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## Melanocytes do not migrate directionally in physiological DC electric fields

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Wounding skin generates an endogenous electric field of 100–200 mV/mm in the immediate vicinity of the wound. When keratinocytes are exposed to direct current electric fields of this magnitude, they exhibit galvanotaxis, or directional migration toward the cathode, suggesting that wound-generated electric fields provide migrational cues that contribute to wound healing. Because melanocytes must also migrate into the healing wound to repigment it, their motility in response to electric fields of physiologic magnitude was examined. Human skin-derived melanocytes, either exposed to 100 mV/mm direct current electric fields or nonexposed controls, both exhibited motility rates of 9  $\mu\text{m}/\text{hour}$ , significantly (three- to five-fold) lower than the motility rates of keratinocytes under identical conditions. However, in sharp contrast to keratinocytes, melanocytes exhibited no directional migration in the electric field. Additionally, neither the number of primary dendrites per cell, nor the orientation of the dendrites with respect to the field vector, nor the average length of the dendrites was significantly different in melanocytes exposed to the electric field as compared to nonexposed controls. Thus, in marked contrast to keratinocytes, human skin-derived melanocytes do not respond to direct current electric fields of physiologic magnitude with either directional migration or reorientation of dendrites. This may account for the delay in repigmentation that often accompanies wound reepithelialization. (**WOUND REP REG 2003;11:64–70**)

Regeneration of wounded skin requires that the cell types normally present in skin migrate from the wound periphery to repair the defect and reconstitute the newly regenerating tissue. Although many physical processes and biochemical factors are involved in stimulating and guiding this migratory response,<sup>1</sup> one of the earliest migratory cues is the electric field generated within skin immediately upon wounding.<sup>2,3</sup> DC electric fields of physiological magnitude (100 mV/mm) can direct keratinocyte migration toward

KGM	Keratinocyte growth medium
MGM	Melanocyte growth medium
NHK	Normal human keratinocytes
NHM	Normal human melanocyte
PBS	Phosphate buffered saline solution

the cathode.<sup>4</sup> Because melanocyte migration into the healing wound is required for restoration of normal pigmentation, we turned our attention to these cells, asking the question of whether human dermal melanocytes would respond to applied DC electric fields with directional migration as do human skin-derived keratinocytes. A logical prediction was that they would. *Xenopus* neural crest cells and their melanocyte derivatives exhibit galvanotaxis when exposed to an electric field.<sup>5</sup> Other dendritic cells of neural crest origin, such as nerve cells, respond to an electric field by turning their dendrites toward the cathode (galvanotropism),<sup>6–11</sup> and immature amphibian melanocytes show cathodal migration in DC electric fields.<sup>5</sup> Thus, our unexpected finding of an absence

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of a directional response in melanocytes and absence of redistribution of their dendrites when exposed to an applied DC field is novel and shows that the galvanotaxis response varies with species and with cell type.

## MATERIALS AND METHODS

Normal human epidermal melanocytes (NHM) were isolated from primary keratinocyte cultures derived from neonatal foreskins, obtained with an approved protocol from the University Institutional Review Board. The initial isolation of the keratinocytes is the same procedure that we have reported previously.<sup>12</sup> Briefly, foreskins were trimmed of excess subcutaneous tissue, cut into small pieces, and trypsinized overnight at 4 °C. Epidermis was scraped from the dermis, dispersed into a single-cell suspension, and plated onto a mitomycin C Treated 3T3 feeder layer, as previously described.<sup>13</sup> After the cells in culture formed 6–12 cells per colony, the medium was changed to keratinocyte growth medium (KGM, Cascade Biologics Inc., Portland, OR). Once the cell culture reached 20% confluency, the melanocytes were isolated by selective trypsinization<sup>14</sup> and plated onto new dishes in melanocyte growth medium (MGM, Cascade Biologics Inc.), an M154-based medium supplemented with penicillin-G, streptomycin, amphotericin B, bovine pituitary extract, fetal bovine serum, bovine insulin, bovine transferrin, basic fibroblast growth factor, hydrocortisone, heparin, and phorbol 12-myristate 13-acetate. After cultures reached about 70% confluency, cells were treated with 100 µg/ml G418 sulfate (Geneticin®, Gibco BRL, Grand Island, NY) for 3–5 days to selectively eliminate any fibroblast contamination.<sup>15</sup> To test the purity of melanocyte cultures, cells were recovered, passed onto collagen I-coated glass coverslips, and probed with the NK1/beteb antibody (Caltag Laboratories, Burlingame, CA) specific to melanocytes.<sup>16</sup> Cultures were only used if they were free of contaminating keratinocytes and fibroblasts. Cells were maintained at 37 °C/5% CO<sub>2</sub>. Cultures between passage 4–12, were derived from two separate donors, tested separately, and the data were averaged.

### Immunostaining of NK1/beteb

NHM were plated on glass type I collagen (Vitrogen, Cohesion Technologies, Palo Alto, CA) coated coverslips. Collagen coating was done by immersing coverslips in 60 µg/ml collagen diluted in Medium 154 at 37 °C for a minimum of 1 hour. Coverslips were then washed three times with Medium 154 before melanocytes were plated. Cells were cultured on coverslips 24–48 hours before immunostaining. Coverslips were fixed in –20 °C acetone for 10 minutes, permeabilized with –20 °C methanol for 5 minutes, and washed with distilled water and phosphate

buffered saline solution (PBS). Nonspecific binding was blocked by incubation in 10% normal goat serum diluted in PBS for 1 hour at room temperature. Coverslips were then incubated in mouse anti-NK1/beteb (1 : 20; Caltag Laboratories) at 37 °C for 1 hour. Coverslips were then washed three times with PBS, incubated for 1 hour at 37 °C in goat anti-mouse Cy3 immunoglobulin (1 : 500, Jackson Immuno Research Labs, West Grove, PA), washed three times in PBS, incubated for 10 minutes in equilibrium buffer, and mounted in SlowFade Light (Molecular Probes, Eugene OR).

### Galvanotaxis

Coverslips and cells were processed following the procedure described previously.<sup>4,17–21</sup> Briefly, melanocytes or a combination of keratinocytes and melanocytes were plated on type I collagen (60 µg/ml)-coated glass coverslips at a density low enough to allow cells to attach singly, without contact with other cells. After cell attachment (2–3 hours), the coverslip was placed into a galvanotaxis chamber. The galvanotaxis chamber is a piece of plexiglass constructed with a reservoir containing medium on either side of the coverslip that allows flow from one side to the other, as previously described.<sup>9</sup> The chambers were sealed with tape and silicone high-vacuum grease to prevent evaporation and pH change. The experimental chamber contained supplemented MGM, which has a final calcium concentration of 2 mM. Medium was first added to one well of the chamber and only after a clear path of the medium flow over the cells was established was the medium added to the second well. Melanocytes were exposed to a constant DC voltage of 100 mV/mm, applied through Ag-AgCl electrodes in each well for 2 hours at 35 °C in room air. Agar-filled glass bridges separated the electrodes supplying the current from the chamber itself to prevent diffusion of electrode products into the medium. As a control, melanocytes on type I collagen-coated glass coverslips were simultaneously exposed to 0 mV/mm using the same experimental conditions. The current was measured at the beginning and end of each experiment with an ammeter in series to ensure that the amperage was kept below 0.6 mA to minimize Joule heating. The temperature of the medium in the chambers was monitored at the beginning and end of the experiment using a YSI 400 analog temperature probe (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) and did not vary by more than 0.5 °C. If greater fluctuations occurred, the experiments were not included in the analysis. Eight separate experiments were performed with two different cell strains per condition for the melanocyte alone experiments. The co-culture experiments were performed twice. Occasionally, cell division was noted during the experiments.

**Recording and data analysis for galvanotaxis**

During exposure to the electric field, cells were observed using either phase contrast or Hoffman optics. Images were captured and recorded every 20 minutes for 2 hours to an image analysis program on a Power Macintosh 8500/120 using a modified version of NIH Image 1.60 and FileMaker Pro 3.0. After each cell was tracked, the data was automatically exported to FileMaker Pro 3.0 where the data was analyzed and stored. Cell migration was quantitatively analyzed for speed, distance, and directionality. To quantitate the directedness of the average cellular translocation, the cosine of the angle at which each cell moved in relation to the anodal-cathodal orientation was calculated. Specifically, a cosine value of 1 would indicate direct cellular movement toward the negative pole (cathode); 0 would indicate movement perpendicular to the field direction or random migration; and -1 would indicate direct cellular movement toward the positive pole (anode). The average cosine  $\phi$  for each experiment was calculated from the formula:  $\langle \cos \phi \rangle = (\sum_i \cos N_i)/N$ ,<sup>4</sup> where  $\sum_i$  is the summation of cosine values obtained from individual cells,  $\phi$  is the angle between the field axis and the cellular translocation direction, and  $N$  is the total number of cells observed for a given experiment. Average cell velocities ( $\mu\text{m}/\text{minute}$ ) were calculated by dividing the sum of each 20-minute translocation distance for each cell by the total time (120 minutes). True distance is defined as the actual distance the cell traveled during the 2-hour experimental period.

**Statistical analysis**

Statistical significance was determined by Student's *t* test (unpaired), with  $p < 0.005$  considered significant. Data is presented as mean  $\pm$  the standard error of the mean (SEM).

**Dendrite analysis**

After galvanotaxis analysis was completed, images for the time points of 0, 60, and 120 minutes were re-analyzed for the parameters of dendrite length, cosine of each dendrite, and number of dendrites per cell. To measure the cosine of each dendrite, the angle of each dendrite with respect to the cell body and the field was measured and from that angle the cosine was calculated.

**QuickTime movie**

A movie was made in QuickTime Pro 4.0 of the co-culture experiments by using images captured every 20 minutes for 2 hours by NIH Image, making a stack of the images and importing the stack into QuickTime. This movie may be downloaded and viewed from the *Wound Repair and Regeneration* Web site (ea.cphs.wayne.edu/wrr/WRR.HTM).

**RESULTS**

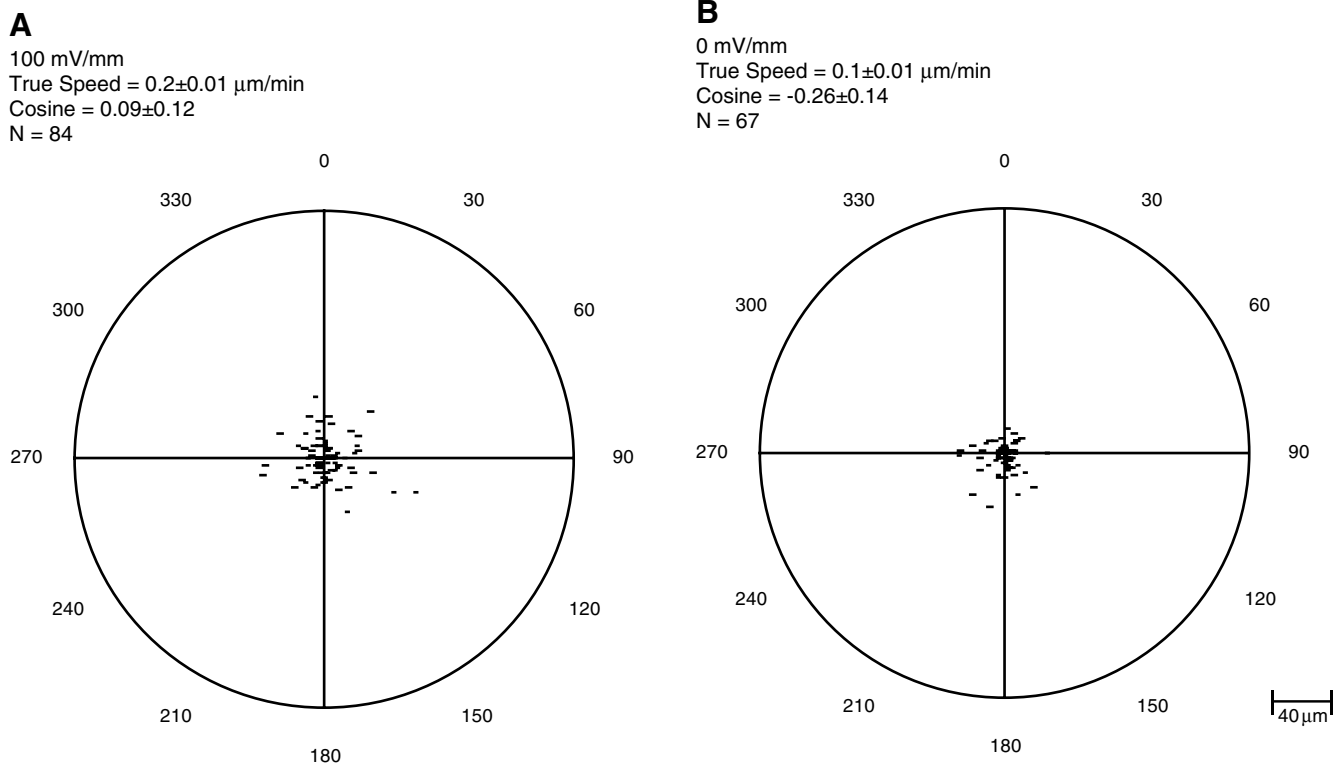
Melanocyte motility in a DC electric field of 100 mV/mm was examined (Figure 1). The cells moved randomly with no sustained directional persistence. Migration was not directional with respect to the field vector; cells exposed to the field had an average cosine of  $0.09 \pm 0.12$ , which was not significantly different from the control cells ( $p < 0.05$ ). The electric field also did not alter the migratory speed of exposed cells. True speed for melanocytes in the field was  $0.17 \pm 0.01 \mu\text{m}/\text{minute}$  which was slightly higher, but not significantly different, than the controls ( $p < 0.05$ ). The total distance traveled by melanocytes exposed to the field (true distance) was  $20.6 \pm 1.7 \mu\text{m}$ , which was slightly higher, but not significantly different, than the control cells ( $p < 0.05$ ). Because the melanocyte cell body averages about 17  $\mu\text{m}$  in diameter, over the 2-hour experimental observation period an average cell displaced about one cell diameter. Data from Figure 1 are summarized in Table 1.

**Response of melanocyte dendrite length and angle to applied DC electric field**

Melanocyte dendrite length and angle were unaffected by the applied DC electric field. Dendrites maintained a random distribution relative to the cell body in both the field and control groups (average cosine  $0.014 \pm 0.02$  and  $0.026 \pm 0.025$ , respectively) (Figure 2). The average dendrite length of cells in the field ( $38.8 \pm 1.4 \mu\text{m}$ ) was not significantly different from that of the control cells ( $37.5 \pm 1.7 \mu\text{m}$ ,  $p < 0.5$ ). Furthermore, the average number of dendrites per cell in the field ( $3.6 \pm 0.17$ ) was not significantly different from that of the control group ( $3.2 \pm 0.13$ ,  $p < 0.05$ ). Data from Figure 2 are summarized in Table 2.

**Keratinocytes maintain directional migration co-cultured with melanocytes**

Negative data (such as the absence of a galvanotaxis response, as we note with melanocytes) raises concerns regarding the fitness of the experimental conditions in general. To test this, NHM and normal human keratinocytes (NHK) were co-cultured and exposed to DC fields. In these experiments, NHK migrate directionally toward the cathode, whereas NHM exhibit random migration (Figure 3). The NHK show a robust directional response, migrating toward the cathode with a true speed of  $0.7 \mu\text{m}/\text{minute}$  and an average cosine of 0.82, indicating strong directional migration toward the cathode. Because the keratinocyte cell body is about 30  $\mu\text{m}$  in diameter, the average cell translocated about three cell diameters over the 2-hour course of observation. On the other hand, NHM



**FIGURE 1.** Cellular translocation distribution of human melanocytes during 2 hours of applied electric field. Images of the migration paths were captured every 20 minutes and the translocation distance and directionality calculated as described. Each cell's position at time ( $t$ ) = 0 minutes is at the origin (0,0), and its final position at the end of the 2 hours exposure to the DC field is plotted as a single point on the graph. The radius of each circle represents 200  $\mu\text{m}$  of translocation distance. The cathode is at the top of each graph ( $0^\circ$ ), and the anode at the bottom ( $180^\circ$ ). In the upper left corner of each circle plot are the average true speed, the average cosine and the total number of cells studied (N). (A) 100 mV/mm and (B) 0 mV/mm.

**Table 1.** Average distance, speed, and cosine of melanocytes exposed to an electric field

Electric field strength (mV/mm)	True distance ( $\mu\text{m}$ )* (N)	True speed ( $\mu\text{m}/\text{min}$ )* (N)	Net cosine (N)*
0	$16.3 \pm 1.3$ (67)	$0.14 \pm 0.01$ (67)	$-0.26 \pm 0.14$ (25)
100	$20.6 \pm 1.7$ (84) ( $P = 0.06$ )¶	$0.17 \pm 0.01$ (84) ( $P = 0.10$ )¶	$0.09 \pm 0.12$ (37) ( $P = 0.06$ )¶

Data taken from experiments described in Figure 1.

\*Values indicate mean  $\pm$  SEM.

@Cosine was calculated from cells with a true speed greater than 0.2  $\mu\text{m}/\text{min}$ .

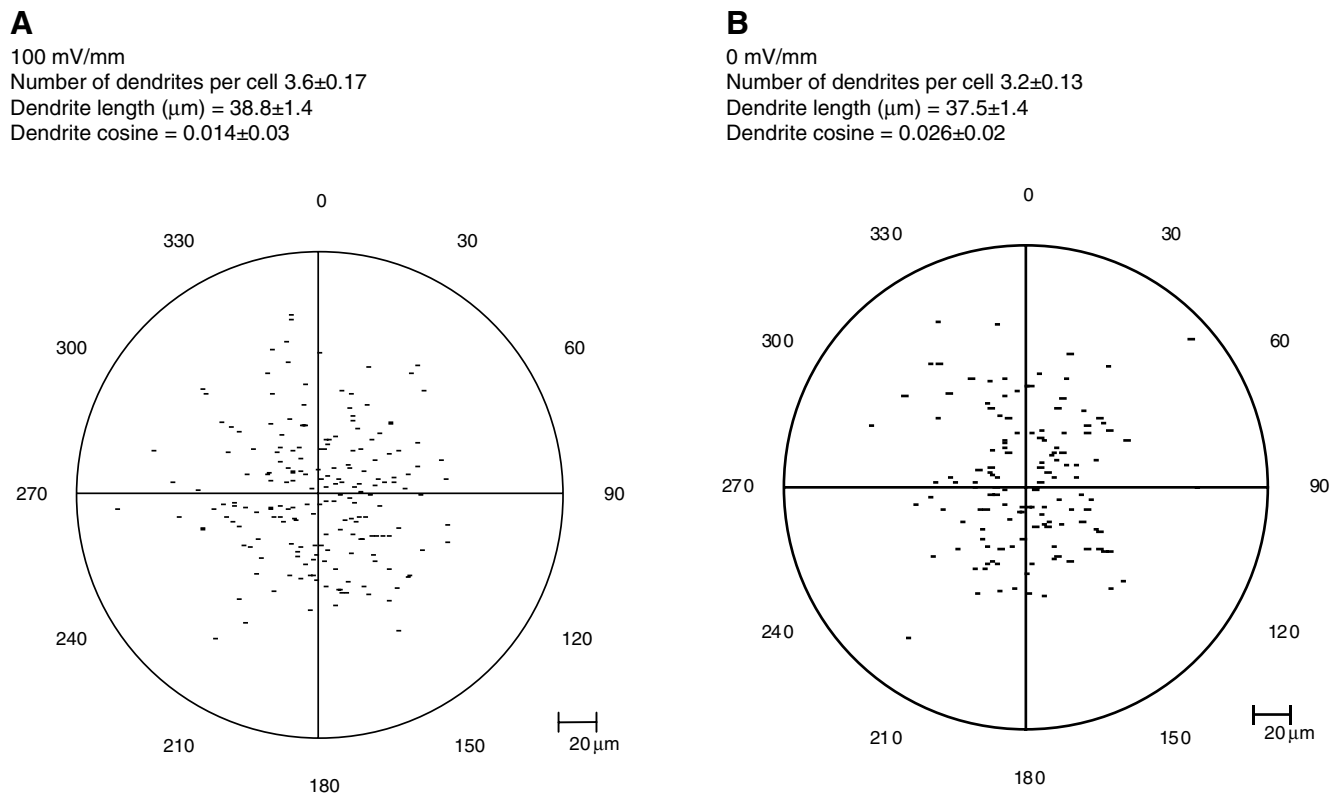
¶ $P$  values were determined by two tailed unpaired Student's  $t$  test.

migrate more slowly than the keratinocytes, with true speeds of 0.2  $\mu\text{m}/\text{minute}$ , equivalent to the speed observed when melanocytes are cultivated in the absence of keratinocytes (Figure 1), and their migratory paths show no evidence of directionality, with an average cosine of 0.22. The robust directional migratory response of the co-cultivated NHK in this system makes the observed sluggish response of the melanocytes unlikely to be a function of experimental artifact. A movie of the time-lapse images taken of the co-cultured keratinocytes and melanocytes over a 2-hour DC field exposure shows that although the melanocytes are motile and exhibit morphologic changes associated with the polarized migratory phenotype, their movement is not relative to the field vector. While the

keratinocytes move toward the cathode, the melanocytes move and extend dendrites toward their neighboring keratinocytes with no relationship to the applied field.

## DISCUSSION

When skin is wounded, an endogenous electric field of 100–200 mV/mm is generated,<sup>3</sup> with the negative (cathodal) pole of the generated field at the center of the wound. Human keratinocytes migrate toward the cathode (galvanotaxis) when exposed to a DC electric field of physiological strength.<sup>4,17–21</sup> Similar studies using corneal epithelial cells have also shown cathodal galvanotaxis,<sup>22</sup> and analogous to skin wounds, corneal epithelium also



**FIGURE 2.** Dendrite length and angle of human melanocytes during 2 hours of applied electric field. The final length and angle of each dendrite was measured as described. Each dendrite's final length and angle is plotted as a single point on the graph. The radius of each circle represents  $120 \mu\text{m}$  of length. The cathode is at the top of each graph ( $0^\circ$ ), and the anode at the bottom ( $180^\circ$ ). In the upper left corner of each circle plot are the average number of dendrites per cell, average dendrite length, average cosine of the dendrites, and total number of cells studied (N). (A)  $100 \text{ mV/mm}$  and (B)  $0 \text{ mV/mm}$ .

**Table 2.** Average number of melanocyte dendrites, dendrite length, and cosine

Electric field strength (mV/mm)	Number of dendrites* per cell (N)	Dendrite length* ( $\mu\text{m}$ ) (N)	Dendrite cosine* (N)
0	$3.2 \pm 0.13$ (53)	$37.5 \pm 1.7$ (53)	$0.026 \pm 0.03$ (53)
100	$3.6 \pm 0.17$ (59) ( $P = 0.06$ ) <sup>@</sup>	$38.8 \pm 1.4$ (59) ( $P = 0.55$ ) <sup>@</sup>	$0.014 \pm 0.02$ (59) ( $P = 0.71$ ) <sup>@</sup>

Data taken from experiments described in Figure 2.

\*Values indicate mean  $\pm$  SEM.

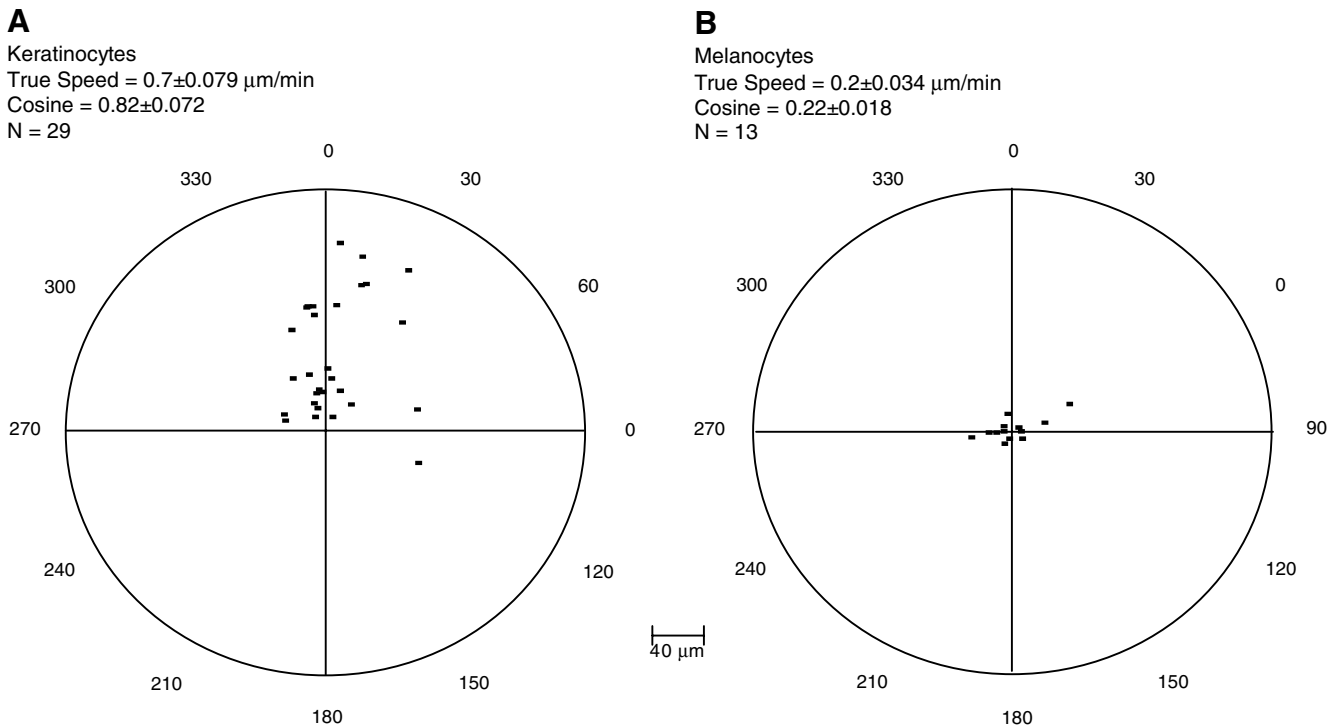
@Values were determined by two tailed unpaired Student's *t* test.

generates endogenous electric fields when wounded.<sup>23</sup> Together, these studies have led to the notion that endogenous wound electric fields provide early cues for directional migration of cells involved in wound repair.<sup>24</sup> In this study, we asked the question of whether another resident cell of the skin, the melanocyte, also responds to an applied electric field of physiologic strength with directed migration. Contrary to predictions based on the previously reported responses of embryonic melanocytes in different species, we found that under the experimental

conditions that allow for robust directional migration of keratinocytes in an applied DC electric field, skin-derived melanocytes are essentially "blinded" to the field. Rather than exhibiting directional, cathodal migration, they move randomly. Furthermore, they do not show dendritic galvanotrophism as do nerve cells in an electric field.

Melanocyte "blindness" to the DC field is noted under the assay conditions used: conditions under which keratinocytes migrate optimally and show a robust galvanotaxis response. It is possible that altering the composition of the underlying extracellular matrix could influence the galvanotaxis response of melanocytes as it does in keratinocytes.<sup>17</sup> Further work will be required to evaluate the effect of other matrix proteins on melanocyte galvanotaxis. However, the type I collagen substrate emulates the skin wound environment, providing the cells with the predominant collagen of the dermis and the wound environment, and is the extracellular matrix protein the melanocyte is most likely to encounter as it migrates to the wounded area.

Wound healing in skin requires cell migration of a number of different cell types to repopulate the wound.



**FIGURE 3.** Cellular translocation distribution of human keratinocytes co-cultured with human melanocytes during 2 hours of applied electric field. Images of the migration paths were captured every 20 minutes and the translocation distance and directionality calculated as described. Each cell's position at time ( $t$ ) = 0 minutes is at the origin (0,0), and its final position at the end of the 2 hours of exposure to the DC field is plotted as a single point on the graph. The radius of each circle represents 200  $\mu\text{m}$  of translocation distance. The cathode is at the top of each graph ( $0^\circ$ ) and the anode at the bottom ( $180^\circ$ ). In the upper left corner of each circle plot are the average true speed, average cosine, and total number of cells (N), combined from two separate experiments. (A) human keratinocytes and (B) human melanocytes.

An early cue for directional migration is the endogenous wound electric field, and keratinocytes respond to this cue with directional migration that can aid in the reepithelialization process. If melanocytes do not respond to the immediately generated endogenous wound electric field, their directed migration or extension of dendrites into the wound may be delayed. An initially depigmented wound would result, and this is often the case, especially in burn wounds.<sup>25-28</sup> Understanding the cues that mediate melanocyte migration into healing wounds will allow rational approaches for enhancing wound healing and normalizing wound pigmentation.

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