Differentiation, cell fusion, and nuclear fusion during *ex vivo* repair of epithelium by human adult stem cells from bone marrow stroma

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To investigate stem cell differentiation in response to tissue injury, human mesenchymal stem cells (hMSCs) were cocultured with heat-shocked small airway epithelial cells. A subset of the hMSCs rapidly differentiated into epithelium-like cells, and they restored the epithelial monolayer. Immunocytochemistry and microarray analyses demonstrated that the cells expressed many genes characteristic of normal small airway epithelial cells. Some hMSCs differentiated directly after incorporation into the epithelial monolayer but other hMSCs fused with epithelial cells. Surprisingly, cell fusion was a frequent rather than rare event, in that up to 1% of the hMSCs added to the coculture system were recovered as binucleated cells expressing an epithelial surface epitope. Some of the fused cells also underwent nuclear fusion.

The source of the cells that repair tissues after injury remains poorly defined and controversial (1). One possible source is stem-like progenitors that are endogenous to injured tissues. Candidate cells for this function have been identified in a variety of tissues, including neural (2), hepatic (3), vascular (4, 5), gastrointestinal (6), pancreatic (7), epidermal (8), and muscular (9). A second possible source is stem-like cells arising from the bone marrow that migrate to sites of tissue injury.

Bone marrow contains at least two multipotential populations of stem cells likely to contribute to tissue repair: hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). In addition to producing the blood cell lineages, HSCs or related cells have been reported to differentiate into many cell types of nonhematopoietic tissues (10–14). Similarly, numerous reports indicate that MSCs or cells related to MSCs differentiate into osteoblasts, chondroblasts, adipocytes, and hematopoietic-supporting stroma both *ex vivo* and *in vivo* (15, 16). Furthermore, they can generate cells of all three germ layers (17–21). *In vivo* evidence indicates that unfractionated bone marrow-derived cells as well as pure populations of HSCs or MSCs can give rise to epithelial cell types in lung and other tissues (12, 19, 22, 23). Also, several recent studies have shown that engraftment of MSCs is enhanced by tissue injury (19, 24, 25).

To examine differentiation of human MSCs (hMSCs), we developed an *ex vivo* model of epithelial repair by coculturing hMSCs with heat-shocked human small airway epithelial cells (SAECs). We found that the cocultured hMSCs rapidly differentiated into cells with the morphological and molecular phenotype of SAECs. Because two recent reports suggested that cell fusion may explain some of the observed plasticity of adult stem cells (26, 27), we examined the cocultures for evidence of cell fusion. To our surprise, fusion between adult stem cells and epithelial cells was a frequent event. Also, some of the cells underwent nuclear fusion.

Methods

Cell Culture and Manipulation. hMSCs were obtained from bone marrow aspirates as described (28). Most experiments were carried out with passage 3 cells that were plated at 100 cells per cm^2 in complete culture medium containing 20% FCS and cultured until they reached ≈60% confluency. To obtain cells expressing GFP, the cells were electroporated with a plasmid expressing enhanced GFP from a cytomegalovirus promotor (pIRESneo; CLONTECH). Cells from a single G418-resistant clone were used for all of the experiments. MSCs from two other donors were dye-labeled (CellTracker Green, CMFDA; Molecular Probes). For coculture experiments primary cultures of SAECs were grown to confluence in T-75 flasks under the conditions suggested by the supplier (Clonetics, San Diego). The cultures were heat-shocked at 47°C for 30 min and cooled for 1–2 h at 37°C, and $2.5–5.0 \times 10^5$ GFP^+ hMSCs or dye-labeled hMSCs were added before incubation for up to 4 wk. Similar conditions were used in cocultures with primary bronchial epithelial cells (Clonetics).

Immunocytochemistry. All primary antibodies were obtained from one source (Chemicon) except for anti-CD24 (Becton Dickinson). $GFP^+/CD24^+$ cells isolated by fluorescence-activated cell sorting (FACS) were examined by deconvolution microscopy (Sensicam, Intelligent Imaging Innovations, Denver) to eliminate overlapping cells.

Immunoblotting. Cell lysates were separated by electrophoresis on 4–12% NuPage bis-Tris gels with Mes buffering, and electroblotted onto poly(vinylidene difluoride) membranes. The blots were incubated with primary antiserum (Chemicon) and then with goat anti-mouse IgG horseradish peroxidase conjugate (Sigma) and detected by a chemiluminescent reaction (29).

Microarray Assays. Total RNA was extracted from GFP^+ cells that were sorted from the cocultures and assayed with a chip containing ≈12,500 human genes [Affymetrix (Santa Clara, CA) HGU95Ab2 array].

Time-Lapse Microscopy. Cultures were incubated in an environmentally controlled chamber, and images were recorded with a charge-coupled device camera (ORCA ER; Hamamatsu, Middlesex, NJ). Microscopic functions were controlled with software (METAMORPH; Universal Imaging, Media, PA).

Fluorescent *in Situ* Hybridization. $GFP^+/CD24^+$ cells were sorted from the cocultures and assayed with a commercial kit (CEP X SpectrumOrange/Y SpectrumGreen DNA Probe Kit, Vysis, Downers Grove, IL).

Additional Methods. For more details see *Supporting Methods*, which is published as supporting information on the PNAS web site, www.pnas.org.

Results

Primary human SAECs grown in defined serum-free SAEC medium formed an integrated confluent monolayer of large, flat

Abbreviations: MSC, mesenchymal stem cell; hMSC, human MSC; SAEC, small airway epithelial cell; FACS, fluorescence-activated cell sorting.

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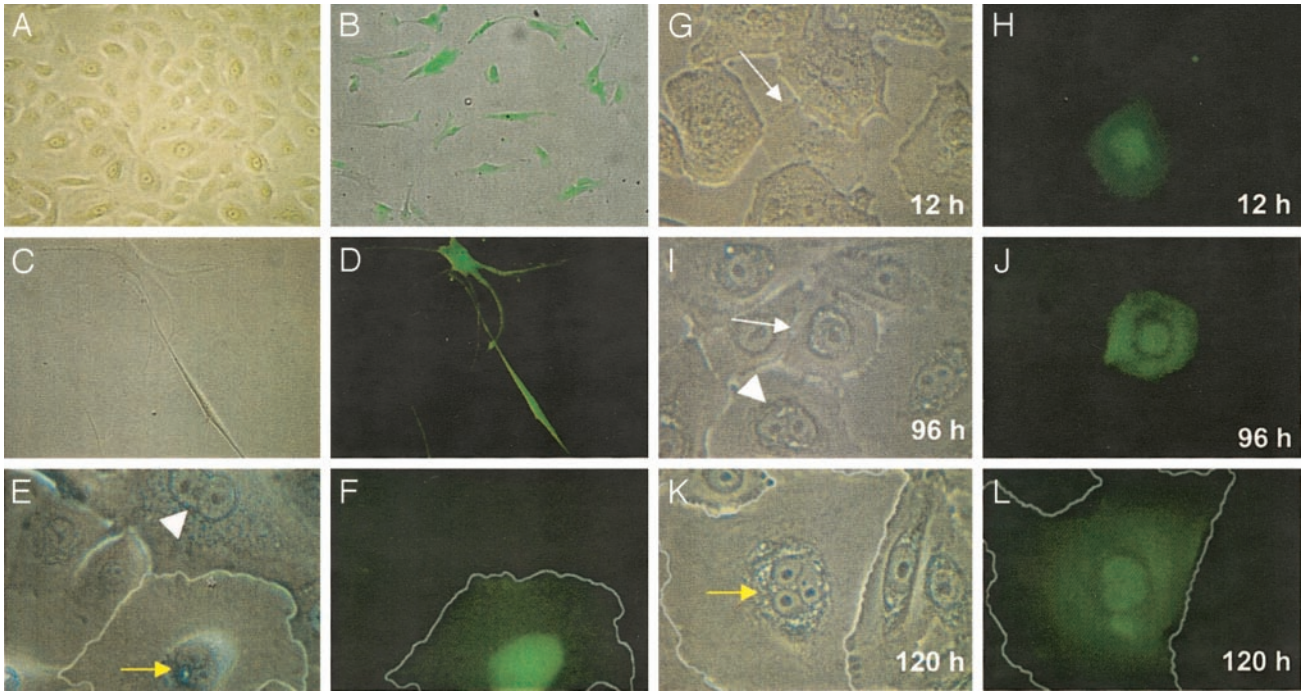

Fig. 1. Phase-contrast and UV microscopy of cultures and cocultures. (*A*) SAECs in SAEC medium. (*B*) GFP$^+$ hMSCs grown in complete MSC medium (FITC overlay on phase). (*C* and *D*) GFP$^+$ hMSCs cultured in serum-free medium for SAECs. (*E* and *F*) Coculture experiment with heat-shocked bronchial epithelial cells at 2 wk. The differentiated GFP$^+$ cell has an epithelial morphology and has repaired the monolayer formed by bronchial epithelium. The cell is binucleated (yellow arrow), as is a GFP$^-$ bronchial cell above it (arrowhead). (*G–L*) Cocultures of GFP$^+$ hMSCs and SAECs after incubation for 12–120 h. (*G* and *H*) GFP$^+$ cell between SAECs undergoing morphological changes (arrow). (*I* and *J*) Differentiated GFP$^+$ cell has an epithelial morphology, has repaired the monolayer formed by the small airway epithelium, and has a single nucleus (arrow). Adjacent SAEC is binucleated (arrowhead). (*K* and *L*) Differentiated GFP$^+$ cell has three nuclei (yellow arrow). (*E*, *F*, *K*, and *L*) The outermost cytoplasmic edges of the GFP$^+$ cells are artificially enhanced. (Magnification: *A–D*, ×10; *E* and *F*, ×40; *G–J*, ×20; *K* and *L*, ×40.)

cells with an elevated perinuclear region (Fig. 1*A*). GFP-expressing hMSCs (GFP$^+$ hMSCs) in complete culture medium containing 20% FCS were fibroblast-like (Fig. 1*B*). Grown alone in SAEC medium, GFP$^+$ hMSCs replicated slowly and developed long, thin processes after a few days (Fig. 1 *C* and *D*).

To test the hypothesis that hMSCs might respond to tissue injury, confluent cultures of SAECs were heat-shocked at 47°C for 30 min to induce cell damage and death. After heat shock, the majority of SAECs remained adherent but many cells lost cell–cell contact as their cytoplasms retracted, opening up holes in the monolayer. GFP$^+$ hMSCs from an isolated clone were added 1–2 h after the heat-shocked SAEC cultures had cooled to 37°C. Within 12 h, ≈1% of the adherent hMSCs began to lose their characteristic fibroblast morphology and became flattened and translucent with an epithelial shape (Fig. 1 *G* and *H*). After 24 h, many of the GFP$^+$ hMSCs were indistinguishable from SAECs by phase-contrast microscopy. By 48–96 h, a continuous monolayer was reassembled in the cultures to which hMSCs were added (Fig. 1 *I–L*). Similar results (not shown) were obtained with hMSCs from two additional donors that were dye-labeled and not cloned. In contrast to the results with cocultures of hMSCs and SAECs, heat-shocked SAECs cultured alone did not consistently regain confluency (not shown). Also, the GFP$^+$ hMSCs added to cultures of SAECs that were not heat-shocked adhered to the surface of the monolayers, primarily at junctions between adjacent SAECs, and showed little evidence of differentiation after several days. In parallel experiments the GFP$^+$ hMSCs were used to prepare cocultures with heat-shocked bronchial epithelial cells. The GFP$^+$ hMSCs also underwent morphological differentiation to bronchial epithelium-like cells (Fig. 1 *E* and *F*).

Of special interest was the appearance of multinucleated GFP$^+$ cells (see yellow arrows in Fig. 1 *E* and *K*), raising the possibility of cell fusion. Many unmodified SAECs and bronchial epithelial cells were also multinucleated (see arrowheads in Fig. 1 *E* and *I*).

The morphologically differentiated GFP$^+$ hMSCs were positive for several epithelial-specific markers including keratins 17, 18, and 19, as well as CC26, a marker of clara, serous, and goblet cells in the lung (Fig. 2*A*). Additionally, immunocytochemistry for E-cadherin and β-catenin demonstrated that differentiated GFP$^+$ hMSCs formed adherens junctions with SAECs (Fig. 2*B*). Many cells that stained for differentiation markers were multinucleated.

The undifferentiated GFP$^+$ hMSCs in the same coculture were negative for keratins and CC26 (see asterisks in Fig. 2*A*). Also, the undifferentiated GFP$^+$ hMSCs did not stain for E-cadherin, stained very lightly for β-catenin, and did not form the pseudostratified epithelioid associations characteristic of SAECs (see asterisk in Fig. 2*B*). The undifferentiated hMSCs contained single nuclei.

To follow differentiation, the cocultures were sorted by FACS to isolate both differentiated and undifferentiated GFP$^+$ cells from the cultures (Fig. 3*A*). By Western blot assays, the isolated GFP$^+$ cells from 3-wk cocultures expressed keratins 17, 18, and 19 (Fig. 3*B*, lane 3); whereas GFP$^+$ hMSCs cultured in complete medium only expressed low levels of keratin 18 (Fig. 3*B*, lane 1). SAECs expressed all three keratins (Fig. 3*B*, lane 2).

To determine the extent of differentiation, mRNA microarrays were used to assay the total population of both differentiated and undifferentiated GFP$^+$ cells. For analysis of the data, we first scanned the genes with the highest signal intensities and selected 20 that are characteristically expressed by epithelial cells (Fig. 3*C*). Correlation analysis of the 20 selected genes indicated a highly significant relationship in expression between the total GFP$^+$ population (epithelial-differentiated, EPI/DIFF) and the SAECs (Spearman rank correlation, two-tailed test at $\alpha = 0.01$, $r = 0.8617$, $P = 0.000001$). Next, we performed a one-sample z test (two-tailed, $\alpha = 0.05$) for all possible two-way comparisons of r values (six r

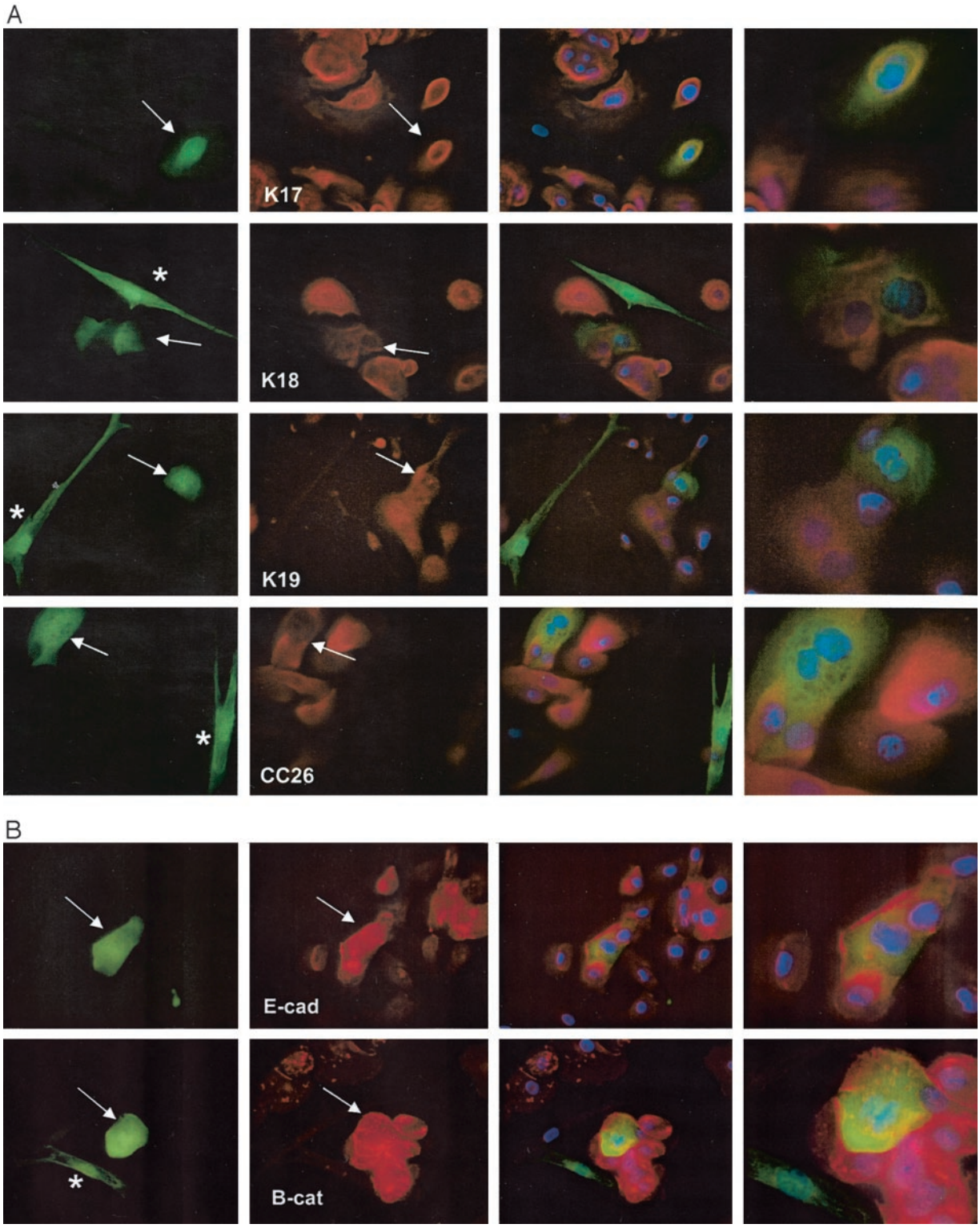

Fig. 2. Immunocytochemistry of GFP$^+$ hMSC and SAEC cocultures. (*A*) Differentiated GFP$^+$ cells express keratins 17, 18, 19, and CC26 (clara cells). (*B*) Markers of adherens junctions; E-cadherin and β-catenin. First column (left to right) UV with FITC filter. Second column, UV with TRITC filter. Third column, merged images with 4′,6-diamidino-2-phenylindole nuclear staining. Fourth column, enlarged merged image. Arrows, double positive cells. *, Undifferentiated GFP$^+$ hMSCs (note single nuclei). (Magnification: $\times$40 for first three columns; $\times$100 for fourth column.)

values, 15 comparisons). Five of the six *r* values were found to be statistically indistinguishable. The remaining *r* for the correlation of gene expression of GFP$^+$ cells isolated from cocultures (EPI/DIFF) with that of SAECs was found to be statistically greater than each of the other five. The results of these analyses support the hypothesis that the gene expression profile of GFP$^+$ cells isolated from cocultures with SAECs more closely resembles that of SAECs than any of the control samples. Interestingly, microarray analyses

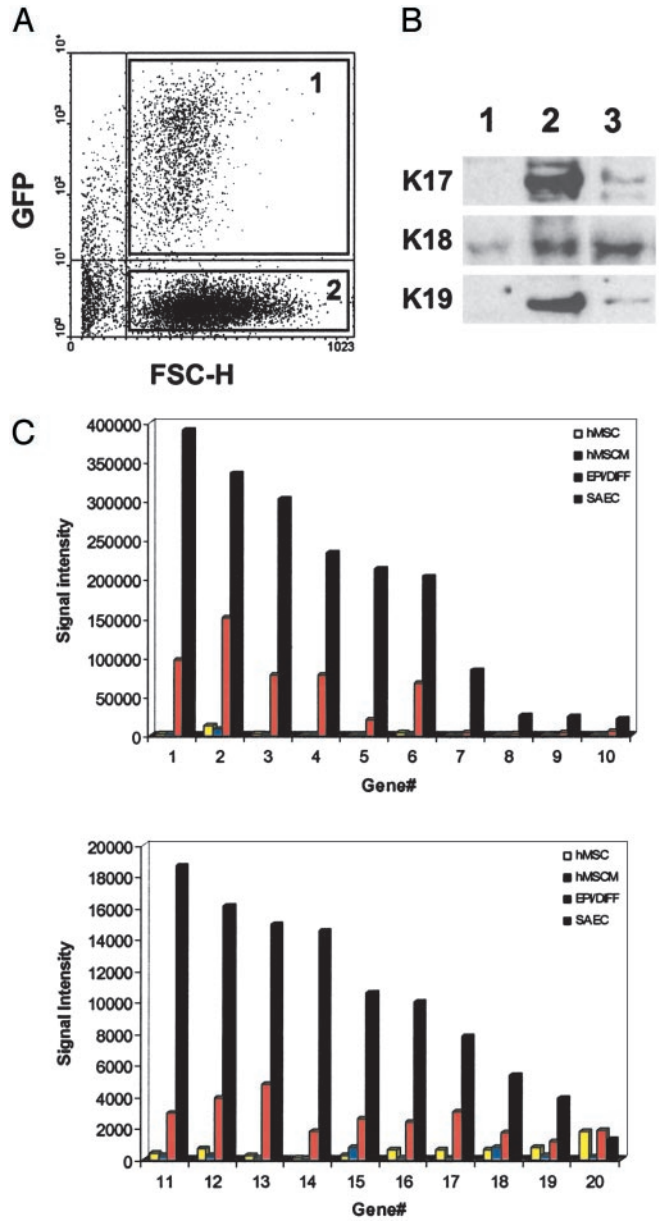

Fig. 3. FACS isolation of cells from the cocultures. (*A*) FACS for GFP and forward scatter of light (FSC-H); GFP^+ cells (gate 1), SAECs (gate 2). (*B*) Immunoblots for keratins 17, 18, and 19 for GFP^+ hMSCs before coculture (lane 1), SAECs (lane 2), and GFP^+ cells isolated by FACS after coculture with damaged SAECs (lane 3; cells isolated with gate 1 from *A*). (*C*) Signal intensities of selected epithelial genes of GFP^+ cells from cocultures assayed by microarrays. hMSC, GFP^+ hMSCs incubated in complete MSC medium (20% serum). hMSCM, GFP^+ hMSCs incubated in SAEC medium (serum-free). EPI/DIFF, GFP^+ cells isolated from the cocultures by FACS. Differentiated GFP^+ cells (EPI/DIFF) express many of the genes expressed by normal SAECs: lane 1, Stratifin (GenBank accession no. X57348); lane 2, keratin 17 (GenBank accession no. Z19574); lane 3, keratin 6 (GenBank accession no. L42611); lane 4, keratin type II (GenBank accession no. M21389); lane 5, keratin 19 (GenBank accession no. Y00503); lane 6, CAN19 (GenBank accession no. M87068); lane 7, keratin 16 (GenBank accession no. 28439); lane 8, Maspin (GenBank accession no. U04313); lane 9, CD24 (GenBank accession no. L33930); lane 10, Claudin-7 (GenBank accession no. AJ011497); lane 11, cornified envelope precursor (GenBank accession no. AF001691); lane 12, Laminin S B3 chain (GenBank accession no. U17760); lane 13, integrin β4 (GenBank accession no. X53587); lane 14, E-cadherin (GenBank accession no. Z35402); lane 15, Laminin-related protein (GenBank accession no. L34155); lane 16, lung amelioride sensitive Na-channel protein (GenBank accession no. X76180); lane 17, P-cadherin (GenBank accession no. X63629); lane 18, Laminin γ-2 chain precursor (GenBank accession no. Z15008); lane 19, NES-1 (GenBank accession no. AF055481); lane 20, Mucin 1 (GenBank accession no. X80761).

of undifferentiated hMSCs revealed the expression of several transcripts commonly found in epithelial cells such as keratin 8 (signal intensity 16,466; GenBank accession no. X14487), cytokeratin 10 (signal intensity 10,491; GenBank accession no. X74929), and keratin 18 (signal intensity 50,650; GenBank accession no. M26326). The mRNAs of these genes are not normally present in differentiated mesenchymal cells and are suggestive of the ability of hMSCs to differentiate across cell lineage boundaries. The microarray data corroborate those of a previous study in which a single cell-derived colony of undifferentiated hMSCs was analyzed by MICROSAGE and found to express keratins 8 and 10, transcripts from endothelial and epithelial cells (31).

In further experiments, GFP^+ hMSCs were added to heat-shocked SAECs and the cocultures were photographed at 20-min intervals for 4 consecutive days (Fig. 4). hMSCs were observed to adhere within 1 h. Within 24 h, GFP^+ hMSCs with single nuclei were observed to approach and contact SAECs (target cells). Some cells differentiated directly after incorporation into the epithelial monolayer but other GFP^+ hMSCs fused with epithelial cells. In some instances, the GFP^+ hMSCs were observed to extend a process to the target cells just before the fusion event. During cell fusion, targeted cells rapidly became GFP^+ within 20-min intervals (Fig. 4 *B*, *G*, and *L*). The hybrid cells were motile and typically seen as a single large flat cell with two nuclei (red arrows in Fig. 4*E*). Over several hours, they reorganized so that both nuclei were adjacent to one another in the elevated perinuclear region characteristic of epithelial cells. Several cells were observed to have three or more nuclei (red arrows in Fig. 4*J*). The GFP intensity in many of the hybrid cells was typically reduced relative to undifferentiated GFP^+ hMSCs, but GFP continued to be expressed for up to 4 wk. Therefore, there was continuing expression of genes from the nucleus derived from the GFP^+ hMSCs. We reverse-tracked 381 GFP^+ cells in the cocultures after 4 days. At least 53 (14%) had participated in cell fusion with an SAEC.

To isolate differentiated GFP^+ cells from the cocultures, we used an antibody to CD24, a mucin-like glycoprotein that is a marker for epithelial cells and not expressed on hMSCs (Fig. 5*A*). After 48 h, 1.3% of the cocultured GFP^+ cells were $GFP^+/CD24^+$. After 1 wk, 4% of the cocultured GFP^+ cells were $GFP^+/CD24^+$. For further examination, $GFP^+/CD24^+$ cells from 1-wk cocultures were sorted into chamber slides, fixed, and nuclear-stained with 4′,6-diamidino-2-phenylindole (Fig. 5 *B–D*, Table 1). Of a total of 754 cells examined from three experiments, 23–26% were binucleated, 2.0–3.3% were trinucleated, and the remainder had a single nucleus (Table 1). However, half or more of the nuclei in the mononuclear cells were large or irregular in shape.

$GFP^+/CD24^+$ cells were isolated from cocultures of male GFP^+ hMSCs and female SAECs and examined by fluorescent *in situ* hybridization for the X and Y chromosomes (Fig. 6). Some of the isolated cells contained a single nucleus with one Y chromosome and one X chromosome, indicating that single-cell differentiation had occurred. Some cells contained nuclei with one Y chromosome and three X chromosomes, indicating that one male MSC nucleus had fused with one female SAEC-derived nucleus (Fig. 6*C*). In addition, a rare nucleus was observed with one Y chromosome and five X chromosomes, indicating that one male MSC nucleus had fused with two female SAEC-derived nuclei (Fig. 6*B*).

Discussion

Current data suggest that MSCs repair tissue *in vivo* by homing to sites of injury. The cells then either differentiate into the phenotypes of the damaged cells (19, 24, 32) or enhance repair by creating a microenvironment that promotes the local regeneration of cells endogenous to the tissue (30, 33). Our results demonstrate that when hMSCs are cocultured with heat-shocked SAECs, a subset of hMSCs rapidly differentiate into epithelial-like cells. Differentiated hMSCs assumed the characteristic morphology of SAECs in culture with a broad, flattened cytoplasm and an elevated perinuclear

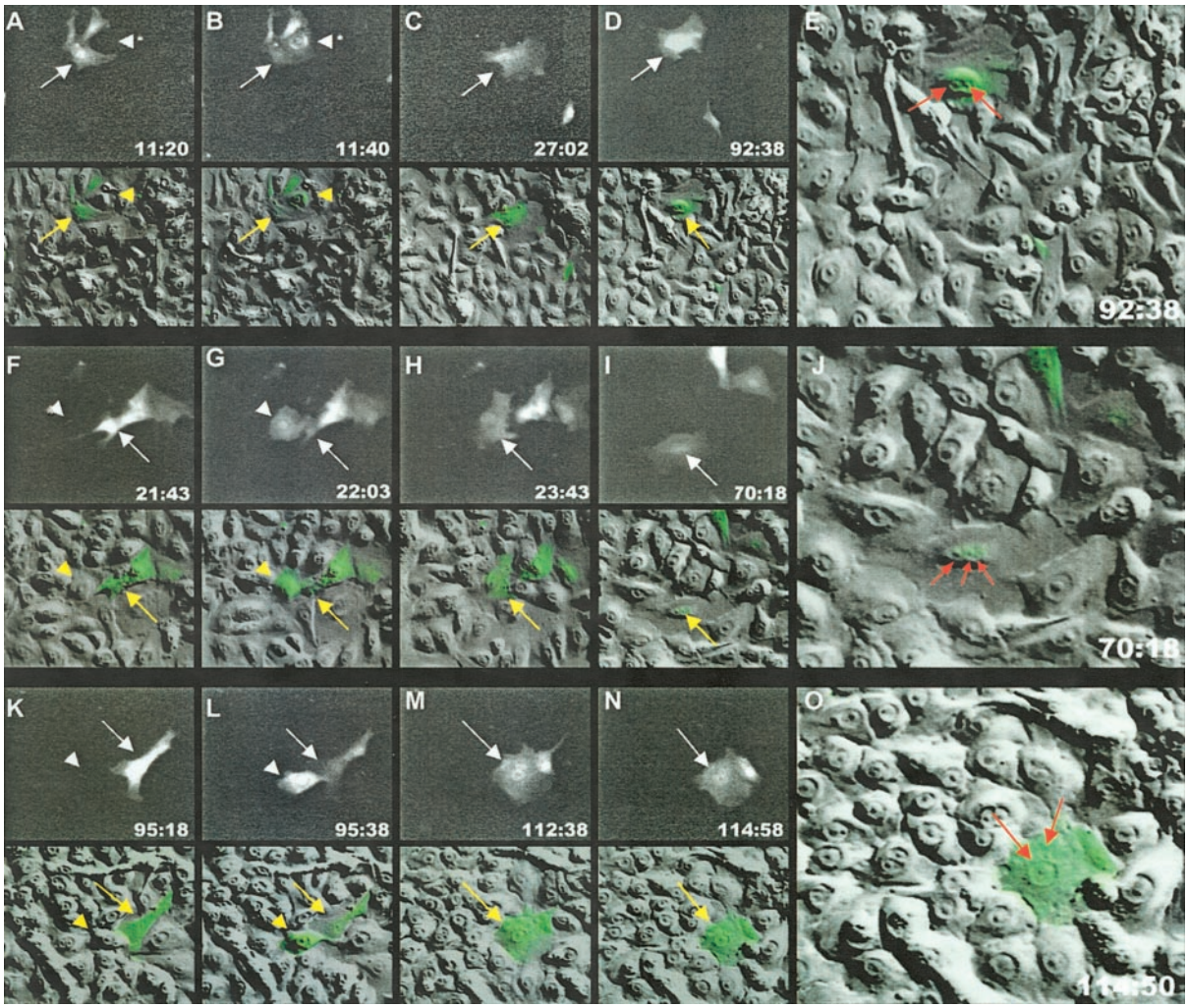

Fig. 4. Time-lapse microscopy of cell fusion in GFP$^+$ hMSC and heat-shocked SAEC cocultures. Three separate fusion events are shown. The same fields were photographed every 20 min by both UV and differential-contrast microscopy. UV photographs are overlain on differential contrast. Selected frames are shown. Note the rapid influx of GFP into target SAECs between the first two frames of each sequence. (*E*, *J*, and *O*) Enlarged images of adjacent frames. White and yellow arrows, GFP$^+$ hMSCs. White and yellow arrowheads, targeted SAECs. Red arrows, multiple nuclei in fused cells. (Magnifications: *A–D*, *F–I*, and *K–N*, ×10; *E*, *J*, and *O*, ×90.)

region. Also, they integrated so as to restore the epithelial monolayer. By several assays, they expressed proteins of epithelial cells: keratins, structural proteins of intermediate filaments; CC26, a lung epithelial marker of clara, serous, and goblet cells; E-cadherin and β-catenin, components of adherens junctions; and CD24, a cell-adhesion molecule. Only a fraction of the GFP$^+$ cells were differentiated, but microarray analyses of the total GFP$^+$ cells recovered from the cocultures showed that the cells expressed mRNAs for many proteins characteristic of normal SAECs. Therefore, the results demonstrated that adult stem cells of mesenchymal origin can be a source of cells to repair damaged epithelium *ex vivo*.

Some of the hMSCs differentiated directly as they integrated into the epithelial monolayer, as demonstrated by immunostaining, FACS of single-nucleated differentiated GFP$^+$/CD24$^+$ cells, and fluorescent *in situ* hybridization analysis of single-nucleated differentiated GFP$^+$/CD24$^+$ cells. However, cell fusion was a frequent rather than a rare event. Two recent reports (26, 27) suggested that cell fusion can explain some of the observed plasticity of adult stem cells. The reports examined spontaneous fusion between embryonic stem cells and either unfractionated bone marrow cells (26), neural stem cells (27), or differentiated neural cells (27). Both experimental protocols required antibiotic selection to identify the resulting hybrid cells. In both reports, the frequency of cell fusion was low and ranged from 10^{-4} to 10^{-5} per cell plated (26, 27). Under the conditions used here, up to 4% of the GFP$^+$ hMSCs added to the cultures were recovered as GFP$^+$/CD24$^+$ cells. Of

Table 1. Nuclear characteristics of isolated GFP$^+$/CD24$^+$ cells

Experiment	No. of cells isolated	Binucleated	Trinucleated	Single nuclei	
				Regular	Large or irregular
1	366	96 (26%)	12 (3.3%)	136 (37%)	122 (33%)
2	246	56 (23%)	5 (2.0%)	93 (38%)	92 (37%)
3	152	40 (26%)	5 (3.3%)	24 (16%)	83 (55%)

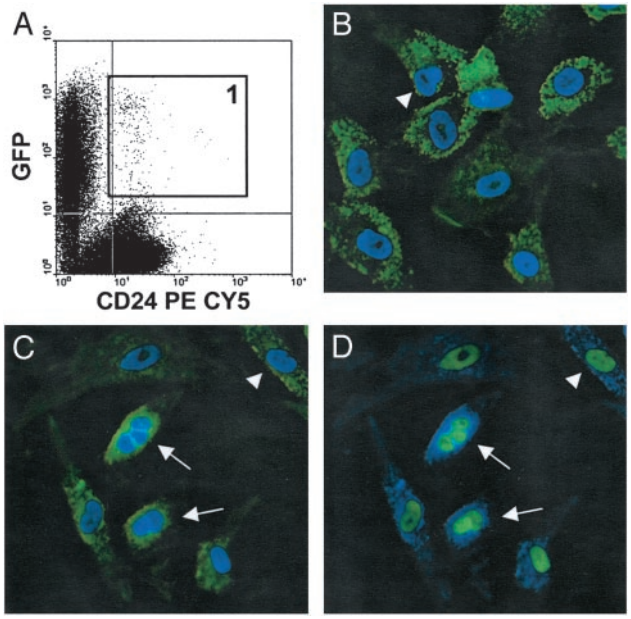

Fig. 5. (*A*) Sorting of GFP$^+$/CD24$^+$ cells from cocultures (gate 1). (*B–D*) Deconvolution microscopy of single cells that were GFP$^+$/CD24$^+$. Three-dimensional images at 1.0-μm intervals through each cell were analyzed to eliminate overlapping cells. The cells were nuclear-stained with 4′,6-diamidino-2-phenylindole. Note multinucleated cells (arrows), cells with single nuclei, and cells with irregular nuclei (arrowheads). Pseudocoloring is reversed in *D* to better visualize cell nuclei from *C*. (Magnification: ×40.)

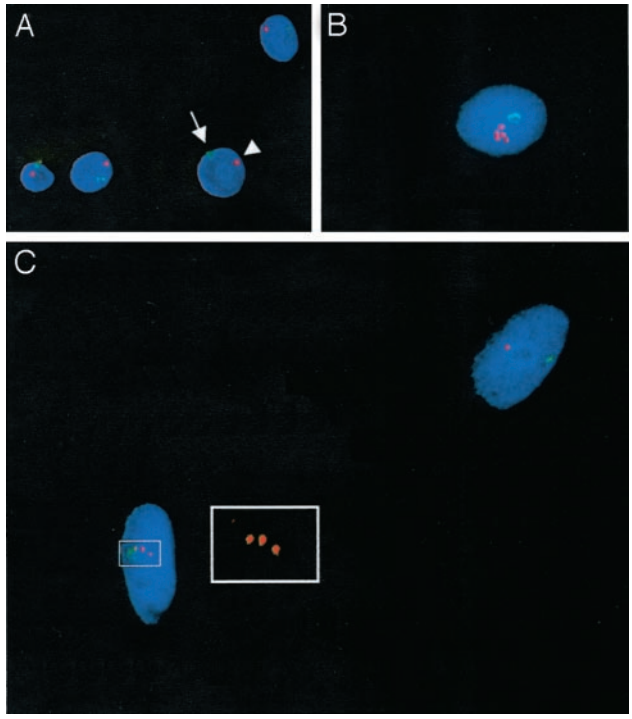

Fig. 6. Fluorescent *in situ* hybridization of GFP$^+$/CD24$^+$ cells isolated from cocultures of male GFP$^+$ hMSCs and female SAECs. Y chromosome, green signal, FITC filter; X chromosome(s), red signal, TRITC filter; 4′,6-diamidino-2-phenylindole nuclear staining, blue, UV filter. (*A*) Control male normal human bronchial epithelial cells (arrow, Y chromosome; arrowhead, X chromosome). (*B*) Hybrid cell nucleus derived from fusion of one male GFP$^+$ hMSC nucleus with two female SAEC-derived nuclei (one green signal, five red signals). (*C*) Hybrid cell nucleus generated from fusion of one male GFP$^+$ hMSC nucleus with one female SAEC nucleus (one green signal, three red signals). (*Inset*) TRITC filter image from hybrid cell. Upper right, single cell-differentiation (GFP$^+$/CD24$^+$, one green signal, one red signal). (Magnifications: *A*, ×63; *B* and *C*, ×100.)

these, about one-quarter were binucleated (Table 1). Therefore the frequency of cell fusion was $\approx 10^{-2}$. Data from experiments with time-lapse microscopy also indicated a high frequency of cell fusion. As demonstrated by fluorescent *in situ* hybridization assays for the X and Y chromosomes, some of the fused cells also underwent nuclear fusion.

The coculture system described here provides an *ex vivo* tool to examine the mechanisms and outcomes of cell differentiation and fusion during tissue repair. Further studies will be required to resolve several of the questions raised by the observations such as which genes from the hMSCs are expressed in the hybrid cells and how frequently cell fusion occurs during tissue repair *in vivo*.

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