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Radiation Induces Diffusible Feeder Cell Factor(s) That Cooperate with ROCK Inhibitor to Conditionally Reprogram and Immortalize Epithelial Cells

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Both feeder cells and Rho kinase inhibition are required for the conditional reprogramming and immortalization of human epithelial cells. In the present study, we demonstrated that the Rho kinase inhibitor Y-27632, significantly suppresses keratinocyte differentiation and extends life span in serum-containing medium but does not lead to immortalization in the absence of feeder cells. Using Transwell culture plates, we further demonstrated that physical contact between the feeder cells and keratinocytes is not required for inducing immortalization and, more importantly, that irradiation of the feeder cells is required for this induction. Consistent with these experiments, conditioned medium was shown to induce and maintain conditionally immortalized cells, which was accompanied by increased telomerase expression. The activity of conditioned medium directly correlated with radiation-induced apoptosis of the feeder cells. Thus, the induction of conditionally reprogrammed cells is mediated by a combination of Y-27632 and a diffusible factor (or factors) released by apoptotic feeder cells. *(Am J Pathol 2013, 183: 1862–1870; http://dx.doi.org/10.1016/j.ajpath.2013.08.009)*

The combination of irradiated feeder cells and a Rho kinase (ROCK) inhibitor Y-27632 conditionally reprograms adult keratinocytes and nonkeratinocyte epithelial cells to an indefinite proliferative state without the use of exogenous viral or cellular gene expression.[1,2] Even epithelial cells that are entering senescence proliferate immediately when transferred to the inductive conditions, which consist of F medium containing the ROCK inhibitor Y-27632 and irradiated Swiss 3T3-J2 mouse fibroblasts.[3–6] Our research group recently demonstrated that these culture conditions induce an undifferentiated, adult stem cell-like state and that this transition reflects a reprogramming of all cells in the culture population, rather than the selective outgrowth of a small subpopulation.[7] Perhaps equally important, the conditionally reprogrammed cells (CRCs) exhibited normal differentiation when the feeder cells and Y-27632 were removed, which demonstrates their maintenance of lineage commitment.[1,2,7] Although the mechanism for the generation of CRCs is still unclear, the combination of feeder cells and Y-27632 appears to provide two distinct activities that promote unrestricted cell proliferation: induction of telomerase and cytoskeletal remodeling and/or interference with the p16/Rb pathway.[1,2]

Calcium- and serum-containing medium rapidly induces terminal differentiation in keratinocytes.[8–11] However, coculturing keratinocytes with feeder cells allows the keratinocytes to bypass these signals for terminal differentiation and to proliferate until they reach cell crisis. Including Y-27632 in the coculture enables the

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keratinocytes to bypass cell crisis and proliferate indefinitely. In the present study, we showed that Y-27632 contributes to the suppression of keratinocyte differentiation in the presence of calcium and serum. Moreover, we used both a Transwell culture system and conditioned medium to demonstrate that direct physical contact between the feeder cells and keratinocytes is not required for the induction of conditional reprogramming and immortalization.

In general laboratory practice, fibroblast feeder cells are mitotically inactivated by irradiation to prevent their overgrowth of keratinocytes in coculture.[5,12–14] Here, we demonstrate that, in addition to preventing fibroblast overgrowth, irradiation of the feeder cells is critical for the production and/or release of one or more diffusible factors that are essential for conditional reprogramming and immortalization. (Hereafter, reference to factors in the plural incorporates the possibility of a single factor.)

Materials and Methods

Culture and Irradiation of J2 Cells

Swiss 3T3-J2 mouse fibroblasts[7] were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 100 μg/mL glutamine (complete DMEM; Life Technologies, Carlsbad, CA). Cultures were passaged when 90% confluent using a 1:4, 1:8, or 1:16 dilution and were given fresh medium every 2 to 3 days. Suspensions of cells in complete DMEM were irradiated at 30 Gy (3000 rads). After irradiation, the cells were plated at a density of approximately 70% in complete DMEM and were allowed to attach for at least 2 hours before addition of keratinocytes.

Keratinocyte Cell Culture

Primary human foreskin keratinocytes (HFKs) were isolated from neonatal foreskins as described previously.[15] For direct-contact coculture, HFKs were seeded on a feeder layer of lethally irradiated J2 fibroblasts[5] in F medium. The F medium consisted of 25% Ham's F-12 nutrient mix (Life Technologies) and 75% complete DMEM, supplemented with 25 ng/mL hydrocortisone, 5 μg/mL insulin, 0.1 nmol/L cholera toxin (Sigma-Aldrich, St. Louis, MO), 250 ng/mL Fungizone (Thermo Fisher Scientific, Waltham, MA), 0.125 ng/mL epidermal growth factor, and 10 μg/mL gentamicin (Life Technologies). In most experiments, cells were cultured in the presence of the ROCK inhibitor Y-27632,[1] at a final concentration of 5 μmol/L (Enzo Life Sciences, Farmingdale, NY). In the absence of feeder cells, HFKs were grown either in F medium containing Y-27632 or in keratinocyte growth medium (KGM) (Life Technologies).

For indirect cocultures, 1.1×10^6 irradiated or nonirradiated J2 feeder cells were seeded on 0.4-μm pore-size Transwell polycarbonate membranes and HFKs were grown on the corresponding 75-mm polystyrene cell culture receiver dishes (Corning Life Sciences, Tewksbury, MA). Growth curves were constructed by plotting population doublings over time.[16]

Short-term proliferation assays were performed by seeding 5.0×10^3 HFKs in six-well tissue culture plates in medium with or without Y-27632. After 6 to 8 days, the cultures were fixed with trichloroacetic acid and stained with sulforhodamine B (Sigma-Aldrich) as described previously.[17] Fixed, stained cultures were imaged by scanning and then were solubilized in 10 mmol/L unbuffered Tris for spectrophotometric quantification of the dye at 564 nm.[17] Cell counting was performed using a Countess automated cell counter (Life Technologies) according to the manufacturer's protocol.

Passaging Epithelial Cells in Direct Cocultures with Feeder Cells

To remove J2 feeder cells, keratinocyte cocultures were rinsed with Dulbecco's PBS and treated with 0.05% trypsin–EDTA (Life Technologies) for 30 seconds at room temperature. The culture vessel was then gently rocked until the feeder cells detached, and the feeder cells were removed by aspiration. The keratinocytes were washed with Dulbecco's PBS and treated with trypsin–EDTA for 3 to 5 minutes at 37°C. The keratinocytes were detached by gentle tapping, and the trypsin was neutralized by adding Dulbecco's PBS containing 10% fetal bovine serum. After centrifugation, the keratinocytes were suspended in F medium and plated on freshly irradiated feeder cells.

Preparation of Conditioned Medium

Irradiated feeder cells (1.0×10^7 to 1.5×10^7) were plated in 175-cm^2 tissue culture flasks (BD Biosciences, San Jose, CA) in 30 mL of F medium. The medium was collected 3 days later and was centrifuged at $1000 \times g$ for 5 minutes at 4°C. The resulting supernatant was passed through a 0.22-μm pore-size Millex-GP filter unit (EMD Millipore, Billerica, MA). Conditioned F medium was frozen and store at -80°C. Three volumes of conditioned F medium were mixed with one volume of fresh F medium; this mixture was supplemented with 5 μmol/L Y-27632 before use.

RT-qPCR

Total RNA was isolated using TRIzol reagent (Life Technologies) and treated with an Ambion DNA-*free* kit (Life Technologies) according to the manufacturer's protocol. cDNA synthesis and quantitative real-time RT-PCR (RT-qPCR)

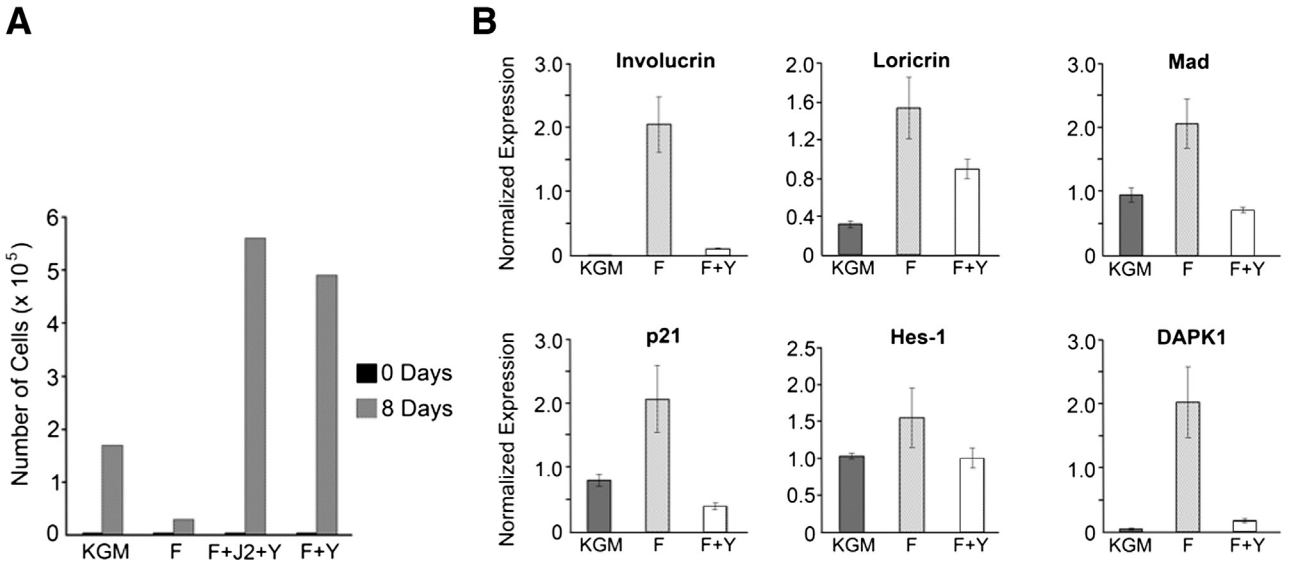

Figure 1 Y-27632 inhibits calcium- and serum-induced keratinocyte differentiation. **A:** Y-27632 stimulates keratinocyte proliferation in medium containing calcium and serum. HFKs were seeded at 5.0×10^3 per well in six-well tissue culture plates under four culture conditions: KGM, F medium (F), F medium with Y-27632 (F+Y), and F medium with Y-27632 in coculture with a layer of lethally irradiated J2 feeder cells (F+J2+Y). The cells were harvested and counted 8 days later. The experiment was performed four times independently; results from one representative experiment are shown. **B:** Y-27632 inhibits expression of differentiation-associated genes in the presence of calcium and serum. 5.0×10^6 HFKs were plated in 10-cm tissue culture dishes under three culture conditions. The cells were harvested 2 days later, and RT-qPCR was used to measure levels of mRNAs characteristic of keratinocyte differentiation. Data are expressed as means ± SD.

were performed as described previously,[18] using iCycler MyiQ and iQ SYBR Green SuperMix (Bio-Rad Laboratories, Hercules, CA). The following forward and reverse primers were used: involucrin, 5′-TCCCAGCAACACACTGCC-3′ and 5′-TGCTCAGGCAGTCCCTTTACAG-3′; p21, 5′-ATGTCAGAACCGGCTGGGGA-3′ and 5′-GCCGTTTTCGACCCTGAGAG-3′; and GAPDH, 5′-TCTCCTCTGACTTCAACAGC-3′ and 5′-GAAATGAGCTTGACAAAGTG-3′. hTERT mRNA was quantitatively measured using published primers and methods.[18] All data were normalized to levels of GAPDH.

Caspase 3/7 Assay

2.0×10^4 irradiated feeder cells were cultured in 96-well plates for 0, 1, 2, or 3 days using DMEM (100 μL per well; Life Technologies). To assay the activity of caspases 3 and 7, 100 μL of Caspase Glo 3/7 reagent (Promega, Madison, WI) was added to the wells, rocked at 500 rpm for 30 seconds and incubated for 30 minutes at room temperature. Next, 100-μL aliquots of the resulting cell lysates were transferred to a white 96-well plate (Sigma-Aldrich) for measurement of luminescence using a Veritas microplate luminometer and software (Turner Biosystems, Sunnyvale, CA).

Statistical Analysis

Standard deviation (SD) was calculated using IQ5 2.0 Standard Edition Optical System software version 2.0.148.060623 (Bio-Rad Laboratories) based the values from three experiments or replicates.

Results

Y-27632 Inhibits Keratinocyte Differentiation Induced by Calcium and Serum

Keratinocytes typically are cultured in low-calcium, serum-free medium[19] or are cocultured in high-calcium, serum-containing F medium with lethally irradiated feeder cells.[5] We have previously shown that the ROCK inhibitor Y-27632 enhances keratinocyte proliferation and indefinitely prevents differentiation in the presence of F medium and feeder cells.[1] To determine the effects of Y-27632 on the proliferation and differentiation of keratinocytes in the absence of feeder cells, HFKs were cultured for 8 days either in low-calcium, serum-free KGM or in F medium in the presence or absence of Y-27632 and feeder cells. There was a significant reduction in HFK proliferation in F medium without feeder cells or Y-27632 (6-fold increase in cells from the initial inoculum) versus KGM (34-fold increase) (Figure 1A). Conversely, the addition of Y-27632 stimulated proliferation 98-fold, which was similar to the 112-fold proliferation seen in the presence of Y-27632 and feeder cells. These results suggest that Y-27632 acts directly on the HFKs to promote proliferation via suppression of differentiation induced by calcium and serum.

The ability of Y-27632 to inhibit keratinocyte differentiation was analyzed in more detail by measuring the level

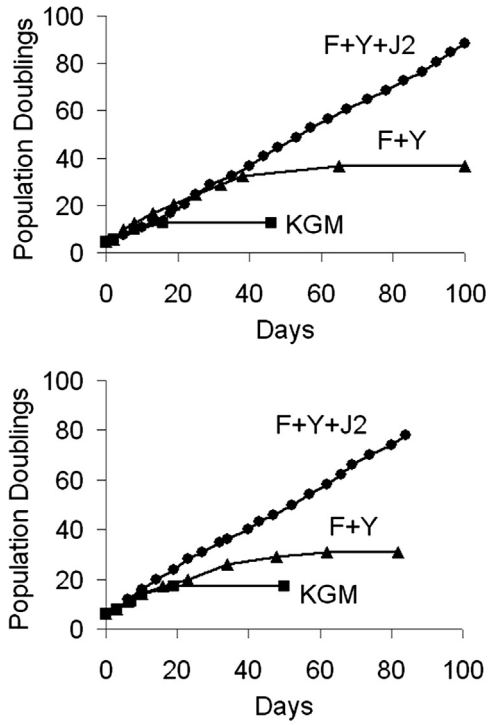

Figure 2 Feeder cells are required for conditional immortalization of keratinocytes. HFKs were passaged for up to 100 days under three culture conditions: KGM, F+Y, and F+Y+J2. Y-27632 alone did not induce conditional immortalization. Two independent experiments with two different HFK strains are shown (p2, **top**; p3, **bottom**).

of mRNAs associated with keratinocyte differentiation in KGM, F medium, and F medium containing Y-27632 (Figure 1B). As expected, transcript levels for the keratinocyte differentiation markers involucrin,[20–22] loricrin,[23,24] Mad,[25,26] p21,[27,28] and Hes-1,[29,30] as well as for the cell death–related gene *DAPK1*,[31,32] increased 2.5-fold to 20-fold when HFKs were transferred from KGM to F medium for 2 days. Importantly, including Y-27632 in the F medium significantly, and in some cases completely, suppressed these increases (Figure 1B).

Feeder Cells Are Required for the Conditional Immortalization of Keratinocytes in Medium Containing Serum and Calcium

Because Y-27632 inhibited keratinocyte differentiation, we asked whether it could immortalize HFKs in F medium in the absence of feeder cells. To investigate this possibility, we performed long-term (100 days) cell culture experiments with two strains of HFKs at passages 2 and 3 (p2 and p3) in KGM, in F medium containing Y-27632, or in F medium containing Y-27632 and feeder cells (Figure 2). HFKs cultured in KGM survived for the shortest period (16 days and 12.6 population doublings). Y-27632 dramatically extended this proliferative capacity (65 days and 36.6 population doublings); however, only HFKs cocultured with feeder cells in the presence of Y-27632 could be propagated indefinitely with no decrease in the rate of proliferation. Therefore, Y-27632 inhibits terminal differentiation and promotes transient cell proliferation, but cannot bypass signals of senescence or crisis.

Conditional Reprogramming and Immortalization Does Not Require Physical Contact between Keratinocytes and Feeder Cells

To determine whether direct contact between feeder cells and HFKs is necessary for conditional immortalization, we used an indirect coculture system that makes use of Transwell permeable support inserts to physically separate the feeder cells from the HFKs while providing the HFKs access to the putative factors released by the feeder cells (Figure 3A). The two strains of HFKs could be passaged indefinitely (at least 100 days and 80 to 90 population

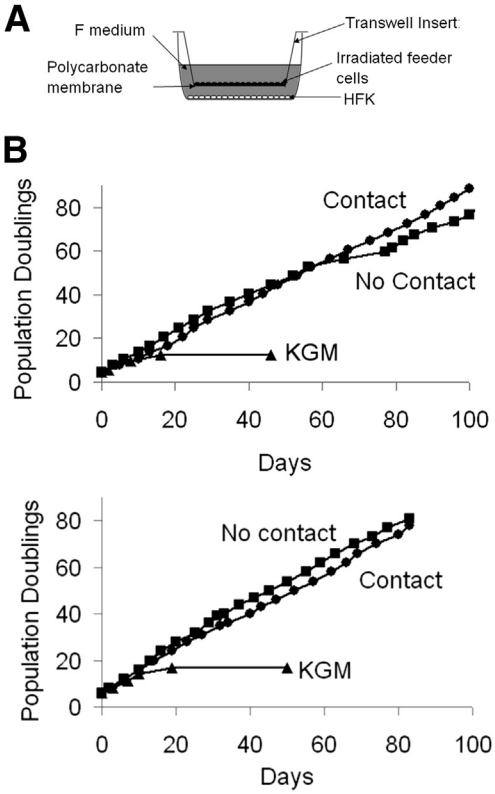

Figure 3 Conditional immortalization of keratinocytes does not require physical contact with feeder cells. **A:** In the indirect coculture system, HFKs were plated on the bottom of the companion dish, and irradiated feeder cells were seeded on the Transwell polycarbonate membrane suspended 1 mm from the bottom. Cells shared the same medium, but were not in direct contact. **B:** HFKs were cultured in direct contact with irradiated feeder cells or in the indirect coculture system without contact for more than 90 days in the presence of Y-27632 in two independent experiments (**top** and **bottom** panels). HFKs showed similar rates of proliferation and conditional immortalization in both culture systems. HFKs grown in KGM served as a negative control for immortalization.

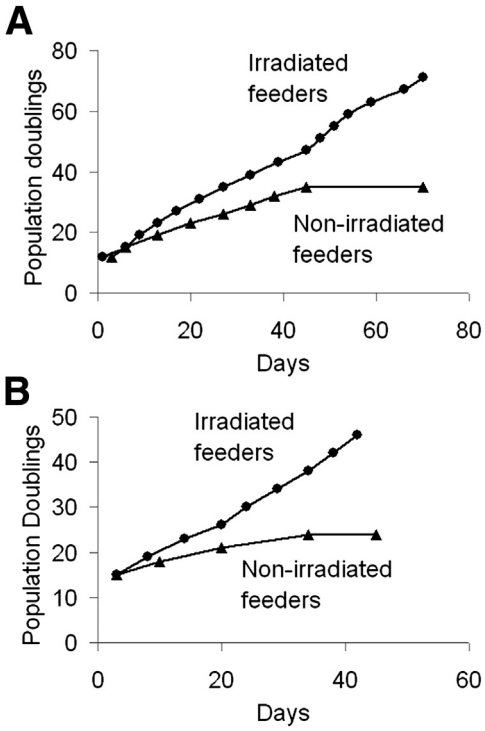

Figure 4 Irradiation of feeder cells induces diffusible factors that conditionally immortalize keratinocytes. HFKs were cocultured with lethally irradiated or nonirradiated J2 feeder cells for more than 70 days in the Transwell indirect coculture system in the presence of Y-27632. Only irradiated feeder cells supported conditional immortalization of the HFKs. The experiment was performed in duplicate, using HFKs at p5 (**A**) or p9 (**B**). Conditional immortalization was more rapidly observed with the late-passage HFKs.

doublings) in F medium containing Y-27632, regardless of whether they were in direct contact with irradiated feeder cells (Figure 3B). Moreover, the HFKs exhibited similar rates of proliferation in the two culture systems. HFKs cultured in KGM, which served as negative control for conditional immortalization, stopped growing after 16 days (12 population doublings). These results clearly demonstrate that the conditional immortalization of HFKs does not require direct physical contact with feeder cells, and imply that the feeder cells release one or more diffusible factors that cooperate with Y-27632 to promote keratinocyte proliferation and survival.

Irradiation of Feeder Cells Is Required for Production of Diffusible Factors That Contribute to Conditional Reprogramming and Immortalization

The use of irradiated fibroblast feeder cells to maintain keratinocytes in coculture is well documented.[5,6] Irradiation is necessary to prevent the fibroblasts from overgrowing the more slowly proliferating epithelial cells. Because our indirect coculture system physically separates the feeder cells and keratinocytes, we used this technique to investigate whether irradiation of the feeder cells is required for the production and/or release of diffusible factors that enable conditional immortalization of the keratinocytes. HFKs at p5 were cocultured with irradiated or nonirradiated feeder cells plated on Transwell inserts for 70 days (Figure 4A). HFKs grown with irradiated feeder cells continued to proliferate at a nearly constant rate throughout the experiment, whereas those cultured with nonirradiated feeder cells lost their proliferative capacity and entered senescence after 40 days. In a similar experiment using HFKs at p9, irradiated feeder cells again supported indefinite proliferation, whereas the growth rate of HFKs cultured with nonirradiated feeder cells decreased immediately and proliferation ceased altogether after the third passage (Figure 4B). These results indicate that irradiation is a vital element in conditional immortalization and is required to stimulate the feeder cells to produce and/or release one or more essential factors.

Conditioned Medium Substitutes for Feeder Cells in Conditional Reprogramming and Immortalization

To confirm that diffusible factors contribute to conditional immortalization, we sought to demonstrate that conditioned medium collected from cultures of irradiated J2 feeder cells could substitute for feeder cells in immortalization assays. First, however, it was important to ascertain, using a short-term proliferation assay, whether Y-27632 is necessary for the release of immortalizing factors by the feeder cells. HFKs were cultured for 6 days in conditioned F medium that was collected from irradiated feeder cells after 3 days in the presence or absence of Y-27632. Then, Y-27632 was added after collection to a portion of the conditioned medium made in its absence (Figure 5). Conditioned medium made without Y-27632 (Figure 5A) gave rise to relatively small colonies of HFKs. In contrast, both the conditioned medium made in the presence of Y-27632 (Figure 5A) and the conditioned medium to which Y-27632 was added after collection (Figure 5A) stimulated proliferation fourfold (Figure 5B). Therefore, Y-27632 does not enhance the release of feeder factors that suppress keratinocyte differentiation and promote proliferation.

We tested the ability of conditioned medium to conditionally immortalize keratinocytes in the presence of Y-27632 by culturing late-passage (p10 and p11) HFKs for 55 days using three different conditions: F medium containing Y-27632, direct coculture in F medium with irradiated feeder cells and Y-27632, and conditioned F medium containing Y-27632 (Figure 6A). Conditioned medium was as effective as direct coculture with irradiated feeder cells for rescuing these HFKs from senescence and inducing rapid, indefinite proliferation in the presence of Y-27632. Similarly, both conditioned medium and direct coculture with irradiated feeder cells conditionally immortalized early-passage (p2) HFKs and induced similar rates of proliferation (in the presence of Y-27632), over a period of 87 days and 60 population doublings (Figure 6B). These results corroborate

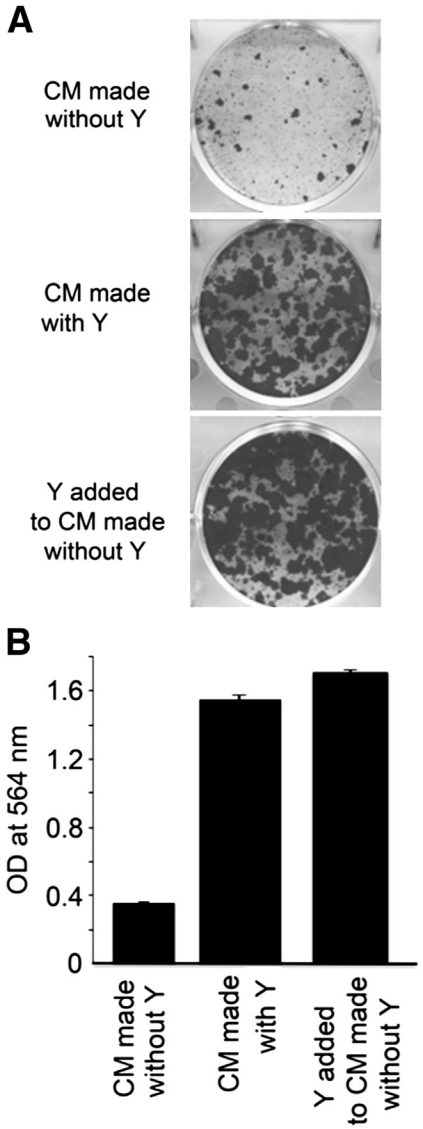

Figure 5 Y-27632 is not required for the production of diffusible factors that stimulate keratinocyte proliferation. **A**: Conditioned F medium was collected from irradiated feeder cells in the absence or presence of Y-27632 or with Y-27632 added to the conditioned medium immediately before use. HFKs (5.0×10^3) were plated in these various media in six-well tissue culture plates and were grown for 6 days. Conditioned medium without Y-27632 did not stimulate proliferation of the HFKs to the extent of that produced with Y-27632 or with Y-27632 added after collection. **B**: For quantification of the proliferation assay, the fixed and stained cells in A were solubilized, and absorbance at 564 nm was measured. CM, conditioned F medium; OD, optical density.

our earlier finding that physical contact with irradiated feeder fibroblasts is not required for the conditional immortalization of keratinocytes, but that diffusible factors released by the feeder cells are essential to this process.

Conditioned Medium Induces Telomerase Expression

It has been reported that telomerase is a critical element in cell immortalization and, moreover, that telomerase is overexpressed in HFKs cocultured with feeder cells,[33] but not in HFKs exposed to Y-27632 in the absence of feeder cells.[1] Because we have shown that irradiated feeder cells release diffusible factors that are essential for conditional immortalization of keratinocytes, we asked whether these factors increase telomerase expression. RT-qPCR was used to measure levels of the telomerase catalytic subunit hTERT in HFKs cultured in KGM, in F medium with irradiated feeder cells, or in conditioned F medium with and without Y-27632 (Figure 7A). The level of hTERT expression was relatively low in KGM, but increased approximately ninefold both in the presence of irradiated feeder cells and in conditioned medium, irrespective of

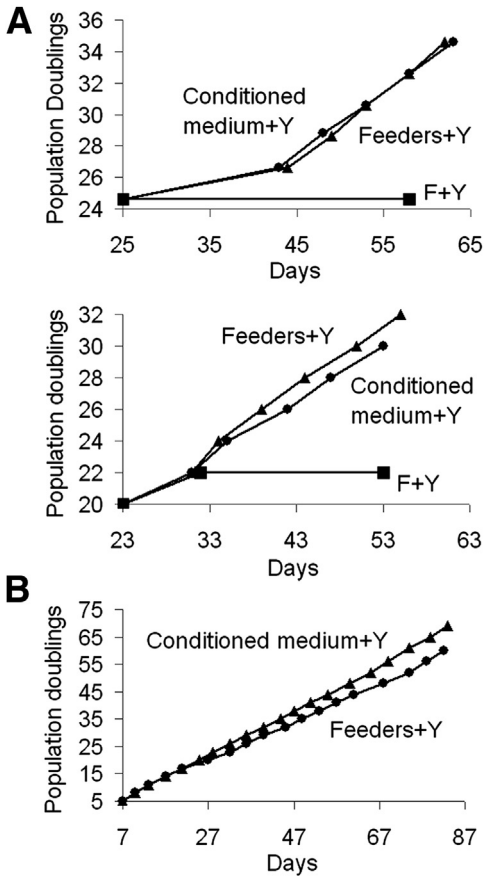

Figure 6 Conditioned medium supports conditional immortalization. **A**: Conditioned medium rescues late-passage HFKs undergoing senescence. Late-passage HFKs (p10, **top**; p11, **bottom**) cultured in F medium containing Y-27632 (as in Figure 2) were plated in F medium using three different conditions: in the presence of Y-27632 (F+Y), in the presence of irradiated feeder cells and Y-27632 (Feeders + Y), and in medium conditioned by irradiated feeder cells containing Y-27632 (Conditioned medium + Y). Y-27632 alone was unable to rescue the HFKs from rapidly undergoing senescence. In the presence of Y-27632, both irradiated feeder cells and conditioned medium were equally able to rescue HFKs from senescence. The experiment was performed in duplicate with two different strains of HFKs (**top** and **bottom** panels). **B**: Conditioned medium conditionally immortalizes keratinocytes. Early-passage HFKs (p2) were cultured for more than 80 days (60 population doublings) in F medium containing Y-27632 in direct contact with irradiated feeder cells or in conditioned F medium containing Y-27632. Under both conditions, the HFKs were efficiently immortalized and exhibited similar rates of proliferation.

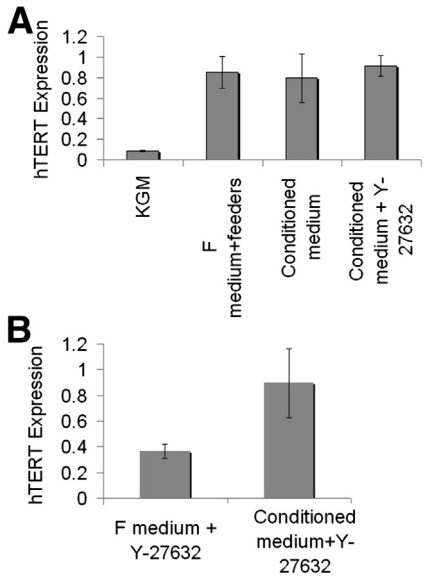

Figure 7 Conditioned medium induces telomerase. **A**: Conditioned medium induces telomerase expression. Levels of hTERT mRNA were measured in HFKs cultured in serum-free medium (KGM), cocultured with irradiated feeder cells in F medium, or cultured in conditioned F medium with and without Y-27632. hTERT was induced equally well by feeder cells and by conditioned medium, irrespective of Y-27632. **B**: Levels of hTERT mRNA were measured in F medium or conditioned medium, both in the presence of Y-27632. F medium did not fully induce telomerase expression.

Y-27632. Elevated hTERT expression was not simply a response of the HFKs to F medium, because expression was threefold greater in conditioned F medium, compared with nonconditioned F medium (Figure 7B). Therefore, diffusible factors released by irradiated feeder cells induce telomerase (through unknown mechanisms), suggesting a possible mechanism by which the feeder cells promote conditional immortalization.

Activity of Conditioned Medium Correlates with Apoptosis of Feeder Cells after Irradiation

Although the conditioned medium routinely used in immortalization experiments was collected 3 days after irradiation of the feeder cells, it was important to define the kinetics of conditioning. Therefore, medium collected from cultures of J2 cells at 0, 24, 48, 72, and 96 hours after irradiation was tested for its ability to stimulate HFK proliferation over a period of 6 days in the presence of Y-27632 (Figure 8A). Essentially no stimulatory activity was detected at 24 hours after irradiation, but activity had increased sharply (eightfold) by 48 hours and continued to increase slightly at 72 hours and at 96 hours. The same general trend was observed if medium was collected at 24-hour intervals from a single culture of irradiated J2 cells with replacement of fresh medium after each collection (Figure 8B). Thus, the observed kinetics of conditioning not only validate our use of 3 days for the routine preparation of conditioned medium, but also interestingly show that there is a 24-hour delay after irradiation before essential factors are released by the feeder cells.

Because irradiation causes DNA damage and triggers apoptosis,[34–36] we compared the timing of apoptosis in irradiated feeder cells to the activity of conditioned medium. Using the activity of caspases 3 and 7 as a marker for apoptosis, we found that the onset of apoptosis closely parallels the release of conditioning factors. Activity of caspases 3 and 7 activity increased dramatically between 24 and 48 hours after irradiation and increased slightly from 48 hours to 72 hours (Figure 8C). The correlation between apoptosis of irradiated feeder cells and the release of feeder factors that promote conditional immortalization suggests

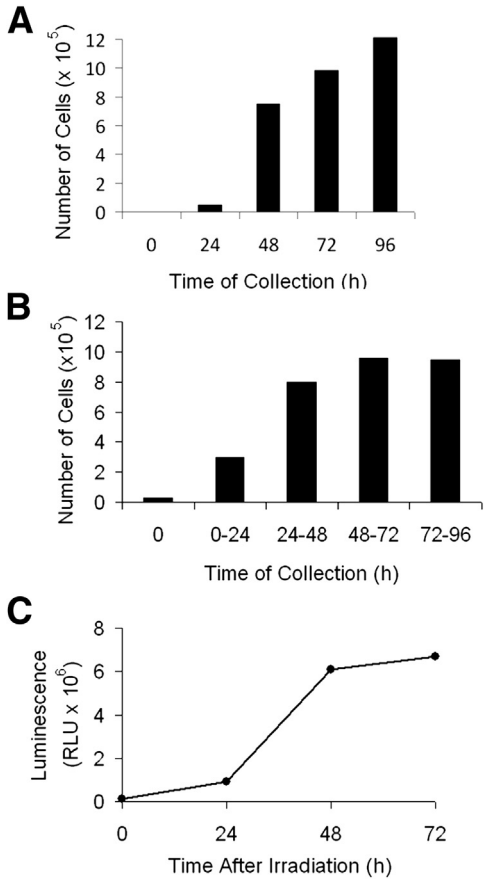

Figure 8 Activity of conditioned medium correlates with apoptosis of irradiated feeder cells. **A** and **B**: Conditioned medium and unconditioned F medium (control) were used to culture 5.0×10^3 HFKs in six-well tissue culture plates in the presence of Y-27632. Cells were counted after 6 days to assess proliferation. **A**: Activity of conditioned medium dramatically increased 24 hours after irradiation. Irradiated J2 feeder cells (1.0×10^7 to 1.5×10^7) were plated with 30 mL of F medium in 175-cm^2 tissue culture flasks. Conditioned medium was collected after 24, 48, 72, or 96 hours. **B**: Conditioned medium was collected from a single flask of irradiated J2 cells every 24 hours (through 96 hours), with replacement of fresh F medium after each collection. **C**: Apoptosis of feeder cells dramatically increased 24 hours after irradiation. Irradiated J2 feeder cells (2.0×10^4) were plated in each well of a 96-well tissue culture plate. Activity of caspases 3 and 7 was measured at 0, 24, 48, and 72 hours after irradiation. RLU, relative luminescence units.

that irradiation induces these factors and/or that the factors are released from dying cells.

Discussion

We have earlier shown that irradiated feeder cells and the ROCK inhibitor Y-27632 reprogram primary human keratinocytes to proliferate indefinitely.[1,2] With the present study, we establish that this cellular reprogramming is a direct result of one or more diffusible factors released from the irradiated feeder cells. Moreover, we show that these factors induce telomerase expression in the keratinocytes, suggesting a possible mechanism by which feeder cells promote conditional immortalization. We show that, in the absence of feeder cells, Y-27632 inhibits calcium- and serum-induced differentiation and transiently increases proliferation but cannot overcome cell crisis and senescence and thus cannot allow keratinocytes to be propagated indefinitely. Growing epithelial cells of interest in coculture is a complex procedure, requiring the irradiation and plating of feeder cells followed by seeding of the plates with epithelial cells. Eventually, the feeder cells need to be replaced, because they are postmitotic and do not survive longer than 3 to 4 days. From our experiments using Transwell permeable culture inserts, it is clear that direct physical contact between epithelial cells and feeder cells is not required for conditional immortalization. Therefore, the coculture system can be simplified greatly by replacing feeder cells with medium that has been conditioned by irradiated feeder cells.

It is important to note that irradiation of the feeder cells is essential for the production and/or release of the factors that impart activity to conditioned medium. These factors are released only after a 24-hour delay after irradiation and continue to be released for the next 72 hours. The timing of this release correlates with the onset of radiation-induced apoptosis of the feeder cells, as evidenced by a dramatic increase in activity of caspases 3 and 7 activity after 24 hours. It is well established that apoptotic cells release factors that promote proliferation in surrounding cells during wound healing and tissue regeneration in mice,[37] *Xenopus laevis*,[38] *Planaria* species,[39] *Hydra* species,[40] and *Drosophila melanogaster*.[41] Irradiated mouse fibroblasts stimulate the proliferation of epithelial, neural, and mesenchymal stem/progenitor cells in coculture; however, irradiated fibroblasts deficient in caspases 3 and 7 are largely defective for this activity.[37] Moreover, both $Casp3^{-/-}$ and $Casp7^{-/-}$ mice exhibit slower healing of skin excision wounds and significantly reduced liver regeneration after partial hepatectomy.[37] Although it would have been informative to add chemical inhibitors of the caspase cascade to the J2 feeder cells to determine whether they abrogate the production of the relevant soluble factors, these inhibitors cannot be removed from the conditioned medium and would have interfered with the interpretation of the experimental data. Our research group is designing studies to generate J2 feeder cells with genetic knockdown of the above caspases, which should clarify their potential role in this phenomenon.

We expect that the use of conditioned medium will significantly enhance the applicability of CRCs to biomedical research. Not only would it simplify long-term maintenance of CRC cultures, but use of conditioned medium could also greatly increase the use of CRCs to investigate the underlying genetic heterogeneity of cancers and allow for drug sensitivity and other testing to be performed in the absence of growth-arrested but metabolically active feeder cells.

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