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Mesenchymal stem cells protect from hypoxia-induced alveolar epithelial-mesenchymal transition

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Mesenchymal stem cells (MSC) in experimental models of acute lung injury (ALI) and pulmonary fibrosis, suggesting the potential interest of MSC in future cell therapies in clinical lung diseases (2, 14, 25–27, 33–35, 42). Intravenous or intra-alveolar administration of MSC attenuates the severity of lung damage, reduces lung inflammation and fibrosis, and increases survival of rodents after bleomycin, endotoxin-induced lung injury, live bacteria, or ventilator-induced injury. The efficacy of MSC therapy has been mostly attributed to paracrine anti-inflammatory, anti-infectious, or immunomodulatory functions (14, 18, 23, 26, 33–34). Interestingly, recent studies reported that MSC could also exert paracrine or juxtacrine protective effects on alveolar epithelial cells (AEC) by ameliorating cell energetic, reducing apoptosis, or restoring vectorial alveolar sodium transport (12, 19, 25, 34). On the basis of previous studies, we and others identified potential soluble factors that could be responsible for the observed beneficial paracrine effects of MSC, such as keratinocyte growth factor (KGF), IL-1 receptor antagonist (IL-1ra), and prostaglandin E2 (PGE2) (12, 25, 33–34).

The protective effect of MSC (or MSC-conditioned media) on stressed AEC is particularly important because AEC or epithelial-mesenchymal transition (EMT), which lead to inefficient or aberrant alveolar repair and contribute to pulmonary fibrosis. EMT is a cellular process during which epithelial cells transdifferentiate into motile mesenchymal cells while losing their epithelial characteristics (24, 50). Indeed, it

epithelial-mesenchymal transition; alveolar epithelial cells; hypoxia; transforming growth factor-β1; keratinocyte growth factor

Recent studies have reported the beneficial effects of bone marrow-derived multipotent mesenchymal stem (stromal) cell (MSC) in experimental models of acute lung injury (ALI) and pulmonary fibrosis, suggesting the potential interest of MSC in future cell therapies in clinical lung diseases (2, 14, 25–27, 33–35, 42). Intravenous or intra-alveolar administration of MSC attenuates the severity of lung damage, reduces lung inflammation and fibrosis, and increases survival of rodents after bleomycin, endotoxin-induced lung injury, live bacteria, or ventilator-induced injury. The efficacy of MSC therapy has been mostly attributed to paracrine anti-inflammatory, anti-infectious, or immunomodulatory functions (14, 18, 23, 26, 33–34). Interestingly, recent studies reported that MSC could also exert paracrine or juxtacrine protective effects on alveolar epithelial cells (AEC) by ameliorating cell energetic, reducing apoptosis, or restoring vectorial alveolar sodium transport (12, 19, 25, 34). On the basis of previous studies, we and others identified potential soluble factors that could be responsible for the observed beneficial paracrine effects of MSC, such as keratinocyte growth factor (KGF), IL-1 receptor antagonist (IL-1ra), and prostaglandin E2 (PGE2) (12, 25, 33–34).

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has been suggested that exposure to hypoxia, a condition usually encountered in alveoli during ALI or chronic lung injury with lung remodeling (51), could promote phenotypic changes in alveolar epithelial cell lines consistent with EMT (57). Whether MSC therapy would limit or prevent alveolar EMT in case of acute or chronic lung injury is currently unknown.

The objective of the present study was therefore to examine in vitro the effects of human MSC (hMSC) on EMT induced in rat primary AEC by prolonged hypoxic exposure and the mechanisms involved. To achieve this goal, we established a coculture system without cell-cell contact, with AEC grown on semipermeable inserts (upper compartment) and hMSC grown on the bottom of the wells (lower compartment). Results reveal that coculture with hMSC markedly attenuated phenotypic changes evoking EMT in rat AEC monolayers exposed to hypoxia (1.5% O2) for up to 12 days. Indeed, coculture with hMSC completely blunted the increase in expression and secretion of the EMT inducer TGF-β1 in hypoxic AEC, as well as the induction of pro-EMT transcription factors ZEB1 and TWIST1 and of connective tissue growth factor (CTGF).

Finally, our data suggest that secretion of KGF by hMSC could be partly responsible for this protective effect.

**METHODS**

*Isolation and culture of rat alveolar epithelial cells.* The procedure of rat AEC isolation accorded with legislation currently in force in France and animal welfare guidelines, and was approved by University Paris 13 institutional committee and the French Agriculture Department (agreement no. B-93-008-01). Alveolar epithelial cells were isolated from young adult male pathogen-free Sprague-Dawley rats (5 wk old, 125 g) by elastase digestion (Worthington, Lakewood, NJ) as previously described (38). The percentage of AT2 cells as assessed by phosphine 3R staining of lamellar bodies was ≥92% of freshly isolated cells, and cell viability was >95%. Cells were seeded onto Transwell/Snapwell (polycarbonate membrane with a pore size of 0.4 μm, Corning, Corning, NY) filters and cultured for up to 13 days in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM d-glucose, 10 mM HEPEs, 23.8 mM NaHCO3, 2 mM L-glutamine, 5% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml gentamycin, 10 μg/ml amphotericin B, and cis-OH-proline (for the first 2 days) (Sigma-Aldrich) to selectively eliminate fibroblasts from AEC culture (53). Media were changed every 2 or 3 days. Transepithelial resistance (Rte) was measured every 3 days by use of a microvolt-ohmmeter (World Precision Instruments, Astonbury, UK). Phenotypic characterization showed that primary AEC under normoxic conditions retained characteristics of AT2 cells with increasing time in culture, such as expression of surfactant protein A, B, and C mRNA transcripts (Fig. 1B), and expression of the thyroid-transcription factor (TTF-1), which was detected by Western blot and by immunofluorescence until day 13 after isolation (Fig. 2, A and B). Aquaporin-5 (AQP-5) protein, a marker of alveolar type 1 epithelial cells (AT1 cells), was not detected by Western blotting at day 1 after isolation but could be detected at day 3, day 6, and until day 13 (Fig. 1C) as previously reported (53), indicating that, with increasing time in culture, AEC exhibit an intermediate phenotype with both type AT2 and AT1 cell characteristics. Concerning mesenchymal markers, α-smooth-muscle actin (α-SMA) was not detected at any time in culture in normoxic primary rat AEC cultures. Vimentin was exceptionally detected by Western blotting or immunofluorescence studies at day 4 or 6, and when this was the case, the cell culture was discarded because of possible fibroblast contamination. Forty separate AEC isolations and cultures were performed in this study, corresponding to 120 rats.

*Extraction, culture, and conditioning of human mesenchymal stem cells.* Bone marrow samples were harvested from washed filters used during bone marrow graft processing for allogeneic transplantation after healthy donor informed consent according to approved institutional guidelines (Assistance Publique-Hôpitaux de Paris, Paris, France). hMSC were cultured as previously described (1). Briefly, healthy donors bone marrow cells obtained after Ficoll (Invitrogen, Cergy-Pontoise, France) were cultured at an initial density of 5×10^4
Human MSC used in this study were seeded 0.5 cells/cm². Adherent cells were then trypsinized, harvested, and cultured by changed. The media were changed every 2 or 3 days until confluence. After 24 – 48 h, nonadherent cells were removed and the medium was

Western blot analyses were performed by using the vital dye 7-AAD (Beckman Coulter) according to the manufacturer’s instruction. Cells were acquired by flow cytometry (FACSCalibur) and analyzed by CellQuest software (BD Biosciences). The expression of vimentin and α-SMA in hMSC under various experimental conditions was assessed by Western blotting (see Western blot analyses below) with anti-vimentin (1/3,000) (Sigma-Aldrich) and anti-α-SMA (1/5,000) (Sigma-Aldrich).

In some experiments, hMSC-conditioned media (hMSC-CM) were used and prepared as follows. hMSC at 80% confluence were rinsed with PBS before use. hMSC-CM were immediately frozen and stored at −80°C. hMSC-CM were diluted to 5% FBS for 24 h. Cell media were then collected and centrifuged at 13,000 rpm for 20 min. Supernatants representing hMSC-CM were immediately frozen and stored at −80°C. hMSC-CM were diluted with 5% FBS before use. In some experiments, hMSC-CM were depleted in KGF using protein G/agarose beads coated with anti-KGF monoclonal antibody (R&D Systems, Lille, France) or with anti-IgG antibodies (as a control) as previously described (12). KGF concentrations in hMSC-CM were measured using specific Quantikine ELISA kits (R&D Systems, Lille, France) before and after KGF depletion to verify the efficacy of the procedure.

E-Cadherin protein
TTF-1 protein
Vimentin protein
α-SMA protein
β-actin protein

- Actin signal (n = 10 separate experiments). Statistical significance was calculated from the raw data by paired t-test. *Significantly different from normoxic value (P < 0.05 and P < 0.001, respectively). NS, not significant.

Densitometric signals were normalized for the corresponding -actin signal (n = 4–5 separate experiments). Statistical significance was calculated from the raw data by paired t-test. *, ***Significantly different from normoxic value (P < 0.05 and P < 0.001, respectively). NS, not significant.
Cocultures of rat alveolar epithelial cells and human mesenchymal stem cells. A coculture system without cell/cell contact was used to test the paracrine effect of hMSC on AEC phenotype (Fig. 1A). On day 1 after AEC isolation, Transwell inserts seeded with AEC (seeding density: 1.3 × 10^6 cells/cm^2) were transferred into plastic dishes seeded with hMSC (80,000 cells/cm^2) at the bottom of the wells. Cocultures of AEC (upper compartment) and hMSC (lower compartment) were maintained in DMEM supplemented with 5% FBS and antibiotics/antifungal treatment for 6 or 12 days, with culture medium changed every 3 days. For 12-day cocultures, hMSC at the bottom of the wells were replaced after 6 days when confluence was reached by "fresh" hMSC from the same donor (seeding density: 80,000 cells/cm^2).

Exposure of cells to hypoxia and drugs. At day 1 after isolation, AEC cultured alone or in coculture with hMSC were placed in a humidified airtight incubator with inflow and outflow valves and exposed to a hypoxic gas mixture containing 1.5% O_2-5% CO_2-94.5% N_2 (oxygen tension in culture media: 45 mmHg) and kept at 37°C for increasing periods of time (from 2 h to 12 days). Control normoxic cells were maintained in a 21% O_2-5% CO_2-74% N_2 humidified incubator for the same periods of time (oxygen tension in culture media: 140 mmHg). In some experiments, AEC cultured alone were incubated at day 1 after isolation with increasing concentrations of human recombinant KGF (rhKGF, R&D Systems; final concentration: 125, 250, or 500 pg/ml) and exposed to hypoxia for 24 or 48 h. In other experiments, AEC were preincubated for 2 h with SB431542, an inhibitor of TGF-β, type I receptor kinase (Sigma-Aldrich; final concentration: 10 μM) (57), before exposure to hypoxia, and treatment with SB431542 was maintained throughout hypoxic exposure for up to 6 days.

Immunofluorescence stainings. AEC monolayers cultured on Transwell filters were harvested after 6 and 12 days of hypoxic/normoxic exposure. Filters were rinsed twice with ice-cold PBS and fixed with 4% paraformaldehyde for 20 min at 4°C, then rinsed with PBS and permeabilized with 0.1% Triton X-100 for 15 min. After being rinsed with PBS, cells were incubated in PBS with 2% bovine serum albumin (BSA) for 30 min to block nonspecific binding sites. Monolayers were exposed to the primary antibodies for 1 h at room temperature. The following antibodies for detection of epithelial markers were used with respective dilutions: mouse polyclonal anti-TFF-1 (Invitrogen) (1/50), rabbit polyclonal anti-zonula occludens-1 (ZO-1) (Sigma-Aldrich) (1/200), mouse polyclonal anti-E-cadherin (Sigma-Aldrich) (1/300), rabbit polyclonal anti-HIF2α (Novus Biotechnologies) (1/500), anti-SNAIL1 (Santa Cruz) (1/500), anti-hypoxia inducible factor (HIF) 1-α (Novus Biotechnologies) (1/500), anti-SNAIL1 (Cell Signaling) (1/500), anti-ZEB1 (Santa Cruz) (1/500), anti-ZEB2 (Santa Cruz) (1/500), anti-β-actin (Sigma-Aldrich) (1/2,000). The signals were quantified by densitometric analysis using NIH image software, normalized by quantification of the β-actin signal in each lane, and expressed in arbitrary units.

Measurement of active TGF-β1 in cell supernatant. AEC grown on Transwell filters were exposed to normoxia or hypoxia (alone or in coculture with hMSC) for 24 h, as indicated previously. The apical supernatants were collected, spun at 12,000 g for 15 min at 4°C, and stored at −80°C for further processing. The concentrations of active TGF-β1 were determined by using specific Quantikine ELISA kits (R&D Systems) according to the manufacturer’s instructions.

RNA extraction and reverse transcriptase-polymerase chain reaction. Total cellular RNA from AEC cells cultured in normoxic or under hypoxic conditions was extracted by using an RNeasy kit (Qiagen, Courtaboeuf, France) following the manufacturer’s instructions. The eluted RNA was quantified by using a Biospec-Nano (Shimadzu, Noisiel, France) at 260 nm. Single-strand cDNAs were synthesized from 0.5 μg of total RNA with a Maxima first-strand cDNA synthesis kit composed by a mixture of oligo(dT) and random hexamer primers according to the manufacturer’s instructions (Fisher Scientific, Illkirch, France). Resulting cDNA samples were amplified by quantitative polymerase chain reaction (PCR) with Absolute qPCR SybrGreen Rox mix (Fisher Scientific) on StepOne system qPCR (Applied Biosystems, Life Technology). Cycle threshold values were normalized to amplification of the β-actin gene. For each transcript, the expression levels were calculated by the 2^ΔΔCT method, as detailed by the manufacturer. Length and sequence of primers used for quantitative real-time PCR are described in Table 1.

Table 1. Sequence and length of primers used for quantitative real-time PCR

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<tr>
<th>Sequence Name (bp)</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>SNAIL1 (120)</td>
<td>GCTTGGCATCTGCCAGGGATTAC</td>
<td>TGGGACTGCCACTGTGGAT</td>
</tr>
<tr>
<td>SNAIL2 (90)</td>
<td>CATTAGCAAACACACTGCGGAAA</td>
<td>TGGGAAGTGGCTGCTGATA</td>
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<tr>
<td>ZEB1 (90)</td>
<td>CTTAGAAGTGGCTGCTGATA</td>
<td>TGGGAAGTGGCTGCTGATA</td>
</tr>
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<td>TWIST1 (108)</td>
<td>CTAGAAGTGGCTGCTGATA</td>
<td>CTAGAAGTGGCTGCTGATA</td>
</tr>
<tr>
<td>CTGF (100)</td>
<td>CTTAGAAGTGGCTGCTGATA</td>
<td>CTAGAAGTGGCTGCTGATA</td>
</tr>
<tr>
<td>TGFβ1 (146)</td>
<td>CGTTAAGTGGCTGCTGATA</td>
<td>CTAGAAGTGGCTGCTGATA</td>
</tr>
<tr>
<td>β-Actin (74)</td>
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<td>GAGTGGAGATGAGACGCCAG</td>
</tr>
<tr>
<td>SPA (135)</td>
<td>GCTGGAGAGTGGAGAGACAA</td>
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<tr>
<td>SPB (76)</td>
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<tr>
<td>SPC (92)</td>
<td>AGCTGAGAAGGAGAAGGAGAATA</td>
<td>AGCTGAGAAGGAGAAGGAGAATA</td>
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RESULTS

Effect of prolonged hypoxic exposure on rat alveolar epithelial cell phenotype. The effects of hypoxia on AEC phenotype were assessed after 6 and 12 days of exposure by changes in $R_{te}$, cell morphology, and expression of a panel of epithelial or mesenchymal markers. Hypoxia induced a progressive and significant decrease in $R_{te}$ (1,635 ± 395 vs. 328 ± 41 Ω·cm² after 6 days of normoxic or hypoxic conditions, respectively; $P < 0.001$), indicating an alteration of AEC tight junctions under hypoxic conditions. Exposure to hypoxia for 6 days did not modify the expression level of E-cadherin as assessed by immunostaining and by Western blot (Fig. 2, A and B). The expression of the junctional protein ZO-1 was detected by immunostaining and remained peripheral in hypoxic AEC (Fig. 2A). By contrast, hypoxia induced a marked decrease in the nuclear expression of the epithelial transcription factor TTF-1 as assessed by immunostaining (Fig. 2A), as well as a 70% decrease in TTF-1 protein expression by Western blotting (Fig. 2B). Indeed, hypoxic exposure induced a 33% decrease in $\alpha$-SMA protein expression levels by Western blot (Fig. 2B). The mesenchymal markers vimentin and $\alpha$-SMA were detected neither in normoxic nor in hypoxic AEC at day 6 (Fig. 2A).

Exposure to hypoxia for 12 days amplified the changes in AEC phenotype (Fig. 2, C and D). Expression of the epithelial markers ZO-1, E-cadherin, and TTF-1 was hardly detected by immunostaining after 12 days of hypoxia (Fig. 2C), and Western blot analysis confirmed that E-cadherin and TTF-1 protein expression were strikingly reduced in hypoxic AEC (Fig. 2D). By contrast, expression of the mesenchymal marker $\alpha$-SMA was detected in hypoxic AEC (Fig. 2, C and D). Indeed, the protein expression level of vimentin was significantly increased in hypoxic AEC compared with normoxic cells (Fig. 2, C and D). All together, these data indicate that prolonged hypoxic exposure induced a profound change in AEC phenotype consistent with EMT. Of note, the progressive decrease in epithelial markers (TTF-1 and $\alpha$-SMA) was also observed when hypoxic exposure started on day 3 (and not on day 1) after isolation, indicating that the phenotypic changes induced by hypoxia were not simply due to the fact that hypoxic exposure began the first day after isolation when AEC in culture were not stable.

Effect of prolonged hypoxia on hMSC phenotype. We tested whether prolonged exposure to hypoxia or coculture with AEC would modify hMSC phenotype and viability (Fig. 3). Prolonged hypoxic exposure didn’t alter hMSC viability either when cultured alone (95.2 ± 0.5% viable cells in normoxia and hypoxia, respectively) or when cocultured with AEC (95.6 ± 0.2%).

Fig. 3. Effect of hypoxia on human mesenchymal stem cell phenotype. hMSC grown on plastic were exposed for 6 days to normoxia (21% O₂) or hypoxia (1.5% O₂), either alone or in coculture with rat AEC. A: morphological appearance of hMSC by light microscopy (hematoxylin staining; original magnification ×100). B: expression of hMSC surface markers by flow cytometry ($n = 3$ separate experiments). C: representative immunoblots showing the expression of $\alpha$-SMA, vimentin, and β-actin.
vs. 92.8% viable cells in normoxia and hypoxia, respectively). Following 6 days of hypoxic exposure, no significant change was detected in hMSC morphology (Fig. 3A), in the expression of hMSC cell surface markers (i.e., CD29, CD44, CD49a to CD49f, CD73, CD90, and CD105) (Fig. 3B), or in the protein expression of α-SMA and vimentin (Fig. 3C), indicating that hMSC did not transform themselves into activated fibroblasts (myofibroblasts) when submitted to chronic hypoxia.

**Effect of hMSC coculture or of hMSC-CM on phenotypic changes of AEC induced by prolonged hypoxia.** Coculture experiments revealed that $R_{w}$ was significantly higher in AEC monolayers cocultured with hMSC than in AEC monolayers without hMSC after a 6-day hypoxic exposure (Fig. 4A). Immunostaining studies showed that coculture of AEC with hMSC allowed AEC to maintain their epithelial phenotype under hypoxic conditions (6-day exposure) (Fig. 4B): ZO-1 and TTF-1 expression were restored in hypoxic AEC monolayers cocultured with hMSC. Similar observations were made when AEC were incubated with hMSC-conditioned media (hMSC-CM) for 6 days under hypoxic exposure (Fig. 4B). Western blot experiments confirmed that TTF-1 protein expression was restored in hypoxic AEC cocultured with hMSC (Fig. 4C). The effect of hMSC coculture on AEC phenotype was also evidenced after a 12-day hypoxic exposure (Fig. 4, D–F). Transepithelial electric resistance was significantly decreased under hypoxic conditions in AEC monolayers cultured alone but not when cocultured with hMSC (Fig. 4D). By immunostaining, the expression of ZO-1 and TTF-1 was completely abolished in hypoxic AEC cultured alone but was clearly visible in AEC cocultured with hMSC (Fig. 4E). Also, hypoxia-induced decrease in the protein expression of E-cadherin and TTF-1 as assessed by Western blotting was completely prevented by coculture of AEC with hMSC (Fig. 4F). Finally, coculture with hMSC tended to decrease vimentin

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**Fig. 4.** Effect of coculture with hMSC on hypoxia-induced alterations of alveolar epithelial cell phenotype. At day 1 after isolation, rat AEC monolayers on Transwell filters were maintained in normoxia (21% O2) (gray bars) or exposed to hypoxia (1.5% O2) either alone (black bars) or in coculture with hMSC at the bottom of the wells (hatched bars) for 6 or 12 days. In some experiments, AEC were incubated with hMSC conditioned media (hMSC-CM) throughout hypoxic exposure. **A:** transepithelial electric resistance ($R_{w}$) values across AEC monolayers after 6 days of exposure ($n = 13$ filters per condition from 6 independent experiments). **B:** representative immunostainings of E-cadherin (nuclei stained with DAPI), and ZO-1 (green) and TTF-1 (red) in AEC monolayers after a 6-day exposure (original magnification $\times 400$). **C:** representative immunoblots showing the expression TTF-1 and β-actin. Densitometric signal for TTF-1 normalized for the corresponding β-actin signal ($n = 5$ separate experiments). **D:** transepithelial electric resistance values after a 12-day exposure ($n = 15$ filters per condition from 9 independent experiments). **E:** representative immunostainings of ZO-1 (green) and TTF-1 (red) in AEC monolayers after a 12-day exposure (original magnification $\times 400$). **F:** representative immunoblots showing the expression TTF-1, E-cadherin (E-Cadh), vimentin (Vim), and β-actin, and densitometric signals normalized for the corresponding β-actin signal ($n = 4–6$ separate experiments). Statistical significance was calculated from the raw data by 1-way ANOVA. ***,++++++**Significantly different from normoxic value ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively) and #significantly different from hypoxic value ($P < 0.05$). NS, not significant.
protein expression in hypoxic AEC (2.09 ± 0.46 vs. 0.97 ± 0.31 arbitrary units in 1.5% O₂ and 1.5% O₂ + hMSC, respectively; n = 3–5; P = 0.10).

Effect of hMSC on hypoxia-inducible factor expression in alveolar epithelial cells. To decipher the cellular mechanism(s) involved in the beneficial effect of hMSC on AEC exposed to prolonged hypoxia, we first evaluated whether coculture with hMSC did modify the expression of HIF1-α and HIF2-α in hypoxic AEC (44). Stabilization of HIF1-α proteins was previously shown to be necessary in AEC for developing hypoxia-induced EMT (57). As shown by Western blotting (Fig. 5, A and B), HIF1-α protein was hardly detected in normoxic AEC but was significantly induced in AEC exposed to hypoxia for 2 and 3 h. HIF2-α protein expression began to increase at 3 h of hypoxia, but this increase was not significant. Both HIF1-α and HIF2-α protein expression levels were significantly increased in hypoxic AEC after a 24-h exposure but went back to normoxic values afterward. Expression levels of SNAIL1 protein was significantly induced by short hypoxic exposures (2 and 3 h) and rapidly decreased to reach normoxic values afterward. Expression levels of SNAIL2 (SLUG) mRNA transcripts decreased by ~50% during the first hours of hypoxic exposure but went back to normal after 16 h of exposure (not shown). Expression levels of ZEB1 and TWIST1 mRNA transcripts were significantly increased after 24 and 48 h of hypoxic exposure, respectively (Fig. 6, C and D). ZEB1 protein expression was not modified by a 24-h hypoxic exposure (data not shown). We then examined whether coculture of AEC with hMSC could modulate the expression of SNAIL1, ZEB1, and TWIST1. Coculture with hMSC had no effect on hypoxia-induced expression of SNAIL1 protein (Fig. 6B). By contrast, coculture completely prevented the increase...
in ZEB1 and TWIST1 mRNA levels in hypoxic AEC (Fig. 6, C and D).

**Effect of hMSC on profibrotic factor expression in alveolar epithelial cells.** We next studied whether coculture with hMSC could affect the production of two profibrotic proteins in hypoxic AEC known to favor EMT: TGF-β1 and CTGF (48, 53). As shown in Fig. 7A, TGF-β1 mRNA levels increased twofold after 24 h of hypoxia and decreased thereafter (not shown). Also, the concentration of TGF-β1 in the apical medium of hypoxic AEC was strikingly increased compared with normoxic conditions (Fig. 7B). The mRNA and protein expression of CTGF, a target of TGF-β1, was induced as well by a 48-h hypoxic exposure (Fig. 7, C and D). Interestingly, coculture with hMSC completely blunted the increase in TGF-β1 mRNA expression as well as TGF-β1 protein apical secretion induced by hypoxia (Fig. 7A and B). Coculture with hMSC suppressed as well the twofold increase in CTGF mRNA expression induced in AEC by a 48-h hypoxic exposure (Fig. 7C) and blunted the increase in CTGF protein expression (Fig. 7D). To study the role of TGF-β1 autocrine signaling in hypoxia-induced alterations of AEC, hypoxic AEC were incubated with SB431542, an inhibitor of ALK5, a TGF-β1 type I receptor kinase (57). As shown in Fig. 7, E and F, SB431542 completely prevented the hypoxia-induced increase in TWIST1 and CTGF mRNA transcripts observed after 48 h of hypoxic exposure. SB431542 also prevented the decrease in TTF-1 expression as assessed by immunostaining after 6 days of hypoxia (Fig. 7G). Taken together, these data strongly support the importance of the TGF-β1 pathway in hypoxia-induced EMT and indicate that hMSC coculture downregulates the expression of TGF-β1 and its target genes.

**Involvement of keratinocyte growth factor secretion in the paracrine effects of hMSC.** KGF, a well-known growth factor for AEC that is secreted by hMSC (25, 36), has been previously shown to be involved in the beneficial effect of hMSC-CM on transepithelial sodium transport across AEC monolayers exposed to hypoxia and inflammatory cytokines (12). Therefore, we investigated the potential role of KGF in our model. Mean KGF concentration measured by ELISA in hMSC-CM was 274 ± 33.8 pg/ml (n = 13). Treatment of hypoxic AEC with increasing concentrations of rhKGF (125, 250, or 500 pg/ml) showed that 250 and 500 pg/ml completely prevented the increase in TGF-β1 mRNA levels induced by a 24-h hypoxic exposure, whereas 125 pg/ml had no significant effect (Fig. 8A). Recombinant human KGF at the concentration of 250 pg/ml was therefore used in subsequent experiments. Treatment with rhKGF for 48 h fully prevented the increase in TWIST1 and CTGF mRNA transcript levels (Fig. 8, B and C) induced by...
hypoxia, whereas it did not affect the increase in ZEB1 mRNA expression at 24 h (data not shown). Also, treatment with rhKGF throughout a 6-day hypoxic exposure completely prevented the hypoxia-induced decrease in TTF-1 protein expression (Fig. 8D).

Finally, Fig. 8E shows that hMSC-CM completely depleted in KGF by use of anti-KGF antibody failed to prevent the hypoxia-induced increase in TWIST mRNA transcripts at 48 h of hypoxia, whereas complete hMSC-CM or hMSC-CM treated with nonspecific anti-IgG did.

DISCUSSION

The main findings of the present study can be summarized as follows. Prolonged exposure to hypoxia induced phenotypic changes in primary rat AEC consistent with EMT, i.e., a change in cell morphology, a progressive decrease in Rte and in the expression of epithelial markers (ZO-1, E-cadherin, AQP-5, and TTF-1) together with an increase in mesenchymal markers (vimentin and α-SMA). Hypoxia induced the expression of hypoxia-inducible factors HIF1-α/HIF2-α and HIF2-α, as well as the expression of transcription factors driving EMT such as SNAIL1, ZEB1, and TWIST1 with a specific time course. Hypoxic exposure also increased TGF-β1 mRNA expression and the secretion of active TGF-β1 in apical medium, and the expression of CTGF, two profibrotic mediators known to induce EMT. Coculture of AEC with bone marrow-derived hMSC (or incubation with hMSC-CM) partially prevented the decrease in Rte and fully prevented the decrease of ZO-1, E-cadherin, and TTF-1 expression induced by hypoxia. Coculture completely blunted the increase in TGF-β1 expression and in ZEB1, TWIST1, and CTGF mRNA levels, whereas it did not affect HIF1-α, HIF2-α, and SNAIL1 protein expression in hypoxic AEC. Finally, incubation with rhKGF at a concentration similar to what was measured in hMSC-CM fully restored the expression of the epithelial transcription factor TTF-1 in hypoxic AEC and prevented the increase in TWIST1, TGF-β1, and CTGF expression. Taken together, our data indicate that hMSC protect primary AEC from hypoxia-induced EMT through the paracrine modulation of EMT signaling pathways and suggest that this effect is partly mediated by KGF secretion.

One limitation of the present study is the fact that the in vitro model used combined cells originating from two different...
species, i.e., primary rat AEC and hMSC. Bone marrow-derived MSC were studied for obvious reasons inasmuch as these cells will likely be used in the future for cell therapy in patients with pulmonary diseases. Unfortunately, human alveolar epithelial cell lines such as A549 show several pitfalls for the study of EMT in the context of lung fibrosis, for instance a very low Rter, basal expression of mesenchymal markers, a short doubling time, and cancerous characteristics (57). Primary rat AEC are usually considered as good surrogates for human AEC, which are very difficult to isolate. Noteworthy, previous studies have shown that hMSC were effective in vivo in several rodent models of injury (5, 28–29), probably because most paracrine factors secreted by MSC (KGF, IL1-ra, or PGE2) show considerable homology across species. Indeed, it is well recognized that recombinant human proteins (rhKGF and rhIL1-ra) have a biological effect on rodent AEC (11, 36). The fact that we observed a clear beneficial effect of hMSC on rat AEC indicates that our model, despite the species difference, still represents a valuable tool for studying in vitro the cross talk between AEC and MSC.

Prolonged exposure to hypoxia (1.5% O2, corresponding to an O2 tension of ~45 mmHg in culture media) was used in this study to induce EMT in primary AEC because alveolar hypoxia is usually associated with acute or chronic lung injuries leading to pulmonary fibrosis, due to pulmonary edema or to distal lung remodeling (6, 31). Indeed, AT2 cells from patients with IPF or from mouse lungs treated with bleomycin specifically express HIF1-α and HIF1-α related genes (51), and alveolar hypoxia may be particularly important during episodes of acute exacerbation in the course of IPF (22). Hypoxia promotes EMT in a large number of cancerous cells, a phenomenon involved in the development of tumors (56). Local hypoxia has also been shown to induce EMT in noncancerous epithelial cells from various organs, including the kidney, skin, and nasal polyps (16, 17, 46). In renal tubular epithelial cells, hypoxia-induced EMT is partly dependent on the expression of HIF1-α and CTGF and could participate in the development of renal fibrosis (15–17). Whereas the role of AEC abnormal secretory phenotype in the pathophysiology of pulmonary fibrosis is well admitted (22), the role of EMT is more contro-
versial, owing to the difficulty to study EMT in vivo. Mouse studies using genetic tools to follow the fate of specific cell types in bleomycin-induced pulmonary fibrosis gave discordant results. For instance, Tanjore et al. (49) reported that approximately one third of lung fibroblasts in the bleomycin model originated from alveolar epithelium, but Rock et al. (41) found no evidence for EMT. Whatever the importance of EMT in pulmonary fibrosis, our data provide evidence that hypoxia at least induces profound phenotypic changes in AEC, which may impair their ability to repair alveolar damage.

In a recent work using mostly alveolar epithelial cell lines, Zhou et al. (57) reported that prolonged hypoxia could also induce EMT in vitro in lung epithelial cells, and that EMT was related to the expression of HIF1-α and to the accumulation of O2 reactive species (ROS) produced by hypoxic mitochondria, both leading to an increase in TGF-β1 expression and secretion (7, 44, 57). TGF-β1 is a profibrotic factor overexpressed in the lung during IPF and ALI, which is considered as one of the major inducers of EMT in lung fibrosis (6, 10, 20, 21, 53).

TGF-β1 has complex biological effects, through both canonical and noncanonical cellular pathways, that can induce SMAD, AKT, ERK, and MAPK signaling pathways leading to EMT (24). Here, in hypoxic rat primary AEC, we observed an increase in HIF1-α and in TGF-β1 expression and secretion in apical medium, consistent with previous findings of Zhou et al. The concentration of active TGF-β1 in apical supernatant of AEC was relatively low, around 50 pg/ml. However, Zhou et al. demonstrated that such concentrations of active TGF-β1 secreted by AEC under hypoxic conditions were able to induce EMT via an autocrine way insomuch as blockade of TGF-β1 type I receptor kinase by SB431542 prevented the hypoxia-induced EMT in these cells (57). Indeed, in the present study, incubation of hypoxic AEC with SB431542 suppressed the hypoxia-induced increase in mRNA transcripts encoding TWIST1 and CTGF and prevented the decrease in TTF-1 protein expression, supporting the major role of TGF-β1 in our model.

In our experiments, the expression of HIF1-α and of HIF2-α proteins was transient (disappearing after 48 h), an observation previously reported for HIF1-α in A549 cells (52). Hypoxia also induced the expression of transcription factors classically involved in EMT such as SNAIL1 first, ZEB1, and TWIST1 with a specific time course (24). Although snail is a target gene for HIF (30), the rapid increase in SNAIL1 protein observed after only 2 h without any change in SNAIL1 mRNA levels suggests a posttranslational regulation of SNAIL1 protein under hypoxic conditions. ZEB1 mRNA upregulation observed at 24 h of hypoxia is consistent with SNAIL1 directly targeting the ZEB1 gene, although ZEB1 expression can also be induced by TGF-β1 (24). However, we could not evidence any increase in ZEB1 protein expression under hypoxia, suggesting either that ZEB1 mRNA upregulation was not followed by translation of the protein or that ZEB1 protein was rapidly degraded under hypoxia. ZEB1 is a short-lived protein (half-life is less than 1 h), expressed at very low levels in epithelial cells and targeted by the ubiquitin proteasome system, which makes it difficult to study (24). The increase in TWIST1 mRNA transcripts at 48 h of hypoxia could be due either to a direct transcriptional effect of HIF1-α or to an indirect upregulation by TGF-β1 (8, 55). Finally, hypoxia induced the upregulation (at both mRNA and protein levels) of CTGF, a target of HIF1-α and TGF-β1, previously shown to favor EMT (3, 16, 45, 48).

The most important finding of our study is that hMSC could prevent hypoxia-induced phenotypic changes evoking EMT in AEC, at least in vitro. This protective effect was reproduced by hMSC-CM, consistent with a paracrine mechanism. Importantly, prolonged exposure to hypoxia did not alter the phenotype of hMSC, which is consistent with the fact that hMSC are physiologically exposed in the bone marrow to low O2 concentrations, ranging from 1 to 7% O2 (9). The fact that when submitted to hypoxia hMSC did not transform themselves into fibroblasts or myofibroblasts (at least in vitro) is of course a prerequisite for the future therapeutic use of these cells in the context of ALI or lung fibrosis. Coculture with hMSC completely blunted the upregulation of some transcription factors driving EMT such as ZEB1 and TWIST1. It also suppressed the induction of TGF-β1 mRNAs as well as the apical secretion of active TGF-β1 induced by hypoxia and blunted the upregulation of CTGF. The decrease in TGF-β1 secretion induced by hMSC is certainly a crucial point since autocrine activation of hypoxic AEC by TGF-β1 can lead to the expression of EMT-driving transcription factors as well as and of the EMT inducer CTGF (3, 8, 24).

Several ex vivo and in vitro studies from our group and other groups have evidenced the involvement of KGF in the beneficial effects of hMSC on injured AEC, namely on transepithelial alveolar sodium transport and alveolar fluid clearance (12, 25, 27). Also, the protective effect of KGF on AEC has been widely demonstrated in vitro and in several models of lung injury in rodents and more recently in the human lung (13, 39, 40, 43, 47). Consistent with these observations, the present study shows that incubation of hypoxic AEC with low concentrations of rhKGF reproduced the effects of coculture with hMSC on TGF-β1, TWIST1, and CTGF. Furthermore, incubation of AEC with hMSC-CM depleted in KGF failed to prevent the hypoxia-induced increase in TWIST1 mRNA transcript, whereas complete hMSC-CM containing KGF did. Interestingly, Pereira et al. (37) previously reported that gene transfer of KGF in the skin reduced the expression of TGF-β and improved wound healing through a mechanism still unknown. Here, we can speculate that KGF, via the activation of Nr2 transcription factor, could upregulate antioxidant responses and reduce ROS accumulation in hypoxic AEC, which in turn could decrease TGF-β1 expression (4, 32, 57). Finally, other paracrine factors distinct from KGF may also contribute to the beneficial effect of hMSC inasmuch as, unlike coculture with hMSC, incubation with rhKGF was unable to suppress the increase in ZEB1 mRNA transcripts observed in hypoxic AEC.

In conclusion, the present study shows that hMSC prevent alveolar EMT induced by hypoxia in vitro by downregulating the expression of TGF-β1 and CTGF and the activation of transcription factors driving EMT, and that this beneficial effect could be due at least in part to the release of KGF. Our study therefore identifies a novel mechanism of action of hMSC on AEC that could contribute to the beneficial effects of hMSC administration evidenced in animal models of acute or chronic lung injuries. This protective role of hMSC may be of particular importance in the perspective of cell therapies using hMSC in patients because alveolar hypoxia is frequently encountered during ALI or during acute exacerbation of IPF, and
because alterations of AEC phenotype might compromise alveolar repair and promote lung fibrosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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