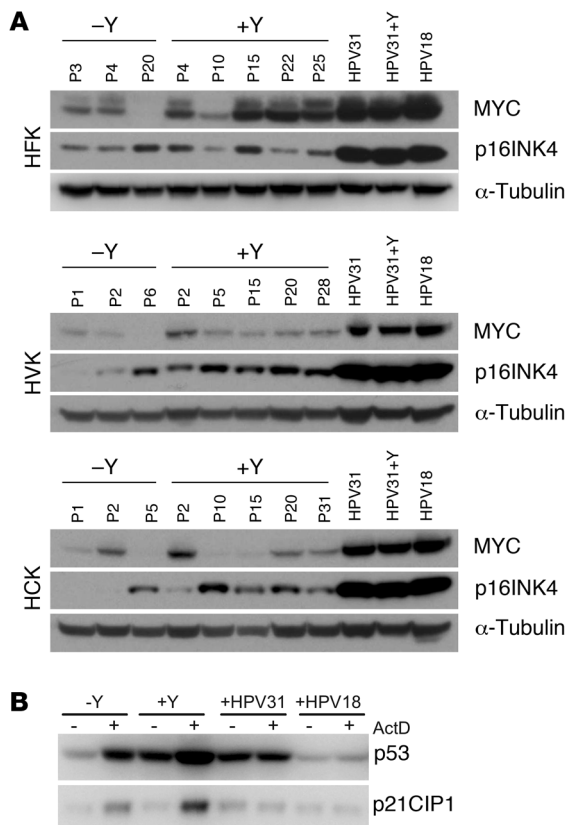


Figure 4

Expression of p16INK4, p53, p21CIP1, and MYC proteins in cells cultured with Y-27632. (A) Immunoblot analysis of MYC and p16INK4 proteins in HFK strain c, HVK, and HCK cells, cultured in the absence or presence of 10 μ M Y-27632 and collected at the pass indicated. Cells containing oncogenic HPV31 and HPV18 viruses are included as controls. α -Tubulin was detected as a loading control. (B) DNA damage was induced by treatment of cells with actinomycin D (ActD). The response was measured by immunoblot analysis of p53 protein levels and those of its downstream target p21CIP1. HFKs grown without Y-27632 were assayed at P4, and those cultured in 10 μ M Y-27632 were assayed at P122.

(22) and HPV E6 requires MYC for cellular immortalization (23). As shown in Figure 4A, Y-27632 has both short-term and long-term effects on MYC expression in all 3 keratinocyte types. MYC protein levels were induced transiently immediately after culture with Y-27632 (compare passage 4 [P4] for HFK and P2 for human ectocervical keratinocyte [HCK] and human vaginal keratinocyte [HVK]). After this initial induction, there was a general decrease in MYC protein levels. The MYC levels then gradually increased with time in all 3 cell types. A preliminary experiment showing the corresponding MYC mRNA expression levels is shown in Supplemental Figure 4C. At very late passages (P107), the level of MYC protein was equivalent to that in an HPV31-containing cell line (Supplemental Figure 5). The long-term increase in MYC levels is consistent with the increase in TERT expression, indicating that increased telomerase expression could be due to MYC induction.

The tumor suppressor gene p53 is expressed in Y-27632-immortalized cells and can mediate a normal DNA damage response. The tumor suppressor gene p53 prevents aberrant proliferation and arrests the growth of cells that have sustained genetic damage. In most cancer-derived or immortalized cell lines, the p53 pathway is either mutated or suppressed to enable cells to proliferate in conditions of aberrant growth regulation. As shown in Figure 4B, p53 protein levels were increased in keratinocytes cultured with Y-27632, but this did not appear to be inhibitory to cell growth, and the downstream effector, p21CIP1, was not induced. To test whether the p53 pathway was functional in the Y-27632-immortalized cells, we analyzed the response of the cells to p53-induced growth arrest mediated by DNA damage. Normal cells exhibit growth arrest when exposed to a mutagen, but this arrest is abrogated in cells immortalized by the HPV E6 and E7 oncoproteins (24, 25). Kera-

tinocytes were treated with 0.5 nM actinomycin D, which induces DNA strand breaks and induces a p53-mediated growth arrest. Early passage keratinocytes and Y-27632-treated keratinocytes exhibited a normal DNA damage response; both p53 and the p53-responsive protein, p21CIP1, were upregulated (see Figure 4B). In contrast, the HPV31-containing cell line, CIN-612, and HPV18-immortalized keratinocytes did not induce p53 levels or the p53 pathway in response to DNA damage. Therefore, Y-27632-immortalized keratinocytes retain a normal DNA damage response.

Y-27632-treated cells retain the capacity to differentiate. McMullan et al. have shown that blocking ROCK function inhibits suspension-induced differentiation of human keratinocytes (15). In monolayer culture in the presence of fibroblast feeders, a fraction of keratinocytes undergo differentiation (26). Therefore, Y-27632 might inhibit differentiation and promote proliferation under these conditions. To examine whether culture with Y-27632 inhibits differentiation in monolayer culture, we assayed for the presence of involucrin in cells cultured in the presence of 10 μ M Y-27632. The levels of involucrin decreased with continued passage in the presence of the ROCK inhibitor (Supplemental Figure 6A), indicating that Y-27632 did reduce the propensity for differentiation in these cultures. We also tested the effect of Y-27632 on the ability of keratinocytes to differentiate into a stratified tissue in organotypic raft culture (Supplemental Figure 6B). Early passage HFKs were seeded onto a collagen matrix containing fibroblasts and cultured as a "raft," either with or without Y-27632 in the raft media. However, no differentiation or stratification was observed when Y-27632 was present in the raft culture medium, confirming the findings of McMullan et al. in a different differentiation system.

However, one very important characteristic of normal keratinocytes is their ability to differentiate and stratify into a tissue

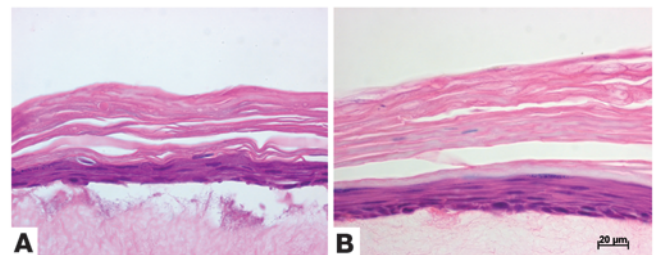
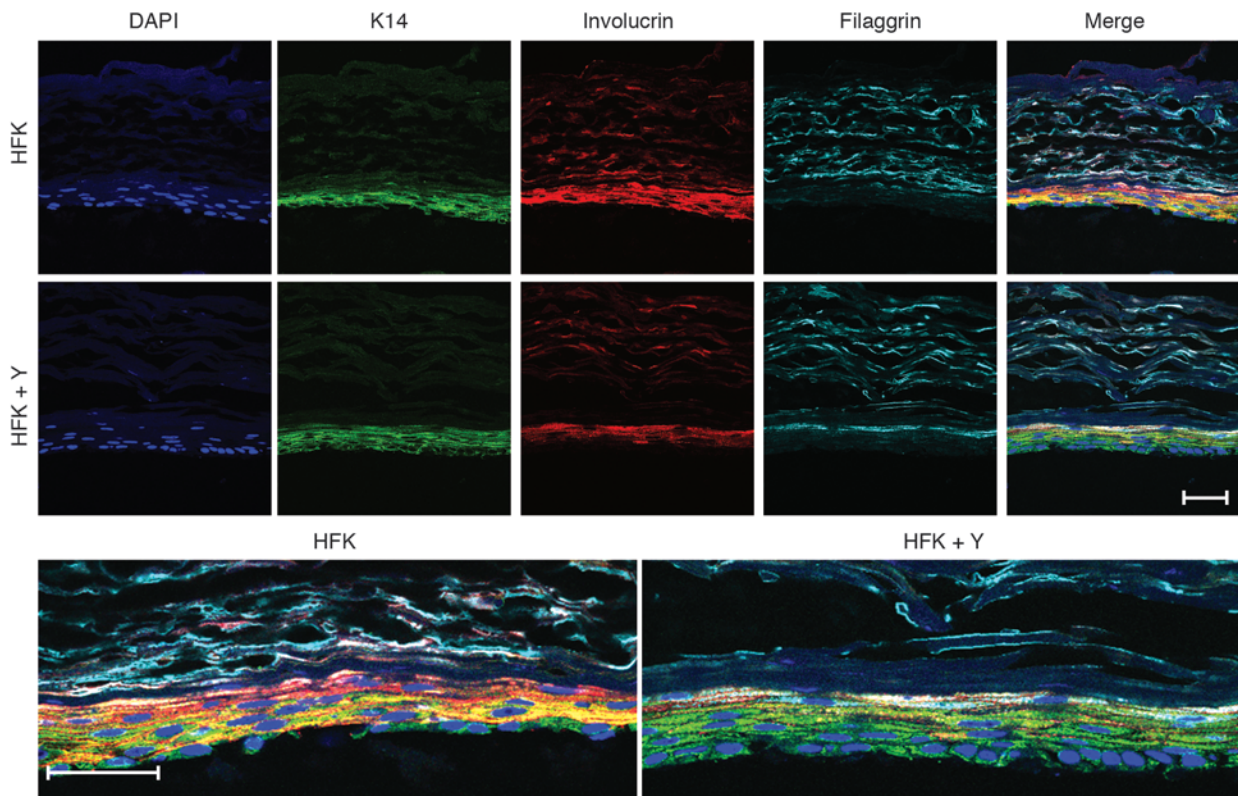


Figure 5

Keratinocytes can differentiate in organotypic raft culture after long-term culture with Y-27632. H&E-stained histological sections of primary keratinocytes at (A) P1 or (B) after 18 passes in 10 μ M Y-27632, cultured in organotypic raft culture for 17 days, are shown. Scale bar: 20 μ m.

**Figure 6**

HFKs express appropriate differentiation markers in organotypic rafts after long-term culture with Y-27632. After 17 days in organotypic raft culture, tissue sections from normal primary keratinocytes at P1 (HFK) or after 18 passes in 10 μ M Y-27632 (HFK + Y) were stained for DNA (DAPI; blue), keratin K14 (green; basal layer), involucrin (red; spinous layer), and filaggrin (cyan; granular and cornified layers). A magnified image from the merge is shown on the bottom row. Scale bar: 50 μ M.

equivalent. To determine whether keratinocytes that had been cultured in Y-27632 for many passages retained their differentiation potential if the ROCK inhibitor was removed, we assayed their ability to form a stratified epithelium in organotypic raft culture. Early passage HFKs and HFKs that had been cultured up to this point in Y-27632-containing medium were seeded onto a collagen matrix containing fibroblasts and cultured as a raft at the liquid-air interphase for 17 days without Y-27632 in the raft media. As can be seen in Figure 5B, keratinocytes that had been previously cultured in the presence of Y-27632 for 18 passages could produce a stratified epithelium in organotypic culture.

To further demonstrate that the stratified epithelial tissue grown from Y-27632-immortalized cells expressed appropriate differentiation markers, we analyzed the expression of keratin 14 (expressed in the basal layer), involucrin (upper spinous layer), and filaggrin (granular/cornified layer), using immunofluorescence on fixed tissue sections. As shown in Figure 6, raft tissue grown from either untreated or Y-27632-treated cells expressed these differentiation markers in the appropriate layer (27). Thus, keratinocytes that had been previously cultured with the ROCK inhibitor retained their capacity to differentiate normally into a stratified epithelial tissue.

Discussion

This report describes the effect of a ROCK inhibitor, Y-27632, on keratinocyte proliferation, immortalization, and differentiation.

Y-27632 treatment was shown to result in the bypass of senescence and immortalization of different types of keratinocytes from human foreskin and vaginal and cervical epithelium. Preliminary experiments indicated that human foreskin fibroblasts treated with Y-27632 did not show enhanced proliferation or bypass senescence (see Supplemental Figure 7), suggesting that this is a keratinocyte specific phenomenon. These observations have far-reaching implications for the study and treatment of various skin diseases; improved culture and extended lifespan of keratinocytes will prove invaluable for both research and therapeutic purposes.

To date, our results show that Y-27632-immortalized cells are functionally equivalent to normal cells. They have a normal karyotype and an intact DNA damage response and are able to form a stratified epithelium in organotypic culture. The immortalized keratinocytes demonstrate upregulated telomerase mRNA levels and telomeres that have shortened but remain at a stable length. MYC protein levels increased with continued passage, and this may be responsible for upregulation of *TERT* mRNA expression. Honma et al. have shown that Y-27632 rapidly induces *MYC* mRNA expression in human keratinocytes (28), and this might explain the initial induction of MYC that we observed in the first passage with Y-27632.

Previous studies have shown that telomerase expression is not sufficient for keratinocyte immortalization and inactivation of p16INK4A may also be required to bypass senescence in kerati-



nocytes (7, 8, 18). Y-27632-immortalized keratinocytes express low levels of the p16INK4A protein, but we assume that the cells are either resistant to its antiproliferative effect or protein levels are too low to mediate an inhibitory response. Rea et al. also observed p16INK4A expression in spontaneously immortalized keratinocytes cell lines (29).

In this study, we have identified 2 factors to be important for keratinocyte immortalization: (a) culture with feeder fibroblasts and (b) exposure to a Rho kinase inhibitor. We have also observed that MYC is consistently upregulated during immortalization. These factors regulate the equilibrium between keratinocyte proliferation and differentiation. Prior studies have shown that culture of keratinocytes with feeder cells enhances lifespan, possibly in part by inducing telomerase (17, 18). However, culture of keratinocytes with feeder cells also results in the expression of differentiation markers in a subset of cells when compared with culture of these cells on plastic (26). As shown in Supplemental Figure 7, Y-27632 reduced this tendency in monolayer culture. Moreover, Honma et al. propose that the Rho-GTPase pathway may control MYC activity (28). MYC has a positive role in keratinocyte proliferation but also can promote differentiation of epidermal stem cells (30). We postulate that the interaction of these pathways is important for Y-27632-mediated immortalization.

Future studies will address the mechanism of Y-27632-mediated immortalization of keratinocytes. ROCK can phosphorylate a number of different downstream targets and has profound effects on cell behavior. We do not yet know exactly which of these pathways are important for keratinocyte immortalization. However, despite the diverse effects of Y-27632, cells cultured with Y-27632 have many normal characteristics and are able to differentiate normally when Y-27632 is removed from the culture medium. Y-27632 can also inhibit other Rho effector kinases, such as citron kinase and PKN, but its affinity for ROCK kinase is 20- to 30-times higher (31). Some of the observed effect of Y-27632 on keratinocytes could also be mediated by effects of ROCK inhibitor on the feeder fibroblasts, since efficient immortalization is only observed in their presence.

The current study shows many parallels between Y-27632-induced immortalization and HPV-mediated immortalization. In both cases, telomerase was induced and the cells efficiently bypassed senescence. Another parallel is the finding by Charette and McCance that both HPV16 E7 protein and Y-27632 treatment of human foreskin keratinocytes resulted in increased cell migration (32). However, Y-27632-immortalized cells have a normal DNA damage response and karyotype compared with HPV-immortalized cells and are therefore preferable for many studies.

Keratinocytes are the natural host cells for all HPVs, but nononcogenic HPVs are difficult to study because they do not immortalize these cells. Thus, Y-27632-treated keratinocytes will be very useful for long-term analysis of nononcogenic HPV-related studies. Although not associated with cancer, these viruses are responsible for a great burden of recalcitrant disease, such as genital warts, respiratory papillomatosis, and cutaneous warts. These lesions can be especially problematic in individuals who are immunocompromised by HIV infection or organ transplantation. Y-27632-immortalized keratinocytes that can differentiate and support the entire viral life cycle will allow testing of antiviral therapies in a system that closely reflects the *in vivo* situation.

Human foreskin keratinocytes are most often used for HPV studies, because of the availability of this tissue from routine cir-

cumcision and difficulties in obtaining sufficient numbers of cells from other tissues. However, these keratinocytes might not be the best host for papillomavirus studies, as HPV infection of the foreskin is often clinically inapparent. A more appropriate cell type for the study of the many HPVs is that of the uterine cervix. We show here that Y-27632 can immortalize vaginal and cervical keratinocytes, making such cells much more amenable to study. Different HPVs have a very specific tropism for different regions of epithelia. Y-27632 treatment and expansion of small numbers of keratinocytes derived from different types of epithelia could greatly increase our understanding and treatment of HPV infection.

The ability to greatly expand and derive tissue from different types of primary human keratinocytes is of great research and therapeutic benefit. In an era in which personalized medicine is becoming increasingly important, patient-derived keratinocytes or tissue equivalents could be used to test therapies specific for the donor. Isolation and rapid expansion of keratinocytes from a small tissue biopsy using culture with Y-27632 could greatly increase the efficiency and time frame of this process. For example, small number of keratinocytes can be isolated, expanded in monolayer culture, and developed into tissue sheets for autologous epidermal replacement of regions with extensive burns or ulcers (33). Keratinocyte-mediated gene therapy is another intensively studied area of research, whereby therapeutic genes can be introduced into autologous keratinocytes and regrafted onto the host (34).

Transplantation of human keratinocytes, with enhanced *in vitro* proliferation, onto human hosts raises concerns of potential tumorigenic conversion. However, our studies have shown that ROCK inhibitor-treated cells have a normal p53-mediated growth arrest response and have no gross genetic abnormalities. Furthermore, there is evidence that ROCK inhibitors prevent rather than promote tumor progression and metastasis in human and animal models (35, 36). The Rho/ROCK pathway has been shown to function in the cardiovascular system, central nervous system, cancers, and embryonic development (37). This pathway is an important therapeutic target, and a ROCK inhibitor (fasudil) is already marketed for cerebral vasospasm after surgery (38) and is currently being tested for the treatment of angina pectoris, acute cerebral thrombosis, and other vascular diseases. Our study demonstrates that ROCK inhibitors may also be useful for quickly generating large numbers of normal keratinocytes and tissue equivalents that may be used in a variety of medical and research applications.

Methods

Cell culture. Keratinocytes were cultured in F medium (3:1 [v/v] F-12 [Ham]-DMEM, 5% FBS, 0.4 μ g/ml hydrocortisone, 5 μ g/ml insulin, 8.4 ng/ml cholera toxin, 10 ng/ml EGF, 24 μ g/ml adenine, 100 U/ml penicillin, and 100 μ g/ml streptomycin) in the presence of irradiated 3T3-J2 feeder cells (39). HFks were isolated from pools of at least 7 neonatal foreskins. Primary HCKs and HVKs were isolated as described previously (40). All human tissues were collected with institutional approval from the Institutional Review Boards at NIH and Pennsylvania State University College of Medicine. In the absence of feeders, keratinocytes were grown in 154 medium supplemented with Human Keratinocyte Growth Supplement and Gentamicin/Amphotericin (Invitrogen) or in Keratinocyte Growth Media (Invitrogen). The HPV31-positive cell line, CIN-612 9E, was obtained from Lou Laimins (Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA). The HPV18 cell line was established by introducing the HPV18 genome into primary HFks, using the Amaxa human keratinocyte nucleofection system. Cells were grown in the pres-



ence or absence of Y-27632 (Alexis Biochemicals) as indicated. Cells were subcultured by removing the fibroblast feeder cells with versene, collecting keratinocytes by trypsinization, and passing them 1:10 to 1:20 onto a 10-cm plate with J2 3T3 feeder cells. Population doubling was calculated as $PD = 3.32 (\log [\text{number of cells harvested}/\text{number of cells seeded}])$.

Immunodetection. Proteins were extracted in 2% SDS, 50 mM Tris-HCl (pH 6.8), and 10% glycerol supplemented with inhibitors Complete and PhosphoSTOP (Roche). Protein samples were resolved on NuPage gels, electrotransferred to Immobilon-P membrane (Millipore), and incubated with the indicated antibodies before detection by chemiluminescence. mAbs were against p53 (DO-1; Santa Cruz Biotechnology Inc.) and α -tubulin (B-5-1-2; Sigma-Aldrich). pAbs against MYC (N-262), p21CIP1 (C-19), and p16INK4A (C-20) were from Santa Cruz Biotechnology Inc.

Real-time quantitative RT-PCR. Real-time quantitative RT-PCR for detection of *p16INK4A*, *MYC*, and *GAPDH* mRNA was performed as described previously (17, 23, 41). Detection of *hTERT* mRNA was performed using the TaqMan Gene Expression Assay for TERT (Assay ID Hs00972646_m1) and RPLPO (large ribosomal protein; assay ID 4326314E) as an endogenous control, on a 7900HT Sequence Detection System (PE Applied-Biosystems). Each reaction was carried out in triplicate using cDNA generated from 400 ng total RNA, using oligo(dT)20 and the SuperScript III First-Strand Synthesis Kit (Invitrogen) for RT-PCR. For each sample, the amount of target and control transcripts was determined from standard curves generated using a pool of cDNA samples prepared from cells with high TERT expression. Values were adjusted according to the level of the endogenous control, RPLPO. The data were analyzed with SDS 2.1 software (Applied Biosystems).

Telomere length assay. Genomic DNA was extracted from cells and the average telomere length was assessed using a modified method of the RT-PCR-based telomere assay described previously (42). Briefly, the ratio (T/S) of telomere repeat copy number (T) to single copy gene *HBG1* number (S) was determined using a Bio-Rad IQ5 thermocycler. Five ng genomic DNA was amplified and detected with SYBR Green Super Mix (Bio-Rad). The primers for telomeres were as follows: Tel-1, 5'-CGGTTT-GTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3', and Tel-2, 5'-GGCTTGCCCTACCCTTACCCTTACCCTTACCCTTACCCT-3'; and *HBG1*, 5'-TGTGCTGGCCATCACTTTG-3', and *HBG2*, 5'-ACCAGC-CACCACTTTCTGATAGG-3'. Reaction conditions were as follows: 1 cycle, 95°C, 5 minutes; 41 cycles, 95°C, 15 seconds; 1 cycle, 60°C 45 seconds. All reactions were carried out in triplicate and compared with a standard curve of 0, 0.2, 1, 5, 25, 125 ng genomic DNA (with telomere length 10.4 kb) from Telo-kit (Roche). The T/S ratio (dCt) for each sample was calculated by normalizing the average HBG Ct value to the average telomere Ct value.

Karyotype analysis. This was conducted by Molecular Diagnostic Services Inc. Metaphase spreads were prepared and stained to observe chromo-

somal G bands. Twenty metaphase spreads were analyzed and 5 complete karyotypes were prepared from each cell line.

DNA genotype analysis. Cellular DNAs were analyzed by Molecular Diagnostic Services Inc., using the PowerPlex 1.2 STR genotyping kit (Promega).

Organotypic raft culture. Organotypic cultures were generated as described previously, with modifications (43). Briefly, 1×10^5 keratinocytes were seeded onto a rat tail type 1 collagen dermal equivalent containing $1-2 \times 10^6$ J2 3T3 feeder cells. The rafts were lifted onto stainless steel grids and were fed by diffusion from below with raft medium (3:1 [v/v] DMEM, F-12; 10% FBS, 0.4 $\mu\text{g/ml}$ hydrocortisone, 0.1 nM cholera toxin, and 5 $\mu\text{g/ml}$ transferrin). Raft cultures were allowed to stratify for 11–17 days and were fixed in formalin for 4 hours, paraffin embedded, sectioned, and stained with H&E or by immunofluorescence as described by Peh et al. (44). Abs used were mouse mAb anti-keratin 14 (Ab-1; Thermo Fisher Scientific) and polyclonal goat antiserum against filaggrin (N-20; Santa Cruz Biotechnology Inc.) and rabbit antiserum against involucrin (H-120; Santa Cruz Biotechnology Inc.). Tissue sections were counterstained with DAPI.

Growth arrest assay. Briefly, $1-2 \times 10^6$ keratinocytes were seeded on a 10-cm plate and, 48 hours later, were treated with 0.5 nM actinomycin D for 24 hours, as described previously (25). Protein extracts were prepared, as described above, and analyzed for p53 and p21CIP1 protein levels.

Statistics. A Student's paired 2-sample *t* test was used to test whether the distribution of the number of population doublings at days 60 and 80 were equal for the control and “plus Y-27632” conditions. In these analyses, all replicates of a specific cell type (foreskin, cervix, etc.) were averaged to create a single population doubling number at day 60 and day 80. Two-tailed *P* values are reported, and *P* values of less than 0.05 are considered significant.

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