# Keratinocytes Act as a Source of Reactive Oxygen Species by Transferring Hydrogen Peroxide to Melanocytes

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**Basal hydrogen peroxide ($H_2O_2$) levels in normal human epidermal keratinocytes (NHEK) and melanocytes (mel) were compared on a per cell basis and found to be significantly higher in keratinocytes. Since $H_2O_2$ is a neutral molecule capable of permeating through cellular membranes, we then investigated the possibility that $H_2O_2$ transfer might occur between these two types of cells. Because the ratio of keratinocytes to mel in skin is 36:1, keratinocytes may act as a source of reactive oxygen species (ROS) even by passive diffusion and, thus, affect melanocytic functions. In order to measure $H_2O_2$ transfer, a fluorescence-based co-culture system was developed in which mel were first pre-labeled with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate (DCFdA). When mel were co-cultured with keratinocytes, fluorescence increased as a function of keratinocyte cell number. Thus, for mel incubated with 1-, 1.5-, and 2-fold the number of keratinocytes, fluorescence increased by 22.6% (± 2.8%), 25.6% (± 4.8%), and 39.9% (± 4.1%), respectively. Separating the cells with a transwell membrane did not prevent the transfer, whereas the addition of catalase to media significantly reduced the transfer of $H_2O_2$ to mel. In conclusion, keratinocytes appear to be a previously unexamined source of ROS that may affect neighboring skin cells, such as mel, and, as a result, may influence the process of melanogenesis or contribute to the progression of vitiliginous lesions.**

Key words: hydrogen peroxide/keratinocytes/melanocytes
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Increased levels of reactive oxygen species (ROS) have been associated with many pathological processes (Sies, 1985; Frenkel, 1992; Packer and Fuchs, 1993; Halliwell and Gutteridge, 1999) and the effects of these reactive intermediates on epidermal cells in skin have been extensively studied. Beginning with Pathak and Stratton (1968), the potential for free radical-mediated damage was established by the observation that melanin radicals were generated in the epidermis after ultraviolet (UV) radiation. Later work by Dixit *et al* (1983) demonstrated an increase in lipid peroxides in UV-irradiated epidermal homogenates, whereas subcutaneous injection of superoxide dismutase (SOD), a superoxide anion radical scavenger, by Danno *et al* (1984), reduced epidermal sunburn cell formation. In addition to ROS production as a result of photodynamic action, influx of ROS into skin also occurs during inflammatory episodes due to the action of phagocytic NADPH oxidase (Babior, 1984). Ischemia/reperfusion during dermatological surgical procedures is yet another situation in which ROS ingression has also been observed (Knight, 1994). The subject of free radicals in skin biology has been reviewed by Darr and Fridovich (1994) and more recently an entire volume has been devoted to this topic (Thiele and Elsner, 2001).

During oxidative stress, molecular oxygen is reduced to form superoxide anion radicals, thereby eliminating the electron spin restriction imposed by the paramagnetic nature of oxygen and, thus, leads to the increased reactivity of oxygen. Further, superoxide anion radicals dismutate to hydrogen peroxide ($H_2O_2$) either spontaneously ($k = 1 \times 10^5$ per M per s) or by the action of SOD ($k = 1 \times 10^9$ per M per s) and there is much evidence that $H_2O_2$ is a mediator of oxidative damage in cells (Davies, 1998). For example, 8-oxo-7,8-dihydro-2′-deoxyguanosine, an oxidative lesion in DNA, was decreased in the presence of catalase (Zhang *et al*, 1997) and membrane damage has been associated with increased levels of hydrogen peroxide (Chatterjee and Agarwal, 1988). Frenkel and Chrzan (1987a, b) also demonstrated an increase in $H_2O_2$ when HeLa cells were treated with a tumor promoter and additional studies showed that leukemic cells could be protected by chemopreventive agents from the effects of tumor promoter-induced $H_2O_2$ (Bhimani *et al*, 1995). In epidermal cells, $H_2O_2$ levels increase in response to oxidative stress from environmental trauma, such as ultraviolet radiation B (UVB, 290–320 nm) (Peus *et al*, 1998), causing damage to biomolecules (Peus and Pittelkow, 2001; Pelle *et al*, 2003). Moreover, there is a basal level of oxidants in cells that is a by-product of normal endogenous processes. Although a system of enzymatic and non-enzymatic antioxidants provides cells with protection against ROS (Lopez-Torres *et al*, 1998), these antioxidant defenses can be overwhelmed during times of oxidative stress and can lead to a disruption of cellular function (Aruoma, 1998).

Yohn *et al* (1991) evaluated enzymatic antioxidant levels in epidermal cells and found higher levels of catalase and

Abbreviations: $H_2O_2$, hydrogen peroxide; mel, melanocytes; NHEK, normal human epidermal keratinocytes; ROS, reactive oxygen species

glutathione peroxidase in keratinocytes as compared with melanocytes. These findings indicated that either catalase was higher in keratinocytes in response to $H_2O_2$ or, alternatively, may have been lower in mel due to the lower steady-state levels of $H_2O_2$. Modulation of $H_2O_2$ in mel appears to be crucial for the process of melanogenesis (Nappi and Vass, 1996), which seems to require a precise redox balance. This latter point is supported by the work of Jimbow *et al* (2001), who demonstrated that, despite the utilization of ROS in melanogenesis, mel are particularly sensitive to oxidative stress. In patients with vitiligo, a disease characterized by the destruction of mel, Schallreuter *et al* (1991) detected low levels of epidermal catalase in affected areas. Successful repigmentation of vitiliginous patches was then achieved by these workers using topical application of a psuedocatalase (Schallreuter, 1999).

Since $H_2O_2$ is diamagnetic (Halliwell and Gutteridge, 1999), it has no unpaired electrons and, as a result, is a neutral species with the ability to permeate membranes (Frenkel and Chrzan, 1987b) and, thus, has the potential to migrate into neighboring cells. In human epidermis, the ratio of keratinocytes to mel is 36:1 (Jakubovic and Ackerman, 1992) and, as a consequence, the potential for transfer of $H_2O_2$ from keratinocytes to mel, even by a passive diffusion mechanism, appears to be quite high. In this study, our first objective was to compare the relative levels of $H_2O_2$ in keratinocytes and mel, whereas our second objective was to measure the transfer of $H_2O_2$ from keratinocytes to mel. Herein, we present evidence demonstrating that human keratinocytes transfer $H_2O_2$ to mel and, therefore, can act as a source of oxidative stress in mel.

## Results

Due to its lipophilic nature, DCFdA readily permeates through cell membranes, whereupon non-specific esterases in the cytosol remove the acetyl moieties and produce non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH), which is trapped inside the cell. In order to measure the ability of $H_2O_2$ to permeate into mel, cells were first incubated in the presence of glucose oxidase in Dulbecco's phosphate-buffered saline (D-PBS) over a range of concentrations (5–50 mU per mL) and as a function of time. Table I shows a linear ($p<0.001$) increase in fluorescence in mel caused by increasing doses of glucose oxidase, which indicates that the product of glucose oxidase, $H_2O_2$ (Rubin and Farber, 1984), was generated *in situ* followed by its migration into mel.

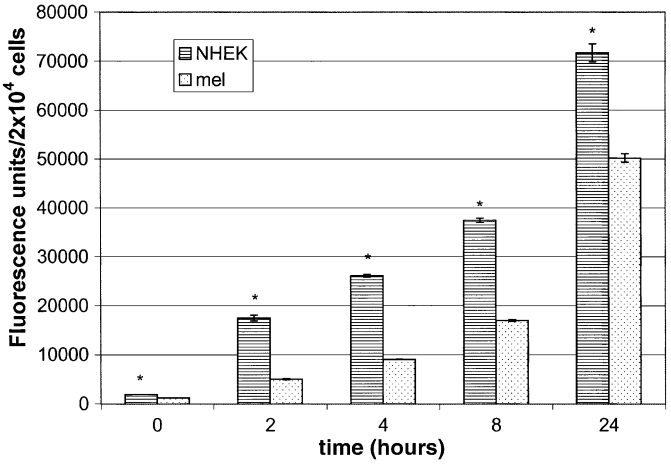


**Figure 1**
**Determination of the basal hydrogen peroxide levels in cells: a comparison between normal human epidermal keratinocytes (NHEK) and melanocytes (mel).** The levels of hydrogen peroxide in NHEK and mel were compared on a per cell basis. Cells were labeled with 10 μM DCFdA, at 37°C for 15 min, followed by addition of sodium azide (catalase inhibitor) to a final concentration of 12.5 mM in Dulbecco's phosphate-buffered saline, and fluorescence was measured over time. Data are expressed as means ± SE ($n=9$, $^*p<0.001$ for each time point).

In Fig 1, the basal levels of hydrogen peroxide in NHEK are compared with those in mel. NHEK had a significantly ($p<0.001$) higher titer of oxidants than mel on a per cell basis at each time point, which indicates the potential for passive transfer of $H_2O_2$ from NHEK to mel due to the imbalance of the basal levels of $H_2O_2$.

To test the hypothesis that NHEK contribute to the oxidative load in mel, mel were co-cultured with increasing numbers of NHEK. Mel were first labeled with 10 μM DCFdA at 37°C and then washed five times with D-PBS in order to remove any unincorporated DCFdA. Increasing numbers of NHEK were then added to the mel, co-incubated overnight, and their fluorescence was measured. The results of these experiments are shown in Fig 2 and demonstrate increasing fluorescence in mel as a function of the incubation with increased numbers of NHEK. Thus, as the number of NHEK increased to 1-, 1.5-, and 2-times the number of mel, the fluorescence intensity in the mel increased 22.6% (± SE 2.8%), 25.6% (± SE 4.8%), and 39.9% (± SE 4.1%), respectively. To further illustrate that fluorescence was

**Table I. $H_2O_2$ measurement in mel**

| | Glucose oxidase (mU per mL) | | | | |
|---|---|---|---|---|---|
| **Time (h)** | **0** | **5** | **10** | **25** | **50** |
| 0 | 89 (± 0.8) | 124 (± 8.1) | 103 (± 5.8) | 109 (± 9.8) | 104 (± 2.2) |
| 1 | 216 (± 1.8) | 1159 (± 53.2) | 1316 (± 64.9) | 1606 (± 66.6) | 1964 (± 54.1) |
| 2 | 365 (± 2.7) | 3555 (± 121.2) | 4118 (± 90.3) | 4911 (± 101.1) | 5903 (± 144.0) |
| 3 | 558 (± 3.6) | 7110 (± 200.4) | 8171 (± 238.4) | 9593 (± 135.5) | 10,814 (± 193.7) |

Human mel were labeled with 10 μM DCFdA, at 37°C for 15 min, followed by addition of $NaN_3$ (catalase inhibitor) to a final concentration of 12.5 mM in D-PBS. Glucose oxidase (0–50 mU per mL) was then added and increases in fluorescence observed as a function of time of the exposure to glucose oxidase. Data are expressed as means ± SE ($n=3$, ANOVA: $p<0.001$).

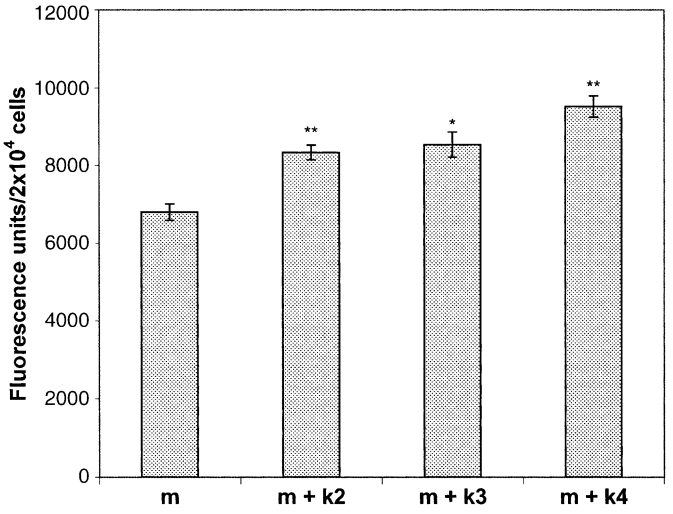


**Figure 2**
**Transfer of hydrogen peroxide from normal human epidermal keratinocytes (NHEK) to melanocytes (mel).** Mel ($2 \times 10^4$) were prelabeled with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate and then co-incubated with increasing numbers of NHEK (2, 3, and $4 \times 10^4$), respectively. Increased levels of $H_2O_2$ (means $\pm$ SE) were correlated with increasing numbers of NHEK ($n = 4$, $^*p < 0.001$, $^{**}p < 0.006$, ANOVA: $p < 0.001$) where m = mel, k = NHEK; k2, k3, and k4 represent 2, 3, and $4 \times 10^4$ NHEK, respectively.

specific only to mel in co-cultures with NHEK, phase contrast microscopy was used to visualize a co-culture of both cell types followed by fluorescence microscopy of the same field. As shown in Fig 3*a*, both mel and NHEK are visible as morphologically distinct entities by phase contrast, whereas in Fig 3*b*, only those cells with dendritic processes, which are characteristic of mel, are fluorescent.

Since mel transfer melanosome-containing melanin to NHEK by direct contact through its dendrites, it was possible that $H_2O_2$ could have been transferred to mel in the reverse direction along its dendritic processes. In order to determine whether direct contact was a prerequisite for $H_2O_2$ transfer, mel were again co-cultured with NHEK but physically separated by a transwell membrane. In this experiment, when mel and NHEK were co-cultured together, there was a 28% ($\pm$SE 3.9%) increase in fluorescence and when co-cultured but separated by a transwell membrane there was an increase of 38% ($\pm$SE 5.5%), which is a nonsignificant difference between both sets. Thus, these results indicate that NHEK did not need to be in direct contact with mel in order to transfer $H_2O_2$. To further prove that it was $H_2O_2$ that was transferred from NHEK to mel, an experiment was performed in which catalase was present in the cell culture medium during co-culture incubation. The results from this experiment show a reduced $H_2O_2$ transfer in the presence of catalase (Fig 4). Although other oxidants are capable of DCFH oxidation, the inhibitory effects of catalase prove that in these experiments it was $H_2O_2$ that was transferred from NHEK to mel.

## Discussion

In this study, we compared oxidant levels of human keratinocytes with those in mel and found that keratinocytes had

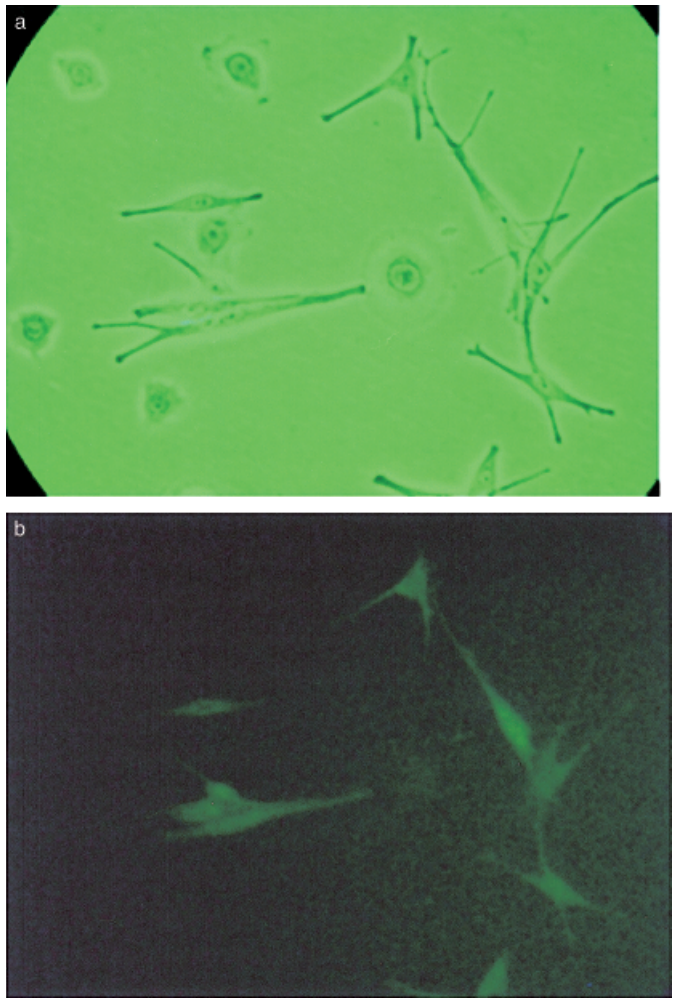


**Figure 3**
**Detection of hydrogen peroxide in melanocytes (mel) grown in co-cultures with normal human epidermal keratinocytes (NHEK) by fluorescence microscopy.** 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate (DCFdA)-labeled mel were co-cultured with NHEK at 37°C for 16 h, and then visualized by fluorescence microscopy ($\times$ 100, 1 cm = 10 $\mu$m). (*a*) Phase contrast of mel and NHEK showed the presence of both types of cells; (*b*) fluorescence of the same field illuminated only DCFdA-labeled mel.

significantly higher basal levels of $H_2O_2$ on a per cell basis by approximately 4-fold under conditions that inhibited catalase. Based on this finding, and the fact that keratinocytes far outnumber mel in the skin (36:1), we assessed the potential of $H_2O_2$ to pass from keratinocytes to mel. Since only mel were labeled with DCFdA in the co-culture system with keratinocytes, we were able to develop a fluorescence-based assay that detected the transfer of $H_2O_2$ in a concentration-dependent manner from keratinocytes to mel. As a result, these data show the potential of keratinocytes to act as a source of ROS in mel. These data also demonstrate a sensitive way to measure the effect that one type of cell has on the oxidative state of a different type of cell since, even at a cell-to-cell ratio of 1:1, a significant increase in ROS (22.6%) was detected in mel. This increase was also visualized by fluorescence microscopy of our co-cultured cells, which clearly depicted the localization of DCFdA only in the mel.

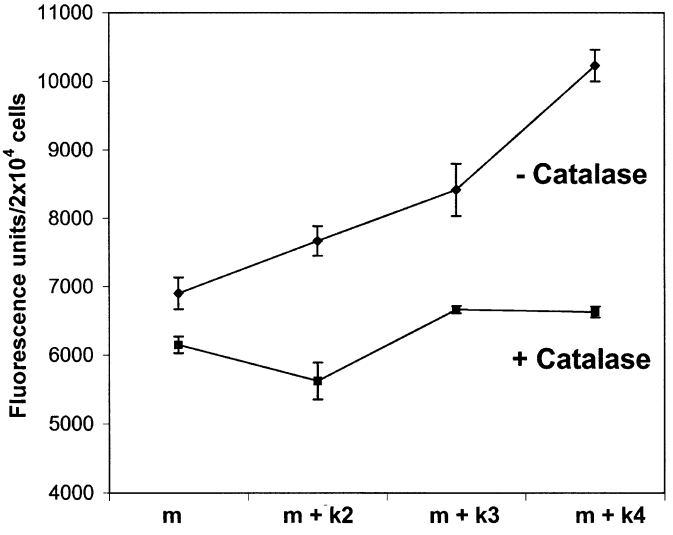


**Figure 4**
**Effects of catalase on hydrogen peroxide transfer from normal human epidermal keratinocytes (NHEK) to melanocytes (mel).** Catalase (5 U per mL) inhibited the rise in fluorescence (means ± SE) in mel ($2 \times 10^4$) co-cultured with increasing numbers of NHEK (2, 3, and $4 \times 10^4$) for 24 h. For each pair of data points the difference from m was: m + k2 ($p < 0.004$); m + k3 ($p < 0.04$); m + k4 ($p < 0.04$), where m = mel, k = NHEK; k2, k3, and k4 represent 2, 3, and $4 \times 10^4$ NHEK, respectively.

Because of the proximity of keratinocytes to mel in human epidermis, the transfer of $H_2O_2$ may have wide-ranging effects *in vivo* on the biology of the melanocyte. One putative effect of $H_2O_2$ transfer may involve the destruction or impairment of mel at the periphery of vitiliginous lesions leading to the characteristic discoloration of skin observed in these lesions. Thus, as $H_2O_2$ passes into the melanocyte, oxidative damage may occur, perhaps due to an increased level of exported $H_2O_2$ from the keratinocyte. This is supported by Schallreuter *et al* (2001), who showed that catalase and tetrahydrobiopterin dehydratase activities are inhibited by $H_2O_2$ and that these enzymes are also lower in patients with vitiligo. Alternatively, vitiligo-involved mel may have a higher sensitivity to normal levels of $H_2O_2$. Since tyrosinase-related protein (TRP-1) has been shown to have peroxidase activity (Schallreuter, 1999a), and autoantibodies to TRP-1 have also been found in the sera of vitiligo patients (Kemp *et al*, 1998), it is possible that an influx of $H_2O_2$ from keratinocytes may render mel vulnerable to oxidative attack due to a defective or incapacitated TRP-1, which is unable to effectively remove $H_2O_2$.

Although other molecules secreted from keratinocytes, such as cytokines, may affect $H_2O_2$ levels in mel, our data show that $H_2O_2$ is directly involved in the increase in oxidation because the presence of catalase in the co-culture medium negated the increase in fluorescence. Moreover, $H_2O_2$ transfer between cells was not contingent upon direct cell-to-cell contact. When keratinocytes were separated from mel by a transwell membrane in our co-culture system, we still observed an increase in fluorescence in mel.

A future area of investigation will be to study the transfer of $H_2O_2$ in co-cultures that contain ratios that reflect the 36:1 ratio and our preliminary data suggest a linear correlation to $H_2O_2$ levels as the keratinocyte to melanocyte ratio increases. Also, since catalase and glutathione peroxidase affect $H_2O_2$ levels, our next steps include determining the relative expression of these two enzymes by RT-PCR in both cell types. Another area of investigation will assess the downstream effects of $H_2O_2$ on cell signaling. Previously, we reported that UVB increased $H_2O_2$ levels in keratinocytes (Pelle *et al*, 2003) and, in a parallel set of experiments, we also observed higher levels of $H_2O_2$ and Activator Protein-1 (AP-1) in mel following UVB irradiation (unpublished observations). Delineating transduction pathways in response to increased levels of $H_2O_2$ in normal as well as abnormal mel in the future may provide insights into the mechanisms of melanogenesis. In summary, this study proves that $H_2O_2$ can be transferred from keratinocytes to mel and demonstrates that keratinocytes can act as a source of ROS, which may contribute to oxidant-related modulation of biochemical processes in the melanocyte.

## Materials and Methods

**Materials** DCFdA was obtained from Molecular Probes (Eugene, Oregon). Transwell membranes (3472, clear) from Corning (Corning, New York) were used for the separation of keratinocytes and mel in co-culture experiments. Glucose oxidase, Grade 1 from *Aspergillus niger,* was obtained from Roche (Indianopolis, Indiana), whereas catalase was purchased from Sigma (St Louis, Missouri).

*Cell culture*

*Keratinocytes* Primary NHEK were obtained from Cascade (Portland, Oregon) as a primary culture of cells isolated from fetal foreskin. Cells were cultured in 10 ml EpiLife (calcium-free) medium containing growth factors and serum (Cascade) using T75 Falcon culture flasks with screw-capped HEPA filters; cells were subcultured by trypsinization. At approximately 50% confluency of the third passage, there were sufficient numbers of cells to perform experiments.

*Mel* Normal human epidermal neonatal mel were obtained from Cascade as tertiary proliferating cultures and grown in Medium 154 supplemented with 1% melanocyte growth serum using T75 Falcon culture flasks with screw-capped HEPA filters. Cells were subcultured by trypsinization and used for experimentation when they were in the log-phase of growth.

*$H_2O_2$ analysis* $H_2O_2$ was determined by adding DCFdA to cells, which was a modification of the technique used by Bhimani *et al* (1995) and Huang *et al* (1994). For the analysis, $2 \times 10^4$ cells were plated into the wells of a 96-well microtiter plate because it was determined that this number of cells attained approximately 50% of confluence after overnight incubation. The next day, the medium was aspirated, 100 μL of a 10 μM DCFdA solution in D-PBS was added to the cells, and incubated at 37°C for 15 min. The DCFdA solution was prepared by adding 100 μL ethanol to a vial containing 50 μg DCFdA and then transferring 50 μL to 5 mL D-PBS to a final concentration of 10 μM. After incubation with DCFdA, 100 μL 25 mM sodium azide ($NaN_3$, a catalase inhibitor) was added and incubated with the cells at 37°C for 2 h. But $NaN_3$ was not added to cells for overnight incubations due to its cytotoxicity. Fluorescence measurements were made in a fluorescence plate reader (Cytofluor, PerSeptive Systems, Framingham, Massachusetts) with a 485/20 nm excitation filter and a 530/25 nm emission filter set at a gain of 75. Although $NaN_3$ is used to inhibit catalase in order to obtain a stronger fluorescent signal, an additional control was also carried out to demonstrate the validity of our technique. This was achieved by substituting $NaN_3$ with 3-amino-1,2,4-triazole, which

inhibits catalase more specifically than $NaN_3$. Cells treated with 12.5 mM $NaN_3$ increased fluorescence over untreated cells by 165% ($\pm$2.4%, SE, n = 3), whereas those treated with the same concentration of 3-amino-1,2,4-triazole increased fluorescence by 352% ($\pm$19.1%, SE, n = 3). Thus, both treatments increased fluorescence and demonstrate the validity of this technique for $H_2O_2$ measurements.

*Microscopy* For microscopic visualization of melanocyte-labeled co-cultures, mel were plated in 60 mm culture dishes at $3 \times 10^5$ cells per dish, treated with DCFdA, washed five times with D-PBS, and then incubated overnight with an equal number of keratinocytes. Cell cultures were then viewed at $\times$ 100 magnification by phase contrast and fluorescence microscopy with an Olympus, BX60 microscope (Olympus, Melville, New York). Microscope fluorescence was generated with a mercury arc lamp and observed through blue light filters.

*Statistics* Probability (p) values were calculated using a Student's two-tailed *t* test assuming unequal variances or one-way analysis of variance (ANOVA) where indicated.

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