

Keratinocyte proximity and contact can play a significant role in determining mesenchymal stem cell fate in human tissue

Raja K. Sivamani,^{*,†} Michael P. Schwartz,^{†,‡} Kristi S. Anseth,^{†,‡} and R. Rivkah Isseroff^{*,1}

^{*}Department of Dermatology, University of California Davis School of Medicine, Davis, California, USA; and [†]Department of Chemical and Biological Engineering and [‡]Howard Hughes Medical Institute, University of Colorado, Boulder, Colorado, USA

ABSTRACT Bone marrow-derived human mesenchymal stem cells (hMSCs) possess multipotent differentiation capabilities and are a potent source of paracrine factors. We show how the epidermal keratinocyte can direct hMSC differentiation selectively. Keratinocytes and hMSCs were either cocultured in physical contact (contact cocultures), or separated without physical contact using a transwell insert (noncontact cocultures). We also delivered hMSCs into an *ex vivo* human excisional wound where subpopulations of the hMSCs were either in contact or were physically separated from the epidermal keratinocytes. In comparison to control hMSCs that were not cocultured, contact cocultured hMSCs adopted an epithelial morphology and expressed keratinocyte markers while noncontact cocultured hMSCs, surprisingly, adopted phenotypes that resembled myofibroblast and early neural lineage, both of which are of dermal origin. Cell fusion was not a requirement in *in vitro* contact cocultures, as determined by fluorescence-activated cell sorting (FACS) and fluorescence *in situ* hybridization analysis (FISH). To the best of our knowledge, this work provides the first example of hMSC differentiation into different lineages depending on their proximity to a single cell type.—Sivamani, R. K., Schwartz, M. P., Anseth, K. S., Isseroff, R. R., Keratinocyte proximity and contact can play a significant role in determining mesenchymal stem cell fate in human tissue, *FASEB J.* 25, 122–131 (2011). www.fasebj.org

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BONE MARROW-DERIVED STEM CELLS possess enormous, near-term, translational potential for regenerative medicine since they have the capacity to differentiate into various cell types (1, 2), have potent secretory capabilities that accelerate wound healing (3, 4), and have been applied successfully to heal wounds that are resistant to other treatment modalities (5, 6, 18). Autologous bone marrow-derived mesenchymal stem cells (MSCs) are particularly promising because they can be expanded *ex vivo* and delivered to the injured tissue, thereby eliminating potential for immune rejection

and disease transmission. MSCs were first shown to be pluripotent for mesenchymal cell lineages, such as osteogenic, chondrogenic, and adipogenic differentiation (7). However, recent findings suggest MSCs can be induced to differentiate toward neuroectodermal (8, 9), mesodermal (10–13), and endodermal lineages (14, 15) based on intercellular interactions with a variety of mature cell types. On delivery, MSCs have been shown to engraft and differentiate into cell types of the tissue of engraftment (16–21), but the cues involved in guiding appropriate MSC differentiation remain unknown. Previous reports studying murine embryonic stem cells on fixed feeder layers have shown that cell-to-cell contact can provide important differentiation cues that are separate from diffusible factors (22). MSC cultures have been shown to differentiate into several cell types when in direct contact, although the interpretation of these results is complicated by potential for cellular fusion events, which would not represent true differentiation (14, 23, 24). Therefore, while a great deal of promise remains for using MSCs in strategies for regenerating several tissue types, the mechanisms involved in inducing specific cellular phenotypes need to be better understood.

The impetus that propelled the work reported here was the desire to generate a bioengineered skin tissue to improve healing for the millions of patients each year who suffer from either severe burns or chronic nonhealing ulcers (25). Wound healing studies with bone marrow aspirate and bone marrow-derived MSCs have shown promising results in the treatment of wounds that were refractory to other standard treatment such as bioengineered skin or skin grafts (5, 6, 18). Therefore, we set out to understand how MSCs might participate in wound

¹ Correspondence: University of California, Davis, School of Medicine, Department of Dermatology, Dermatology Research, TB 192, One Shields Ave, Davis, CA 95616, USA. E-mail: rriisseroff@ucdavis.edu

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healing since these cells are expandable, thereby allowing for multiple treatments from a single bone marrow aspirate and the potential for providing a large amount of tissue. Since murine MSC differentiation into epidermal keratinocytes had been reported *in vitro* and *in vivo* (26), we hypothesized that human mesenchymal stem cells (hMSCs) would also differentiate down an epithelial pathway that could either be incorporated into an engineered tissue or directly into healing skin. Indeed, we found that hMSCs could be induced to adopt an epithelial phenotype either through *in vitro* contact coculture with keratinocytes or *ex vivo* incorporation into reepithelializing human skin. Furthermore, we definitively showed *in vitro* that a large population of hMSCs acquired the epithelial phenotype by differentiation rather than by fusion with neighboring keratinocytes. Unexpectedly, we discovered that hMSCs cocultured with keratinocytes without allowing the cells to physically touch (noncontact coculture) did not differentiate down epithelial pathways, but they expressed markers suggestive of early neural and myofibroblast lineages, cell types typically found in the dermis. From the standpoint of fundamental stem cell biology, a particularly notable result from this work is that a single cell type, the epidermal keratinocyte, has the capacity to induce differentiation of hMSCs down multiple lineages. In addition, hMSCs are a particularly promising stem cell type for cutaneous wound healing, as they could provide an autologous, expandable source for cell types found in both dermal and epidermal tissue.

MATERIALS AND METHODS

Cell culture

Primary hMSCs and green fluorescent protein (GFP)-transfected hMSCs (GFP-hMSCs) were obtained as material transfers from the Tulane University Center for Gene Therapy (New Orleans, LA, USA). Although there are no specific surface markers for MSCs, it has been accepted that these cells display a specific morphology, a set of coexpressed surface markers, and have characteristic differentiation capabilities when placed in environments to promote chondrogenic, adipogenic, and osteoblastic differentiation. The Tulane University Center for Gene Therapy has analyzed these cells rigorously for their colony-forming abilities, their marker expression, and their differentiation capacity. Therefore, these cells met the current accepted definition for MSCs. Preliminary experiments were also performed with hMSCs obtained from Cambrex (East Rutherford, NJ, USA), and similar results were observed for cells obtained from both sources. hMSCs were cultured in growth medium consisting of low-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen), 1% penicillin/streptomycin (Invitrogen), 0.25% gentamicin (Invitrogen), and 0.25% fungizone (Invitrogen) and grown in humidified chambers at 37°C and 5% CO₂. Primary neonatal human keratinocyte (NHK) cultures were isolated from neonatal human foreskins using an adapted procedure from Rheinwald and Green (27). Briefly, the foreskin was placed in

trypsin to separate the epidermis from the dermis. The separated epidermis was then centrifuged and resuspended in serum-free keratinocyte growth medium (KGM; Cascade Bioiocs, Inc., Portland, OR, USA) and cultured with a feeder layer of mitomycin-treated 3T3 fibroblasts. Cells were subsequently passaged and cultured in KGM in humidified chambers at 37°C and 5% CO₂.

hMSC-NHK coculture

For direct coculture, NHKs were grown to 90% confluence and then labeled with Cell Tracker Blue [CTB; 7-amino-4-chloromethylcoumarin (CMAC); Invitrogen]. The NHKs were grown either in Petri dishes (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for fluorescence-activated cell sorting (FACS) experiments or on sterile glass coverslips (Thermo Fisher Scientific) that were coated with 50 µg/ml collagen, type I (BD Biosciences, San Jose, CA, USA) for 1 h at 37°C for immunocytochemistry experiments. The NHKs were then treated with 10 µg/ml mitomycin C (EMD, San Diego, CA, USA) for 1 h to inhibit proliferative activity. Green fluorescent hMSCs were obtained by either using GFP-hMSCs or by staining hMSCs with Cell Tracker Green [CTG; 5-chlorofluoromethylacetate (CMFDA); Invitrogen], according to the manufacturer's protocol. The GFP-hMSCs were derived from a different female donor than the hMSCs that were labeled with CMFDA. The green fluorescent hMSCs were grown to 50% confluence and then trypsinized and plated on a lawn of the CTB-labeled NHKs at 5000 cells/cm² in KGM. All contact cocultures were grown in KGM. hMSCs maintained in KGM without exposure to NHKs served as controls. Two keratinocyte strains that were derived from different male donors were used for the cocultures.

For noncontact coculture, NHKs were seeded into the bottom of 6-well cell culture insert companion plates (BD Biosciences) and grown to 50–75% confluency. Then, 1-µm-pore 6-well cell culture inserts (BD Biosciences) were seeded with hMSCs that were placed in a second 6-well cell culture insert companion plate to avoid potential contamination of the NHKs with hMSCs, and the inserts were then placed into the 6-well plates that contained the NHKs (Supplemental Fig. 1B). Initial studies indicated that no changes were observed at d 5 for noncontact cocultures. However, previous literature in which exogenous delivery of growth factors led to epidermal or epidermal-like differentiation of stem cells followed a 21-d time course. Therefore, for this work, noncontact cocultures were cultured out to 21 d in accordance with previous differentiation studies (1, 28, 29). All noncontact cocultures were cultured in KGM, and hMSCs grown in KGM and on transwell inserts without exposure to NHKs served as controls.

Quantitative RT-PCR

Total ribonucleic acid (RNA) was extracted from samples using the Qiagen RNAeasy Mini Kit (Qiagen, Valencia, CA, USA) and quantified using the RiboGreen assay (Invitrogen) as described by the manufacturer. The RNA was converted to complementary deoxyribonucleic acid (cDNA) using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time RT-PCR was used to analyze gene expression levels of Keratin 14, ΔNp63α, α-smooth muscle actin (αSMA), and βIII-tubulin (Tuj1) by utilizing Taqman Gene Expression Assay probes and primers (Applied Biosystems, Foster City, CA, USA). 18S rRNA was used as an internal control to normalize expression to cell number. Reported values for RNA

expression were normalized further relative to control hMSCs that were cultured alone as described above.

FACS

Culture dishes of NHKs (90% confluent) derived from a male donor were labeled with CTB and treated with 10 $\mu\text{g}/\text{ml}$ of mitomycin C. Green fluorescent hMSCs derived from female donors, labeled either by GFP or by CTG, were trypsinized and plated on a lawn of the blue fluorescent NHKs and contact cocultured. After 5 d, the cells were lifted gently with sterile modified PBS (0.9 mM CaCl_2 , 0.5 mM MgCl_2 , 10 mM EDTA, and 1% FBS in Dulbecco's Ca^{2+} , Mg^{2+} PBS; Invitrogen) and stained with mouse anti-CD104 (β_4 -integrin) primary antibodies and donkey anti-mouse R-PE conjugated secondary (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Cells were sorted on the Dako MoFlo high-performance cell sorter (Dako, Pirenteria, CA, USA) and analyzed by Summit software (Dako). Sort gates for R-PE, CTG, and CTB were set by analyzing 50,000 cells from CTG-labeled hMSCs, unlabeled hMSCs, CTB-labeled keratinocytes, unlabeled keratinocytes, and R-PE-labeled keratinocytes, such that no bleedthrough occurred in each of the gates. CTG^+CTB^+ were sorted into chamber slides and visualized under fluorescence microscopy to confirm that these cells arose from fusion and consisted of multiple nuclei (Supplemental Fig. 2). The contact cocultured cells were also analyzed for expression of both CTG and the β_4 -integrin. Cells that were $\text{CTG}^+\beta_4^+$ were then additionally gated for expression of CTB, since $\text{CTG}^+\beta_4^+$ cells that express CTB most likely arise from fusion with a neighboring keratinocyte. All cell-sorting experiments were also performed with GFP-labeled hMSCs. GFP-labeled hMSCs were sorted similarly to the CTG-labeled hMSCs, and similar results were obtained.

Fluorescence *in situ* hybridization (FISH)

$\text{CTG}^+\beta_4^+\text{CTB}^{(-)}$ cells isolated by FACS and the expression of the X and Y chromosomes were assayed with the CEP X Spectrum Orange /Y Spectrum Green DNA probe kit (Abbot Molecular, Des Plaines, IL, USA) following the manufacturer's instructions.

Immunocytochemistry

Immunocytochemistry was performed on cells that were grown on collagen-coated coverslips as follows. Sterile glass coverslips (Thermo Fisher Scientific) were placed in 24-well dishes and were coated with 50 $\mu\text{g}/\text{ml}$ collagen, type I (BD Biosciences) for 1 h at 37°C. The coverslips were washed 3 times with KGM before NHKs and green fluorescent hMSCs were seeded as described above. The cocultures were cultured in KGM, and the medium was changed daily. Coverslips were immunostained after 5 d of coculture, based on previous studies of hMSC contact cocultures (14, 30). Coverslips were fixed with 4% paraformaldehyde for 10 min and were permeabilized with 0.1% Triton-X for 5 min. Cells that were to be stained with anti-NK1-beteb were fixed with acetone at -20°C for 10 min and permeabilized with methanol at -20°C for 5 min. Coverslips were then blocked for 1 h in 10% goat serum to match the same species as the secondary antibody. Primary antibodies (outlined in **Table 1**) were diluted in 1% goat serum. Goat anti-mouse AF 594 (Invitrogen) secondary antibodies were diluted at 1:200 in 1% goat serum and incubated for 2 h at room temperature. Coverslips were mounted onto slides with Prolong Gold Antifade with DAPI (Invitrogen), which provided counterstaining for the nuclei. Primary antibodies were omitted for negative controls. Slides were allowed to dry overnight and were visualized on an inverted fluorescent Nikon Diaphot microscope using $\times 20$ and $\times 40$ panfluor objectives (Nikon, Tokyo, Japan). Images were captured using Retiga-EX cameras (QImaging, Burnaby, BC, Canada) and pseudocolored green for GFP or CTG, red for AF 594, and blue for DAPI using Improvion Openlab software (Improvion, Lexington, MA, USA).

Fluorescent immunocytochemistry was technically difficult with the transwells, because the porous surface binds DAPI, resulting in large background fluorescence values. Therefore, the cells on the transwells were stained by a peroxidase visualization method using the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA, USA). The cells were visualized by NovaRed (Vector Labs), and the nuclei were counterstained with Vector Hematoxylin QS (Vector Labs). The transwells were visualized on an inverted Nikon TE-DH100W, and the images were captured using Diagnostic Instruments Spot RTKE camera using Spot Advanced software (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

TABLE 1. Primary antibodies

Antibody	Manufacturer	Species	Dilution	Incubation
Pan-cytokeratin, clone AE1/AE3	Zymed Laboratories (South San Francisco, CA, USA)	Mouse monoclonal	1:200	1 h at RT (ICC)
Keratin 14 Ab-1, clone LL002	Lab Vision Corp. (Fremont, CA, USA)	Mouse monoclonal	1:100	1 h at RT (ICC); 30 min at RT (IHC)
p63, clone 4A4	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	Mouse monoclonal	1:200	1 h at RT (ICC)
CD104 (β_4 -integrin)	BD Biosciences (Franklin Lakes, NJ, USA)	Mouse monoclonal	1:100	1 h at RT (ICC); overnight at 4°C (IHC); 45 min on ice (FACS)
αSMA , clone 1A4	Abcam Inc. (Cambridge, MA, USA)	Mouse monoclonal	1:75	Overnight at 4°C (ICC, IHC)
TuJ1	Stemcell Technologies, Inc. (Vancouver, BC, Canada)	Mouse monoclonal	1:100	Overnight at 4°C (ICC, IHC)
NK1-beteb	Monosan (Uden, The Netherlands)	Mouse monoclonal	1:20	1 h at 37°C (ICC)

ICC, immunocytochemistry; IHC, immunohistochemistry; RT, room temperature.

Ex vivo human skin wounds

We adapted an *ex vivo* human wound healing model developed by Emanuelsson and Kratz (31). Normal human skin was obtained from cosmetic surgical abdominoplasty under an approved exemption granted by the Internal Review Board at the University of California, Davis. An excisional wound through the epidermis and mid-dermis was created using a 3-mm punch tool. Green fluorescent hMSCs, tagged either by GFP or CTG, were then encapsulated and delivered into the *ex vivo* wounds in 5 mg/ml fibrinogen/1 U/ml thrombin solutions that were then allowed to form a fibrin gel in the wound. The wounded pieces of skin with the delivered hMSCs were placed in wells of a 12-well dish and tissue cultured in 10% low-glucose DMEM in humidified chambers at 37°C and 5% CO₂ that were protected from light. At d 5 and 7 of tissue culture, the excisional wounds treated with fibrin gels were fixed in 10% formalin and embedded in paraffin. At d 5, fibrin was still present, and the hMSCs still suspended in the fibrin layer were analyzed for up-regulation of α SMA and TuJ1 with laser capture microdissection (LCM), since the physical separation from the epidermis simulated the *in vitro* noncontact cocultures. The d 7 samples were analyzed for epithelial markers only, since the fibrin was degraded and therefore any remaining hMSCs presumably had an opportunity to make contact with the epidermis and incorporate into the reepithelializing epithelium.

Immunohistochemistry

Sections (5 μ m) were cut from paraffin-embedded d 7 *ex vivo* wounds and rehydrated. An antigen retrieval step was performed prior to primary antibody incubations, as outlined in Supplemental Material. The sections were then incubated with goat anti-mouse AF 594 secondary antibodies (Invitrogen) for 2 h at room temperature, and the sections were mounted with coverslips using Prolong Gold Antifade with DAPI and visualized on an inverted Nikon TE-DH100W. The images were captured using the Diagnostic Instruments Spot RTKE camera.

LCM

Formalin-fixed paraffin-embedded sections from d 5 *ex vivo* human skin wounds with hMSCs delivered in fibrin gels were microdissected using the Veritas Microdissection System (Molecular Devices, Mountain View, CA, USA). Total RNA was extracted and amplified into aRNA using the Paradise Reagent System (Molecular Devices). The aRNA was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR was used to analyze gene expression levels of α SMA and TuJ1. Taqman Gene Expression Assay probes and primers were purchased (Applied Biosystems), and the primers used spanned exon-exon junctions to eliminate amplification of any genomic contamination. PCR was conducted using the standard protocol as provided by Applied Biosystems for TaqMan probes using the iCycler Real-Time PCR machine (Bio-Rad). Expression levels for the genes of interest were normalized to β -actin to account for differences in cell number. hMSCs that were cultured in fibrin gels that were not delivered into the *ex vivo* human skin wounds served as controls for further normalization.

RESULTS

To explore the influence of keratinocytes on hMSC differentiation, we first set up contact cocultures (Supple-

mental Fig. 1A). To distinguish hMSCs from cocultured keratinocytes, the hMSCs were fluorescently labeled, either genetically by expression of GFP, or by uptake of the dye CTG. By d 5, a subpopulation of the contact cocultured hMSCs retained a fibroblastic morphology, while another subpopulation had assumed a keratinocyte-like, polygonal morphology and expressed epithelial cell markers pancytokeratin, K14, p63, and β_4 -integrin (CD104) (Fig. 1A). We observed that many GFP⁺ hMSCs that expressed epithelial markers in contact cocultures appeared to contain a single nucleus (Fig. 1A) while others were multinucleate (Supplemental Fig. 2A, B). While we did not note any further proliferation of the hMSCs (only isolated single cells were observed with no evidence for cell division), significant proliferation might not be expected due to contact inhibition by surrounding basal keratinocytes, which were seeded at a higher density.

Previously, it has been suggested that transdifferentiation of adult stem cells down epithelial lineages may be due to cell fusion rather than true differentiation (14, 23, 24), and thus the presence of multinucleate cells for hMSCs in direct contact with keratinocytes here might similarly indicate fusion rather than true differentiation. To demonstrate hMSC differentiation due to contact coculture with keratinocytes, and to rigorously rule out fusion as being the only cause of the observed epidermal phenotype, we used fluorescence activated cell sorting (FACS) to separate fused cells from nonfused cells and analyzed expression of keratinocyte markers and integrins. To separate cells, we fluorescently labeled hMSCs with CTG or used GFP-expressing cells, while keratinocytes were labeled with CTB prior to contact coculture (Fig. 1B). Fused cells would be labeled both green and blue, while hMSCs that had not fused would be labeled green only. Dissociated cocultured cells were FACS sorted, separating the green⁺blue⁺ fused hMSCs from the green⁺blue⁻ nonfused cells. The nonfused hMSCs demonstrated an increased expression in epithelial mRNA transcripts K14 and Δ Np63 α compared to control hMSCs (Fig. 1C), suggesting differentiation along the epithelial lineage. To characterize hMSC differentiation further in response to cues provided by contact coculture with keratinocytes, we used FACS to isolate CTG-labeled hMSCs from the hMSC/keratinocyte contact cocultures and analyzed their expression of the keratinocyte surface marker β_4 -integrin (CD104; Fig. 2A–E). Of the contact cocultured CTG-hMSCs, 61% significantly increased their expression of β_4 -integrin, compared to 0% of noncocultured CTG-hMSCs (Fig. 2C, D), and thus this subpopulation represented the cells that adopted some features of an epithelial phenotype. To correct for cell fusion, these double-positive (CTG⁺ β_4 -integrin⁺) hMSCs were additionally gated against the blue fluorescence of the keratinocytes, and 56% of the CTG⁺ β_4 ⁺ cells were CTB⁺ (cells derived from fusion) while 44% were CTB⁻ (no fusion) (Fig. 2E).

To demonstrate more rigorously that the nonfusion,

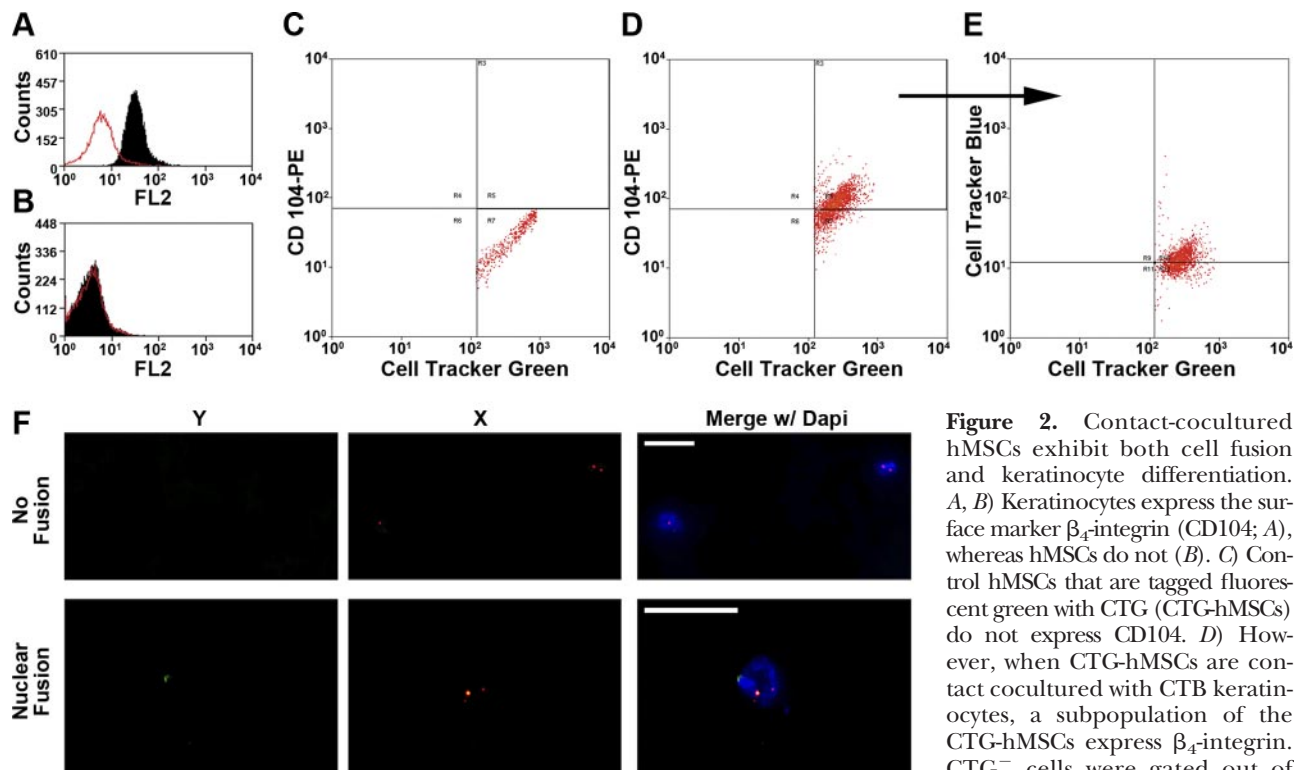


Figure 2. Contact-cocultured hMSCs exhibit both cell fusion and keratinocyte differentiation. *A, B*) Keratinocytes express the surface marker β_4 -integrin (CD104; *A*), whereas hMSCs do not (*B*). *C*) Control hMSCs that are tagged fluorescent green with CTG (CTG-hMSCs) do not express CD104. *D*) However, when CTG-hMSCs are contact cocultured with CTB keratinocytes, a subpopulation of the CTG-hMSCs express β_4 -integrin. CTG⁻ cells were gated out of this analysis, and therefore no

CTG⁻ β_4 ⁺ cells are visible. *E*) To assess the role of cell fusion further, CTG⁺ β_4 ⁺ cells (top right quadrant) were gated against their expression of CTB fluorescence. 56% of the CTG⁺ β_4 ⁺ cells were CTB⁺ (top right quadrant) and represented cells derived from cell fusion, whereas 44% of the CTG⁺ β_4 ⁺ cells were CTB⁻ (lower right quadrant) and represented hMSCs that had truly differentiated. We obtained similar results when we contact cocultured GFP-labeled hMSCs with CTB keratinocytes (data not shown). Fluorescence gates defined in panel *C* were used for subsequent analyses (*D, E*). *F*) GFP⁺ β_4 ⁺CTB⁻ or CTG⁺ β_4 ⁺CTB⁻ were sorted onto glass slides by FACS and subsequently analyzed by FISH. Red spot represents a probe annealed to an X chromosome; green spot represents a probe annealed to the Y chromosome. MSCs were derived from a female donor and only contained X chromosomes; keratinocytes were derived from a male donor and contained an X and Y chromosome. FISH analysis showed that 92% of the cells were composed of cells that only contained X chromosomes (representative cells shown in top panel), confirming that these GFP⁺ β_4 ⁺CTB⁻ or CTG⁺ β_4 ⁺CTB⁻ cells were not due to fusion. Interestingly, some of the fusion cells also exhibited nuclear fusion, as evidenced by the presence of one nucleus containing 3 X chromosomes and one Y chromosome (bottom panel shows representative cell shown in lower panel). Scale bars = 50 μ m.

p63, or β_4 -integrin by immunohistochemistry (Supplemental Fig. 5); and did not express detectable KI4 and Δ Np63 α mRNA transcripts, unlike control hMSCs grown in KGM (data not shown). However, RT-PCR analysis of the noncontact-cocultured hMSCs demonstrated that mRNA transcripts for α SMA (chosen as a myofibroblast marker) and TuJ1 (chosen as an early neural marker) were increased at 21 d of coculture relative to the control population (Fig. 3*B*). Immunohistochemistry confirmed the RT-PCR results for noncontact-cocultured cells. Fibroblastic cells exhibited increased α SMA expression, while TuJ1 expression was elevated for the spindle bi- and tripolar cells (Fig. 3*C*). Our noncontact-coculture results are consistent with previous observations that keratinocytes synthesize and secrete a host of growth factors and signaling molecules that induce MSC differentiation into neural and myofibroblast phenotypes (38–42). Thus, in contrast to observations for direct contact cocultures, hMSCs noncontact cocultured with keratinocytes did not express an epithelial phenotype but

they might express markers consistent with dermal cell types.

In vitro cell culture results indicated that hMSCs adopt very different phenotypes depending on the nature of their physical interaction with keratinocytes in a culture dish. However, many of the biological cues and matrix interactions of the 3-dimensional tissue environment are absent for *in vitro* experiments. We therefore sought to determine whether similar programming of hMSC lineage differentiation could be demonstrated in a more complex, biologically relevant system. In particular, we wanted to demonstrate potential utility for human tissue. Therefore, the model system we chose to utilize was an *ex vivo* human skin wound model in which normal human skin is wounded and allowed to reepithelialize while being maintained in organ culture (25). GFP-labeled hMSCs were encapsulated and delivered within a fibrin gel into a defect formed by a punch biopsy wound made in the explanted skin. Fibrin mimics the structure of the provisional wound

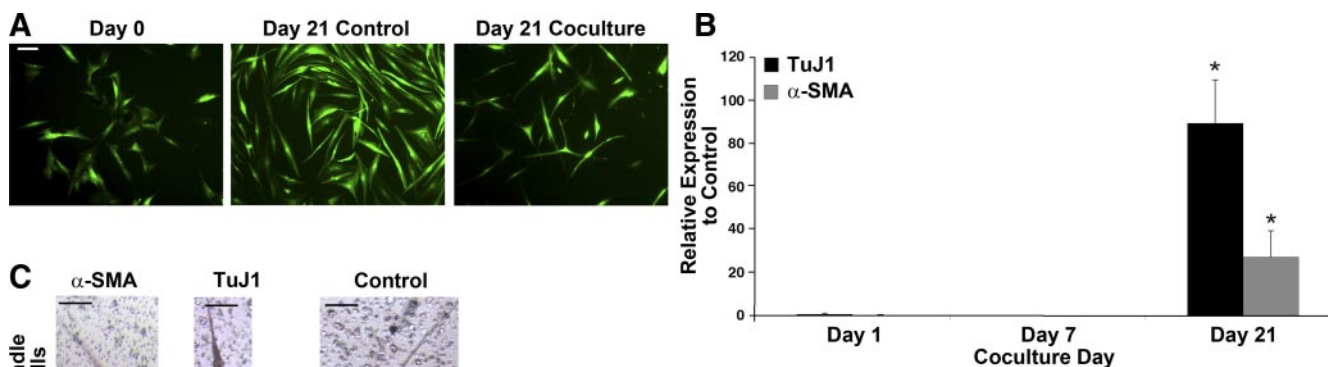


Figure 3. Noncontact-cocultured hMSCs differentiate into α SMA- and TuJ1-expressing cells. GFP-labeled hMSCs were seeded onto transwells and cultured in KGM alone (control) or in noncontact coculture with keratinocytes for 21 d. *A*) Cocultured hMSCs could be morphologically discriminated into 2 cell populations: one that exhibited a spread, myofibroblastic phenotype and another that exhibited a spindly, bi-, and tripolar phenotype. Also, the cocultured cells proliferated more slowly than the control cells, possibly due to differentiation into a nonproliferative cell type. *B*) RT-PCR analysis of expression of the mRNA for the myofibroblast marker α SMA and the early neural marker TuJ1 demonstrates a significantly increased expression of these markers in the noncontact-cocultured MSCs by d 21. RNA expression is reported relative to expression in control hMSCs that were grown under identical conditions except that they were

not cocultured with keratinocytes. mRNAs for K14 and Δ Np63 α were undetectable. Data are reported as means \pm SE. * P < 0.001. *C*) Immunohistochemistry of the d 21 cocultured hMSCs with antibodies against α SMA and TuJ1. Positive cells are red-brown; cells were counterstained with hematoxylin, making nuclei and negative cells appear purple. Spread cells expressed the myofibroblast marker α SMA; spindle cells did not. Conversely, spindle cells expressed TuJ1, whereas spread cells did not. Cells that were not exposed to primary antibody served as controls. Scale bars = 50 μ m.

matrix formed immediately on wounding skin (26), and hMSC encapsulation resulted in the cells being initially sequestered away from the epidermis while allowing for eventual incorporation into the wound site through normal protease and/or migration-related processes.

After 7 d of *ex vivo* culture, some of the GFP-hMSCs had engrafted into the reepithelializing epidermis, while others engrafted into the dermal layers of the wound (Fig. 4A, B). Notably, GFP-hMSCs were found in the basal layer of the reepithelializing epidermis, and they coexpressed β_4 -integrin (CD104) and K14, two markers for basal keratinocytes (Fig. 4A, B). At d 5 postwounding (the fibrin is degraded by d 7), the fibrin matrix was removed selectively from histological sections using LCM (Fig. 4C–F), thus allowing isolation and analysis of hMSCs that had not come into direct contact with keratinocytes. Subsequent RT-PCR analysis of these hMSCs demonstrated an increase in expression of α SMA and TuJ1 (Fig. 4G) relative to control hMSCs that had been fibrin encapsulated but were not introduced into the skin wounds. Thus, as with *in vitro* coculture experiments, hMSCs appear to adopt properties that are dependent on proximity to epithelial cells, with cells in contact with the epithelial layer expressing epithelial markers and those physically separated from the epithelial layer expressing dermal makers.

DISCUSSION

MSCs possess the pluripotent ability to differentiate into many different cell types (1, 2) with the micro-

environment playing a key role in directing their fate (32). Previous studies have suggested that MSCs are capable of epithelial differentiation (14, 15, 21, 28, 33–35), although the epithelial transdifferentiation potential of MSCs has been controversial and it has been suggested that these observations may be confounded by cell fusion (14, 23, 36, 37). Here, we show definitively that hMSCs cultured in direct contact with keratinocytes can express properties at both the mRNA and protein levels consistent with a keratinocyte phenotype. In contrast to the outcome of contact cocultures, noncontact cocultures stimulate hMSCs to differentiate along α SMA- and TuJ1-expressing lineages (the noncontact-cocultured hMSCs were not positive for epidermal lineage cell markers, for melanocytes, or Merkel cells, data not shown). Thus, keratinocytes might direct hMSC differentiation through both short- and long-range interactions, with lineage specification being determined by proximity to the epidermal layer.

The keratinocyte is only one of many cell types that populate skin, with other resident cell types, such as endothelial cells, melanocytes, and fibroblasts, likely providing their own unique sets of contact-mediated and long-range differentiation cues that appropriately influence wound healing. Also, previous contact coculture studies have shown that different mesenchymal cells influence MSCs in a unique fashion (30). Thus, in the *in vivo* environment, a complex network of soluble and insoluble signals from multiple cell types could potentially direct MSC differen-

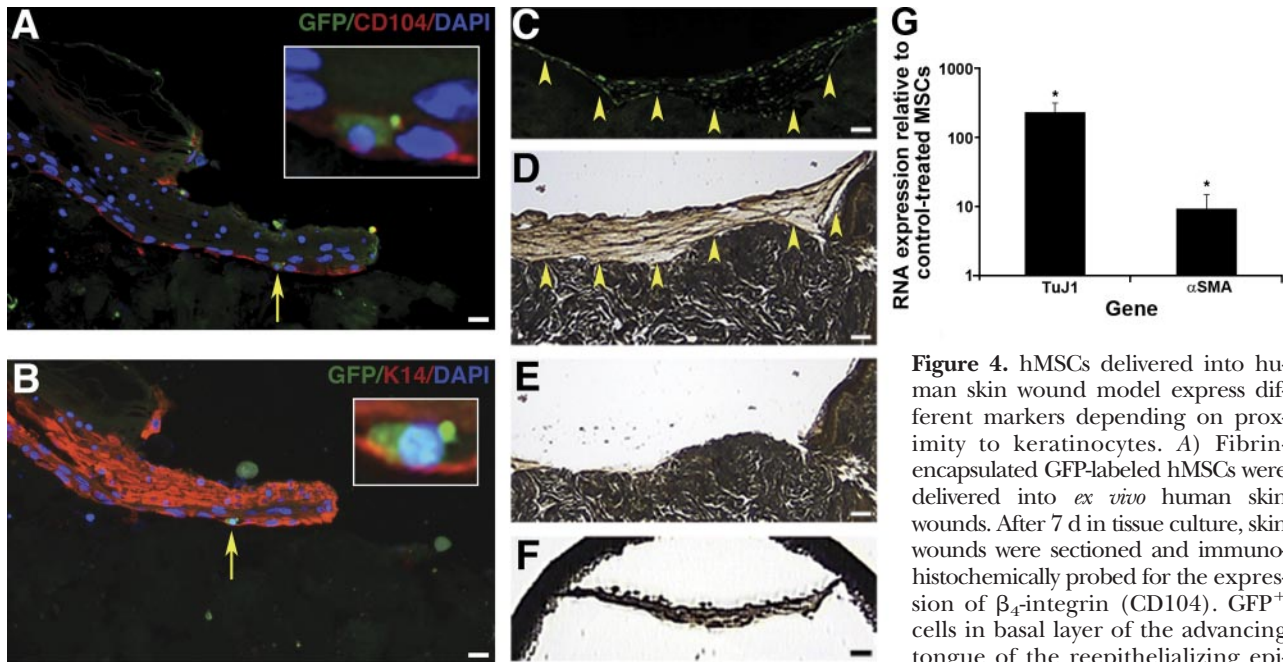


Figure 4. hMSCs delivered into human skin wound model express different markers depending on proximity to keratinocytes. **A)** Fibrin-encapsulated GFP-labeled hMSCs were delivered into *ex vivo* human skin wounds. After 7 d in tissue culture, skin wounds were sectioned and immunohistochemically probed for the expression of β_4 -integrin (CD104). GFP⁺ cells in basal layer of the advancing tongue of the reepithelializing epidermis were found to express

CD104, a characteristic phenotypic marker of the basal keratinocyte (yellow arrow). **B)** Cells that were doubly positive for GFP and K14 were also present in the reepithelializing keratinocyte layer (yellow arrow) Inset: magnification of GFP-labeled hMSC that has engrafted into the basal layer as a K14⁺ cell. **C–F)** Cross section of skin wound showing GFP-labeled MSCs that had been encapsulated in fibrin (yellow arrowheads) and delivered into the wound after 2 d in tissue culture. **C)** LCM was used to selectively remove the fibrin (yellow arrowheads) from the dermal portion of the wound on d 5 postwounding. **D, E)** Delivered fibrin layer was visualized (**D**) and removed (**E**) by LCM. **F)** Retrieval of the fibrin layer was confirmed by visualization of the LCM cap. **G)** RNA collected from the excised fibrin plug in 5 serial skin sections demonstrated an increase in the expression of the early neural marker TuJ1 and the myofibroblastic marker α SMA as compared to MSCs that had been fibrin encapsulated but had not been exposed to the *ex vivo* human skin wound environment. Data are reported as means \pm se. * $P < 0.01$. Scale bars = 50 μ m (**A**); 100 μ m (**B–E, F**); 200 μ m (**G**).

tiation and integration into tissue. Therefore, we investigated how hMSCs interact with skin cells in a more complex *ex vivo* skin model. Our results suggest that the observed *in vitro* differentiation of hMSCs down epidermal as well as TuJ1- and α -SMA-expressing lineages occurs in explanted human skin as well, indicating that hMSCs retain their differentiation potential, even in a more complex microenvironment containing signaling cues of both the epidermis and the dermis. However, proliferation of transdifferentiated cells was not evident in these models.

While our *ex vivo* results demonstrate hMSC expression of a keratinocyte phenotype within the basal layer of the epidermis, further studies will be needed to determine the extent of transdifferentiation compared to cell fusion since we did not investigate the contribution of each here. Further, MSCs are known to be potent secretors of growth factors that can promote wound healing and angiogenesis (3, 4, 43), and hMSCs derived from adipose tissue and bone marrow can influence skin cells through both contact and paracrine mechanisms unrelated to direct differentiation (47, 48). Therefore, much work still needs to be done to understand the potential contribution of hMSCs to healing of human skin. However, our findings indicate that therapeutic strategies utilizing hMSCs are an excit-

ing approach for providing new wound healing options.

In summary, we have demonstrated that a single cell type, in this case a keratinocyte, might direct hMSC differentiation down multiple distinct lineages depending on the proximity of their interaction. Notably, our results indicate that the differentiation potential of hMSCs is maintained in the more complex microenvironment of human skin, where epidermal and possibly dermal expression is also observed. The ability of hMSCs to participate in the formation of new tissue structures that cross lineage boundaries, and the fact that we have demonstrated these results directly in human tissue, have important implications for both stem cell biology and regenerative medicine applications. Therefore, it is our hope that the findings presented herein will help to better understand the role of adult stem cells in forming new tissue and will also mold therapeutic strategies to better address the challenges of regenerative medicine and wound healing. FJ

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