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Hypoxia-Inducible Factor 1α Signaling Promotes Repair of the Alveolar Epithelium after Acute Lung Injury

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During the acute respiratory distress syndrome, epithelial cells, primarily alveolar type (AT) I cells, die and slough off, resulting in enhanced permeability. ATII cells proliferate and spread onto the denuded basement membrane to reseal the barrier. Repair of the alveolar epithelium is critical for clinical recovery; however, mechanisms underlying ATII cell proliferation and spreading are not well understood. We hypothesized that hypoxia-inducible factor (HIF)1α promotes proliferation and spreading of ATII cells during repair after lung injury. Mice were treated with lipopolysaccharide or hydrochloric acid. HIF activation in ATII cells after injury was demonstrated by increased luciferase activity in oxygen degradation domain—Luc (HIF reporter) mice and expression of the HIF1α target gene *GLUT1*. ATII cell proliferation during repair was attenuated in ATII cell—specific HIF1α knockout (*SftpcCreERT2*$^{+/-}$*;HIF1α*$^{f/f}$) mice. The HIF target vascular endothelial growth factor promoted ATII cell proliferation *in vitro* and after lung injury *in vivo*. In the scratch wound assay of cell spreading, HIF stabilization accelerated, whereas HIF1α shRNA delayed wound closure. *SDF1* and its receptor, *CXCR4*, were found to be HIF1α-regulated genes in ATII cells and were up-regulated during lung injury. Stromal cell-derived factor 1/CXCR4 inhibition impaired cell spreading and delayed the resolution of permeability after lung injury. We conclude that HIF1α is activated in ATII cells after lung injury and promotes proliferation and spreading during repair. *(Am J Pathol 2017, 187: 1772—1786; http://dx.doi.org/10.1016/j.ajpath.2017.04.012)*

The alveolar epithelium is comprised of cuboidal alveolar type (AT)II cells and squamous ATI cells. ATI cells play a critical role in barrier function because they cover 98% of the alveolar surface and in gas exchange by virtue of their thin morphologic structure. In the acute respiratory distress syndrome (ARDS), alveolar epithelial cells, primarily ATI cells, die and slough off, leaving a denuded basement membrane and resulting in increased permeability, which in turn leads to the influx of edema fluid and refractory hypoxemia.[1,2] The extent of epithelial injury determines the severity of ARDS,[3,4] and epithelial repair is critical for the resolution of edema and survival.[5,6] ATII cells, which are relatively resistant to injury, reepithelialize the denuded alveolar surface by proliferating to replace lost cells and then spreading into cells that are morphologically intermediate between ATII and ATI cells and ultimately into squamous ATI cells (transdifferentiation).[7–10] Although it has long been recognized

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that reepithelialization after lung injury depends on ATII cell proliferation and spreading, the molecular signaling pathways that promote these processes are not well understood. Hence, no specific therapies are currently available to enhance epithelial repair in ARDS, and management is mainly limited to supportive care.[2]

Here, we examined a potential role for hypoxia-inducible factor (HIF) in these processes. HIF signaling is known to be activated under inflammatory conditions.[11] HIF promotes cell proliferation and motility during tumorigenesis and embryogenesis.[12,13] In addition, HIF is strongly implicated in keratinocyte migration during wound repair,[14,15] barrier protection in the gut,[16,17] and tissue preservation after ischemic injury in multiple organs.[18,19] HIF signaling is involved in alveolarization,[20,21] can attenuate lung injury under some[22] but not all[23] circumstances, and has recently been suggested to be involved in ATII cell proliferation during the pathogenesis of pulmonary fibrosis.[24] Vascular endothelial growth factor (VEGF) is an established HIF target gene that mediates the role of HIF in alveologenesis, angiogenesis, and tumorigenesis.[13,21,25]

The chemokine receptor CXCR4 and its ligand stromal cell-derived factor (SDF)1 (CXCL12) are known for their role in leukocyte migration, including homing to hematopoietic organs and recruitment to inflammatory foci.[26,27] SDF1/CXCR4 signaling also induces cancer cell migration during metastasis[28] and intestinal epithelial spreading and migration during barrier restitution.[29] In addition, SDF1/CXCR4 signaling can be activated during tissue injury, including in the lung, where it induces neutrophil and fibrocyte recruitment.[30,31] *CXCR4*[32] and *SDF1*[33] are both *bona fide* HIF1 target genes that partially mediate the role of HIF1 in cell motility.

On the basis of the potential roles for HIF signaling in cell proliferation and motility, as well as known activation of HIF in inflammatory foci, we hypothesized that HIF signaling may be activated and may promote ATII cell proliferation and spreading during repair after inflammatory injury in ARDS. We further hypothesized that VEGF and SDF1/CXCR4 signaling may mediate the role of HIF in ATII cell proliferation and spreading, respectively, during epithelial repair.

Materials and Methods

Human Tissue

Paraffin-embedded lung tissue from autopsy specimens of de-identified patients with diffuse alveolar damage and noninjured control lungs rejected for lung transplantation were obtained from the archives of University of Colorado Denver Department of Pathology. This tissue was deemed exempt from the requirement for informed consent by the Colorado Multiple Institutions Review Board.

Animal Studies

All animal protocols were approved by the Animal Care and Use Committee at National Jewish Health. Mice and rats were maintained in a pathogen-free environment on a 12-hour light/dark cycle with full access to food and water. *SftpcCreERT2* mice[34] were crossed to *HIF1α*$^{f/f}$ or *Rosa26-mTmG* (abbreviated mTmG) mice (The Jackson Laboratories, Bar Harbor, ME). Genotyping was performed by nonquantitative PCR on gDNA isolated from tail clips using the primer sequences listed in Table 1. *SftpcCreERT2*$^{+/-}$;*HIF1α*$^{f/f}$ or *SftpcCreERT2*$^{+/-}$;*mTmG*$^{+/-}$ mice were administered tamoxifen, starting at 4 weeks of age. Mice were fed tamoxifen citrate 400 mg/kg chow (Harlan, Indianapolis, IN) for 2 weeks or treated with tamoxifen 20 mg/mL in corn oil at a dose of 0.25 mg/g of body weight i.p. every other day for three doses. Tamoxifen administration was followed by a washout period of 4 weeks. Lungs of naive *SftpcCreERT2*$^{+/-}$;*HIF1α*$^{f/f}$ mice or littermate controls lacking one of the transgenes were digested as previously described.[35] Briefly, after euthanasia, the chest was opened, and lungs were perfused with 10 mL of phosphate-buffered saline through the right ventricle. Lungs were instilled with 3 mL of dispase (Corning, Corning, NY), followed immediately by 0.5 mL of low melting point agarose. Ice was placed on the lungs for 2 minutes. Lungs were removed and incubated in 1 mL of dispase at 37°C for 7 minutes. Five milliliter Dulbecco's modified Eagle's medium (DMEM) and 120 U/mL DNase were added, and lungs were minced for 8 seconds on a gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbac, Germany). Cell suspension was filtered through 100-, 40-, and 20-μm strainers. Cells were

Table 1 Primer Sequences

Gene name	Forward	Reverse
Cre	5′-ATGTCCAATTTACTGACCG-3′	5′-CGCGCCTGAAGATATAGAAG-3′
Hif1α Flox	5′-GCAGTTAAGAGCACTAGTTG-3′	5′-GGAGCTATCTCTCTAGACC-3′
m*Cxcr4*	5′-TCCAGACCCCACTTCTTCAG-3′	5′-AGTGACCCTCTGAGGCGTTT-3′
m*Glut1*	5′-GAGACCAAAGCGTGGTGAGT-3′	5′-GCAGTTCGGCTATAACACTGG-3′
m*Vegfa*	5′-TGCGGATCAAACCTCACCAA-3′	5′-GGTCTGCATTCACATCTGCTG-3′
r*Vegfa*	5′-CTGGACCCTGGCTTTACTGC-3′	5′-ACTTCACCACTTCATGGGCTT-3′
r*Sdf1*	5′-AGCCTTAAACAAGAGGCTCAA-3′	5′-GCAGAGGAAGTGGCTATGGG-3′
r*Glut1*	5′-CGGGCATCAATGCTGTGTTC-3′	5′-GATGGTGGCATACACAGGCT-3′
r*Cxcr4*	5′-GCCATGGAAATATACACTTCGGA-3′	5′-TTTTCATCCCGGAAGCAGGG-3′

stained with anti−epithelial cell adhesion molecule (EpCAM)-647 (catalog number 118212; BioLegend, San Diego, CA) and anti−CD45-BV510 (catalog number 103138; BioLegend). The EpCAM$^+$, CD45$^-$ population was purified using a MoFlo (Beckman Coulter, Brea, CA) cell sorter, which yields approximately 90% ATII cells, as determined by immunofluorescent staining of cytospins for prosurfactant protein C (proSPC; catalog number AB3786; Millipore, Billerica, MA) (data not shown). gDNA was isolated from ATII cells, and PCR was performed by GeneTyper Mouse Genotyping Services (New York, NY) using primers that flank exon 2. Deletion of exon 2 results in a 300-bp product; intact exon 2 results in a predicted 820-bp product, which is often too large to amplify. *SftpcCreERT2*$^{+/-}$*;mTmG*$^{+/-}$, C57BL/6 (Charles River Laboratories/NCI, Wilmington, MA), *FVB.129S6-Gt(Rosa)26Sor*$^{tm2(HIF1A/luc)Kael}$ [oxygen degradation domain (ODD)-Luc; The Jackson Laboratories], or female *SftpcCreERT2*$^{+/-}$*; HIF1α*$^{f/f}$ mice (or littermate controls lacking one of the transgenes), aged 8 to 12 weeks, were treated with 2 μg/g body weight lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4; List Biological Laboratories, Campbell, CA) or 2 μL/g body weight hydrochloric acid (HCl) 0.075N, pH 1.18, in 50 μL of saline by i.t. instillation and euthanized at various time points. In indicated experiments, C57BL/6 mice were administered AMD3100 (catalog number 5602; Sigma-Aldrich, St. Louis, MO) 200 μg or SU5416 (catalog number 13342; Cayman Chemical, Ann Arbor, MI) 20 mg/kg of body weight in 500 μL of phosphate-buffered saline by i.p. injection daily, beginning at day 3 after i.t. LPS. In indicated experiments, mice were treated with 1 mg of bromodeoxyuridine (BrdU; catalog number 557892; Becton Dickinson, Franklin Lakes, NJ) or 0.5 mg of 5-ethynyl-2′-deoxyuridine (catalog number C10340; Thermo Scientific, Waltham, MA) in 100 to 200 μL of phosphate-buffered saline i.p. 24 hours before euthanasia. Bronchoalveolar lavage (BAL) was performed using three 0.8-mL lavages. Cell counts and albumin enzyme-linked immunosorbent assay (Bethyl Laboratories, Montgomery, TX) were performed on BAL fluid. In indicated experiments, 400 μL of BAL fluid was ultracentrifuged at 100,000 × *g* for 2 hours, the pellet was lyzed in 25 mmol/L Tris, 10% glycerol, 1% IGEPAL, pH 7.5, supplemented with Complete Mini Protease Inhibitor Cocktail (Roche, Indianapolis, IN), and subjected to Western blot analysis for T1α (catalog number 11936; Abcam, Cambridge, UK). Lungs were inflation-fixed at 25 cm of H_20 pressure with 10% phosphate-buffered formalin, embedded in paraffin, and cut into 4-μm sections, or lungs were digested as described above. In indicated experiments, lungs were frozen in liquid nitrogen, and RNA was isolated using the mirVana Isolation Kit (Invitrogen, Carlsbad, CA) or luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI) on a Synergy Mx microplate reader (BioTek, Winooski, VT).

Immunostaining

Lung sections were deparaffinized in xylene, hydrated with graded ethanol solutions, and equilibrated to water. When indicated, hematoxylin and eosin staining was performed. For immunostaining, antigen retrieval was performed by boiling slides in Target Retrieval Solution (Dako, Santa Clara, CA) for 30 to 50 minutes. Sections were then blocked in 5% goat serum in Tris-buffered saline with 0.05% Tween, followed by incubation with anti−glucose transporter 1 (GLUT1; catalog number 40084; Abcam), anti-SDF1 (catalog number MAB350; R&D Systems, Minneapolis, MN), anti-VEGFA (catalog number 52917; Abcam), and/or anti−proSPC (catalog number AB3786; Millipore, Billerica, MA) antibody, and then Alexa 488- or 555- or 647-conjugated anti-mouse or anti-rabbit secondary antibodies (Invitrogen). Slides were mounted with Vectashield HardSet mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Digital images were acquired using a Zeiss Axiovert 200 microscope. Costaining of GLUT1 and proSPC was determined by Pearson's Correlation Coefficient[36] using ZEN 2012 colocalization software (Zeiss, Jena, Germany). Total fluorescence intensity of SDF1 or VEGF per high power field was quantified by ImageJ software version 1.48 (NIH, Bethesda, MD; *http://imagej.nih.gov/ij*).

Flow Cytometry of Lung Cells

After lung digest as described above, cells were stained with anti−EpCAM-647 (catalog number 118212; BioLegend) or −fluorescein isothiocyanate (catalog number 118207; BioLegend), anti−CD45-BV510 (catalog number 103138; BioLegend), −peridinin-chlorophyll protein and cyanine 5.5 (catalog number 45-0451; eBioscience, San Diego, CA), or -A700 (catalog number 56-0451-82; eBioscience), anti−BrdU-647 (catalog number 557892; Becton Dickinson), anti−5-ethynyl-2′-deoxyuridine-647 (catalog number C10340; Thermo Scientific), anti−Ki-67-660 (catalog number 50-5698; eBioscience) or −fluorescein isothiocyanate (catalog number 11-5698; eBioscience), and/or unconjugated anti-GLUT1 (catalog number 40084; Abcam) or anti-proSPC (catalog number AB3786; Millipore), followed by anti-mouse-647 secondary (Invitrogen) antibodies and analyzed on the LSRFortessa X-20 cytometer (BD Biosciences, San Jose, CA).

Cell Culture

MLE-12 cells (ATCC, Manassas, VA) were grown in DMEM (Invitrogen) containing 44 mmol/L $NaHCO_3$, 1 mmol/L sodium pyruvate, 4 mmol/L L-alanyl-glutamine, 90 μg/mL streptomycin, 40 μg/mL penicillin, and 10% fetal bovine serum (FBS; Hyclone, San Angelo, TX) or DMEM:F12 containing 0.005 mg/mL insulin, 0.01 mg/mL transferrin, 30 nmol/L sodium selenite, 10 nmol/L hydrocortisone, 10 nmol/L β-estradiol, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 90 μg/mL streptomycin, 40 μg/mL penicillin, and 2% FBS. ATII cells were isolated from male Sprague-Dawley rats weighing 175 to 199 g (Charles River Laboratories) as previously described.[37] Freshly isolated or frozen cells were plated on tissue culture plastic in DMEM with 10% FBS or on Millicell

transwell inserts (Millipore) coated with 20% Matrigel (BD Biosciences) and 80% rat tail collagen in DMEM with 5% rat serum (Pel-Freez Biologicals, Rogers, AR) and 10 ng/mL keratinocyte growth factor (Amgen, Thousand Oaks, CA) in air—liquid interface. Media were changed on day 1 and then every other day thereafter.

In Vitro Proliferation

MLE-12 cells (0.4×10^6 per well) were plated in a 24-well plate. The next day, cells were treated with 5 μg/mL mitomycin for 24 hours, then trypsinized and counted. Rat ATII cells were grown to confluence, subjected to scratch wounding, and then cultured in 1% charcoal stripped FBS with 1 μmol/L BrdU. In indicated experiments, rat ATII cells (1×10^6 per well) were plated in a 12-well plate and cultured in 1% charcoal stripped FBS with 1 μmol/L BrdU in the presence or absence of 10 ng/mL recombinant (r) human keratinocyte growth factor (Amgen) or 20 ng/mL recombinant murine (rm) VEGF (PeproTech); alternatively, rat ATII cells were cultured in 10% FBS with 1 μmol/L BrdU in the presence or absence of SU5416 or dimethyl sulfoxide as a control. After 24 hours, cells were trypsinized, counted, stained with anti-BrdU antibody (catalog number 557892; Becton Dickinson), and analyzed on an LSR II flow cytometer (BD Biosciences).

Transfection

MLE-12 cells were grown to 70% to 80% confluence and transfected with a hypoxia response element (HRE) Firefly luciferase reporter plasmid[38] and a Renilla luciferase reporter plasmid (Promega) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 0.4×10^6 cells were cultured per well in a 12-well plate. The next day, 1.6 μg of HRE plasmid and 100 ng of Renilla plasmid with 4 μL of Lipofectamine was incubated for 20 minutes and then added to each well and incubated for 18 hours. Cells were lyzed in Passive Lysis Buffer (Promega), and Renilla and Firefly luciferase activity were determined using the Luciferase Assay System (Promega) on a Synergy Mx microplate reader (BioTek).

Lentiviral Transduction

MLE-12 cells were grown to 30% confluence in a 24-well tissue culture plate, then incubated with 400 μL of lentivirus containing shRNA to HIF1α (catalog number 0000232223; TRC, Cambridge, MA) or a nonsilencing shRNA (SHC216) (Functional Genomics Facility, University of Colorado, Boulder, CA) and 8 μg/mL polybrene for 16 hours. At 48 hours after transduction, cells were cultured in media with puromycin 4 μg/mL for at least 72 hours.

Adenoviral Transduction

Freshly isolated rat ATII cells were transduced with adenovirus expressing green fluorescent protein (GFP; Ad*GFP*), LacZ (Ad*LacZ*), or a constitutively active mutant *HIF1α* construct (Ad*HIF1α*[39]) at multiplicity of infection 20 in DMEM with 2% FBS at 37°C with gentle rocking for 3 hours and then plated on 80% rat tail collagen/20% Matrigel in DMEM with 5% rat serum. RNA was harvested 24 hours later.

qPCR/Microarray

Cells were lyzed in Buffer RLT (Qiagen, Hilden, Germany), and RNA was purified using the RNeasy Mini QIAcube Kit on a QIAcube (Qiagen) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using the QuantiTect Kit (Qiagen) according to the manufacturer's instructions. cDNA was analyzed by real-time quantitative PCR (qPCR) using primers for *GLUT1*, *VEGF*, *SDF1*, and *CXCR4* (Table 1). qPCR was performed for 40 cycles on the CFX96 (Bio-Rad, Hercules, CA) using iQ SYBR Green Supermix (Bio-Rad). Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.[40] In indicated experiments, cDNA was subjected to a GeneChip Rat Gene 2.0 ST array (Affymetrix, Santa Clara, CA).

Immunoblotting

Cells were lysed in 25 mmol/L Tris, 10% glycerol, 1% IGEPAL, pH 7.5, supplemented with Complete Mini Protease Inhibitor Cocktail (Roche). Nuclear extracts were prepared using the NE-PER Nuclear Protein Extraction Kit (Pierce, Waltham, MA) according to the manufacturer's instructions. Whole cell lysates or nuclear extracts were analyzed by SDS-PAGE and immunoblotting for HIF1α (catalog number 100-134; Novus Biologicals, Littleton, CO), CXCR4 (catalog number 13854; Abcam), TATABox Binding Protein (catalog number 818; Abcam), or actin (catalog number 3280; Abcam). Densitometry was performed using ImageJ software (NIH).

Scratch Wounding

Rat ATII cells or MLE-12 cells ± HIF1α shRNA were grown to confluence on tissue culture plastic and subjected to scratch wounding with a pipette tip. Immediately after the wound was made, cells were treated with 0.5 to 1 mmol/L dimethyloxalylglycine (DMOG; Cayman Chemical) or 500 ng/mL rmSDF1 (PeproTech) or 1 μg/mL mitomycin or 0.1 μg/mL cytochalasin D or 5 μg/mL AMD3100, or 100 to 200 μmol/L blebbistatin (catalog number 203389; Millipore) or 50 μmol/L NSC23766 (Tocris Bioscience, Bristol, UK) or dimethyl sulfoxide as a control in DMEM with 1% to 10% FBS. Images were obtained on an Olympus (Tokyo, Japan) DC70 microscope immediately after the wound was made and 24 hours later. Wound width was calculated using ImageJ software (NIH), and wound repair was expressed as the percentage of initial wound remaining at 24 hours. In indicated experiments, images were also captured >1 mm away from the wound.

Hypoxia/DMOG Exposure

Cells were exposed to hypoxia (1% O_2, 5% CO_2, 94% N_2) or normoxia (21% O_2, 5% CO_2, 74% N_2) in a Modular Incubator Chamber (Billups-Rothenberg, Inc., San Diego, CA) or treated with DMOG 0.5 to 1 mmol/L for 16 to 48 hours.

Phalloidin Staining

Cultured cells were fixed in 3% paraformaldehyde, permeabilized in 0.2% Triton X-100, blocked in 1% bovine serum albumin, and stained with phalloidin-Alexa 594 (Invitrogen) for 25 minutes.

Statistical Analysis

Data are expressed as means ± SEM. Statistical analysis was performed by paired or unpaired *t*-test. Multiple comparisons were performed by one-way analysis of variance with post hoc Bonferroni's multiple comparison test. $P < 0.05$ was considered significant. GraphPad Prism software version 5.04 (GraphPad Inc., San Diego, CA) was used for all statistical calculations. *In vivo* data were analyzed from ≥2 independent experiments with ≥5 mice/group. *In vitro* data were analyzed from ≥3 independent experiments each performed in duplicate or triplicate.

Results

HIF Is Activated in ATII Cells in Lung Injury

LPS and HCl, models of infection and aspiration, respectively, both caused injury to the alveolar epithelium, including death and sloughing of ATI cells, as measured by detection of the ATI cell marker T1α in the BAL sediment (Supplemental Figure S1A), which has previously been shown to correlate with loss of ATI cells by electron microscopy.[41] This injury was accompanied by epithelial permeability and inflammation, as measured by increased BAL albumin and inflammatory cell accumulation, respectively (Figure 1A and Supplemental Figure S1B). Injury peaked at day 3.

To determine whether HIF signaling was activated in mouse models of lung injury, we used a bioluminescent HIF1α reporter mouse, ODD-Luc. These mice expressed the ODD of *HIF1α* fused to firefly luciferase. Because hydroxylation of the ODD targeted HIF1α or the luciferase fusion protein for ubiquitination and proteosomal degradation, detectable luciferase activity reflected HIF1α stabilization.[42] In the naive lung, some luciferase activity was detectable (Figure 1B), likely reflecting the presence of low levels of HIF1α, which is known to be constitutively synthesized and rapidly degraded under normoxic conditions.[12] However, luciferase activity in the whole lung homogenate markedly increased after lung injury induced by LPS or HCl (Figure 1B and Supplemental Figure S2A). To confirm that HIF signaling was activated in ATII cells after lung injury, we assessed ATII cell expression of *GLUT1*, a prototypical HIF1 target gene.[43] *SftpcCreERT2*$^{+/-}$;*mTmG*$^{+/-}$ mice, in which ATII cells are induced by tamoxifen to express GFP, were treated with LPS or saline as a control. We confirmed that the GFP^{+} cells were indeed ATII cells, as demonstrated by expression of the ATII cell marker proSPC (Supplemental Figure S2B). Moreover, GLUT1 expression by ATII cells was up-regulated after lung injury induced by LPS (Figure 1C and Supplemental Figure S2C). Increased expression of GLUT1 in ATII cells was confirmed by costaining lung sections from LPS- and HCl-treated mice for GLUT1 and proSPC (Supplemental Figure S2D). Finally, we detected increased levels of VEGF, another established HIF1 target gene,[13] in the lung after injury (Figure 1D and Supplemental Figure S2E).

HIF Promotes Epithelial Repair

A critical stage of repair after epithelial injury in human ARDS patients was ATII cell proliferation[7,8] (Supplemental Figure S3).

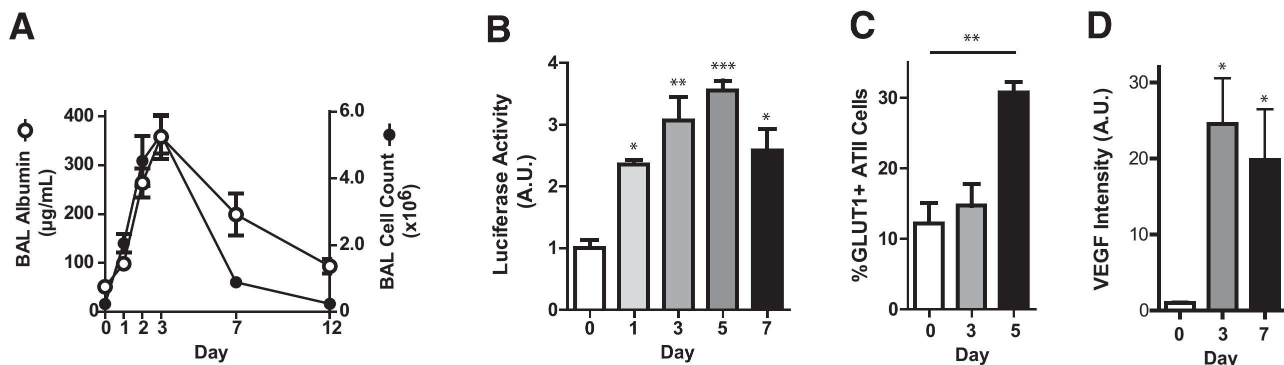

Figure 1 HIF signaling is activated in mouse models of lung injury. **A–D:** C57BL/6 (**A** and **D**) or ODD-Luc (**B**) or *SftpcCreERT2*$^{+/-}$;*mTmG*$^{+/-}$ (**C**) mice were treated with i.t. LPS and euthanized at the indicated time points. **A:** Cell counts and albumin were measured in the bronchoalveolar lavage (BAL). **B:** Luciferase activity was measured in whole lung homogenates. **C:** Lung digests of *SftpcCreERT2*$^{+/-}$;*mTmG*$^{+/-}$ mice were stained for GLUT1 and analyzed by flow cytometry. GLUT1 expression in the GFP^{+} (ATII cell) population is shown. **D:** Lung sections were stained for VEGF and intensity of staining was quantified by ImageJ software version 1.48 (NIH, Bethesda, MD; *http://imagej.nih.gov/ij*) analysis. Data are expressed as means ± SEM (**B–D**). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. ATII, alveolar type II; A.U., arbitrary unit; BAL, bronchoalveolar lavage; GFP, green fluorescent protein; GLUT1, glucose transporter 1; HIF, hypoxia-inducible factor; LPS, lipopolysaccharide; ODD, oxygen degradation domain; VEGF, vascular endothelial growth factor.

To determine the role of HIF1 in ATII cell proliferation after lung injury in animal models, we generated ATII cell−specific, inducible *HIF1α* knockout mice by crossing *SftpcCreERT2* mice to $HIF1\alpha^{f/f}$ mice. Deletion of the *HIF1α* gene from ATII cells was confirmed by PCR using primers designed to detect the null allele performed on genomic DNA extracted from freshly isolated ATII cells (Supplemental Figure S4A). At baseline, $SftpcCreERT2^{+/-}$;$HIF1\alpha^{f/f}$ mice treated with tamoxifen displayed no abnormalities in alveolar structure or barrier integrity and no inflammation (Supplemental Figure S4, B and C). Moreover, no significant difference was found in the initial injury induced by LPS or HCl, as assessed by inflammation and permeability on day 3 (Supplemental Figure S4, D and E), which was the peak of injury (Figure 1A and Supplemental Figure S1B). To assess the role of HIF1α in ATII cell proliferation during repair after lung injury, we first determined the time course of ATII cell proliferation after LPS (Figure 2A and Supplemental Figure S5) and found that ATII cell proliferation was temporally correlated with HIF activation (Figure 1). Importantly, in the ATII cell−specific *HIF1α* knockout (KO) ($SftpcCreERT2^{+/-}$;$HIF1\alpha^{f/f}$) animals, there was attenuated proliferation at the peak of the proliferative response on day 5 (Figure 2B). (After HCl-induced injury, the KO mice had a 38% mortality rate, compared with a 17% mortality rate in the wild-type animals, with animal death occurring between day 4 and day 7. This was thought to be too high to be conducive to further study.) Because HIF is a transcription factor, we questioned whether the prototypical HIF target gene VEGF may be a potential mediator of the role of HIF1α in ATII cell proliferation. Accordingly, freshly isolated rat ATII cells were cultured in the presence of rVEGF or SU5416, a VEGF receptor antagonist. Entry of cells into S phase of the cell cycle and cell division were determined by BrdU uptake and total cell number, respectively. rVEGF enhanced (Figure 2C), whereas SU5416 prevented (Figure 2D) ATII cell proliferation. Notably, the cells treated with SU5416 were deemed viable by trypan blue staining (data not shown) and appeared healthy (Supplemental Figure S6). To confirm that VEGF was critical for ATII cell proliferation during repair after lung injury, LPS-treated mice were administered SU5416 daily, beginning at day 3 after LPS, after injury was established. VEGF receptor blockade attenuated ATII cell proliferation during repair (Figure 2E).

To determine whether HIF1α also promoted ATII cell spreading during epithelial repair, we used the *in vitro* scratch wound assay, which has been previously suggested to be an assay of cell spreading.[44,45] We confirmed that scratch wounds in cultured primary ATII cells and MLE-12 cells, a cell line that has properties of ATII cells such as SPC expression,[46] closed by cell spreading/migration and did not require cell replication. Mitomycin blocked proliferation (Figure 3A) but did not delay wound closure (Figure 3B) in a wounded monolayer of MLE-12 cells. In contrast, treatment with cytochalasin D, which inhibited actin polymerization and cytoskeletal reorganization, prevented wound closure (Figure 3C). Primary ATII cells proliferated minimally under the low serum conditions in which they are cultured during the 24-hour period of wound closure, and there was no increased proliferation in response to scratch wounding (Figure 3D). Instead, the cells that have reepithelialized the wound displayed a spread morphologic structure (Figure 3E and Supplemental Figure S7). Thus, scratch wounds in this system closed by ATII cell spreading.

To determine whether HIF signaling promoted ATII cell spreading, we used shRNA to knockdown HIF1α in MLE-12 cells. This yielded 60% ± 18% knockdown (Figure 4A) and inhibited up-regulation of the prototypical HIF target genes *VEGF* and *GLUT1* in response to the HIF stabilizer (prolyl hydroxylase inhibitor) DMOG (Figure 4B). HIF1α knockdown delayed wound closure (Figure 4C). To confirm these findings in primary cells, a rat ATII cell monolayer was subjected to scratch wounding, followed by treatment with DMOG, which also effectively induced expression of VEGF and GLUT1 in these cells (Figure 4D). HIF activation by DMOG accelerated wound closure in rat ATII cells (Figure 4E). Similarly, in MLE-12 cells, DMOG, which stabilized HIF1α (Figure 4F) and increased HIF transcriptional activity (Figure 4, G and H), accelerated wound closure (Figure 4I). Because *in vitro* epithelial scratch wounds were repaired by cell spreading (Figure 3 and Supplemental Figure S7) and HIF1α promoted wound closure (Figure 4), we concluded that HIF1α promoted ATII cell spreading.

HIF1 Induces CXCR4 and SDF1 Expression in ATII Cells

To identify HIF1α-dependent genes that might mediate the role of HIF1α in ATII cell spreading and migration during repair, we performed a genome-wide microarray on rat ATII cells transduced with an adenovirus containing a constitutively active mutant *HIF1α* construct (Ad*HIF1α*). We observed up-regulation of established HIF target genes, including those involved in oxygen supply (*HMOX1*), glycolysis (phosphofructokinase, aldolase C, phosphoglycerate kinase, phosphoglucomutase, enolase, *PFKFB3*), glucose transport (*GLUT1*), pH regulation (*CAIX*, *XII*), and growth (VEGF, *IGFBP2*) (Figure 5A). Complete results of the array are available at Gene Expression Omnibus (*https://www.ncbi.nlm.nih.gov/geo*; accession number GSE87555). Up-regulation of several of these genes in response to adenoviral-mediated HIF1α expression was confirmed by qPCR (Supplemental Figure S8). Of the genes that were up-regulated in response to HIF1α overexpression, *CXCR4* and its ligand *SDF1* (Figure 5A) have been previously implicated in cell motility.[26,29−31,33] Accordingly, we confirmed that CXCR4 and SDF1 were up-regulated by HIF1α expression in rat ATII cells by qPCR (Figure 5B); in fact, the fold change up-regulation for both genes was much greater by qPCR than by the hybridization array. HIF stabilization via DMOG also induced CXCR4 and SDF1 expression in rat ATII cells (Figure 5C). CXCR4 mRNA and protein expression were also increased in MLE-12 cells in response to stimulation with DMOG or exposure

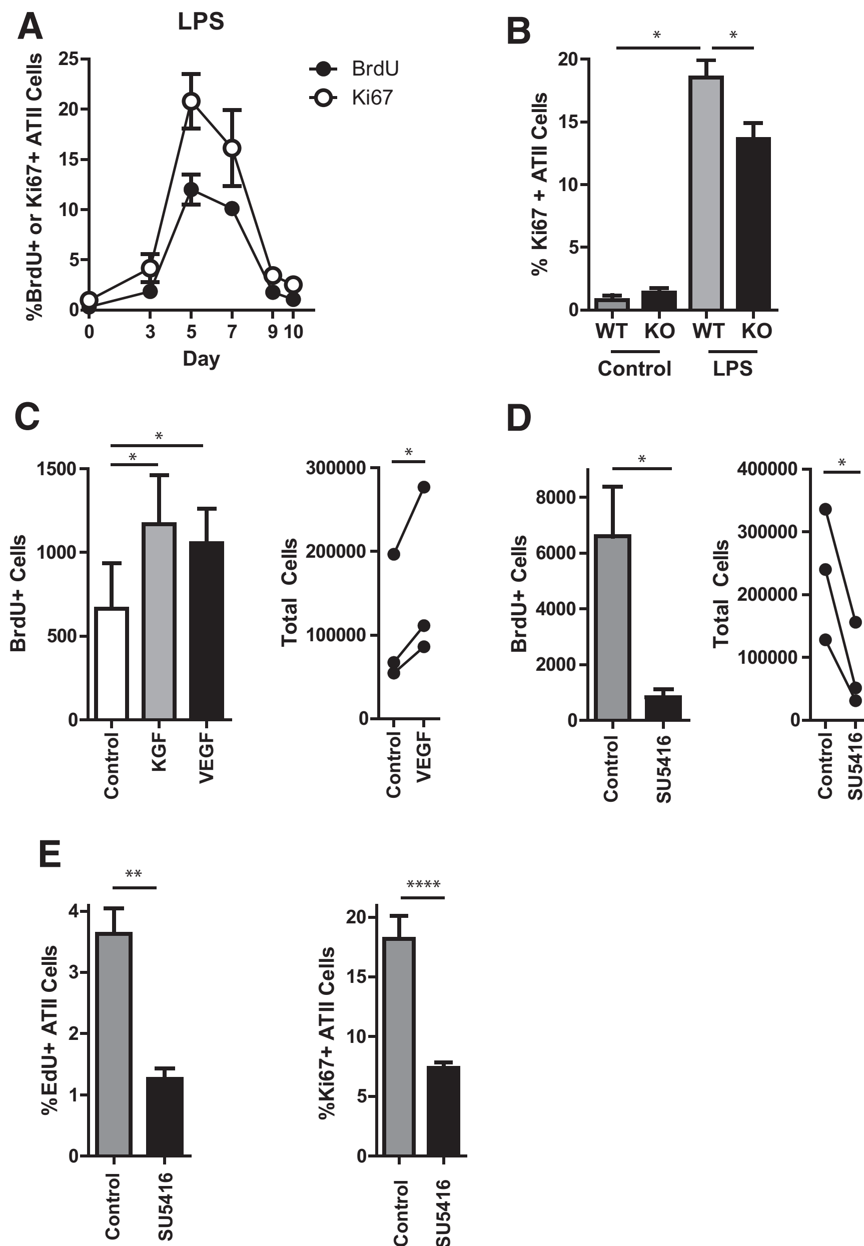

Figure 2 HIF promotes ATII cell proliferation during epithelial repair. **A:** *SftpcCreERT2*$^{+/-}$;*mTmG*$^{+/-}$ mice were treated with i.t. LPS, followed by i.p. BrdU 24 hours before euthanasia. Mice were euthanized at the indicated time points, and lungs were digested, stained for BrdU and Ki-67, and analyzed by flow cytometry. BrdU/Ki-67 expression in the GFP+ (ATII cell) population is shown. **B:** WT or ATII cell–specific *HIF1α* KO (*SftpcCreERT2*$^{+/-}$;*HIF1α*$^{f/f}$) mice were treated with i.t. LPS and euthanized 5 days later. Lungs were digested, stained for EpCAM, proSPC, CD45, and Ki-67. Ki-67 expression in the CD45^{-} EpCAM^{+} proSPC^{+} (ATII cell) population is shown. **C:** Rat ATII cells were cultured in DMEM with 1% charcoal stripped FBS in the presence of 10 ng/mL KGF or 20 ng/mL VEGF and BrdU. **D:** Rat ATII cells were cultured in DMEM with 10% FBS in the presence of 20 μmol/L SU5416 and BrdU. **C** and **D:** At day 3, cells were trypsinized, counted, stained with anti-BrdU antibody, and analyzed by flow cytometry. Total cell numbers were normalized to the control value for each experiment for statistical analysis. **E:** *SftpcCreERT2*$^{+/-}$;*mTmG*$^{+/-}$ mice were treated with LPS, followed by daily i.p. administration of SU5416, beginning at day 3, and i.p. EdU 24 hours before euthanasia. Mice were euthanized at day 5, and lungs were digested, stained for EdU and Ki-67, and analyzed by flow cytometry. EdU/Ki-67 expression in the GFP^{+} (ATII cell) population are shown. Data are expressed as means ± SEM (**B**–**E**). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$. ATII, alveolar type II; BrdU, bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium; EdU, 5-ethynyl-2′-deoxyuridine; EpCAM, epithelial cell adhesion molecule; FBS, fetal bovine serum; GFP, green fluorescent protein; HIF, hypoxia-inducible factor; KGF, keratinocyte growth factor; KO, knockout; LPS, lipopolysaccharide; proSCP, prosurfactant protein C; VEGF, vascular endothelial growth factor; WT, wild-type.

to hypoxia, and this was prevented by HIF1α shRNA knockdown (Figure 5, D and E).

CXCR4/SDF1 Signaling Promotes ATII Cell Spreading

Given that HIF1α promotes ATII cell spreading during epithelial repair (Figure 4) and that *CXCR4* and *SDF1* are HIF1 target genes (Figure 5), we hypothesized that SDF1/CXCR4 signaling may mediate the role of HIF1α in ATII cell spreading during epithelial repair. Inhibition of SDF1/CXCR4 signaling using the CXCR4 antagonist AMD3100 delayed repair of a scratch wound in primary ATII cells as well as MLE-12 cells (Figure 6A). Conversely, rSDF1 promoted cell spreading (Figure 6B) and actin polymerization at the leading wound edge (Figure 6C). CXCR4 is known to regulate cell motility by signaling through Rac1[47] and myosin IIA.[48] Accordingly, inhibitors of myosin II and/or Rac1 also inhibited cell spreading (Figure 6, D and E). Incidentally, rVEGF had no effect on wound closure (data not shown).

CXCR4/SDF1 Signaling Promotes the Resolution of Epithelial Permeability after Lung Injury

To assess the potential relevance of SDF1/CXCR4 signaling during repair after lung injury *in vivo*, we first sought to determine whether those genes are up-regulated after LPS or HCl. CXCR4 and SDF1 mRNA expression was increased in the whole lung homogenate at day 3 after lung injury *in vivo*

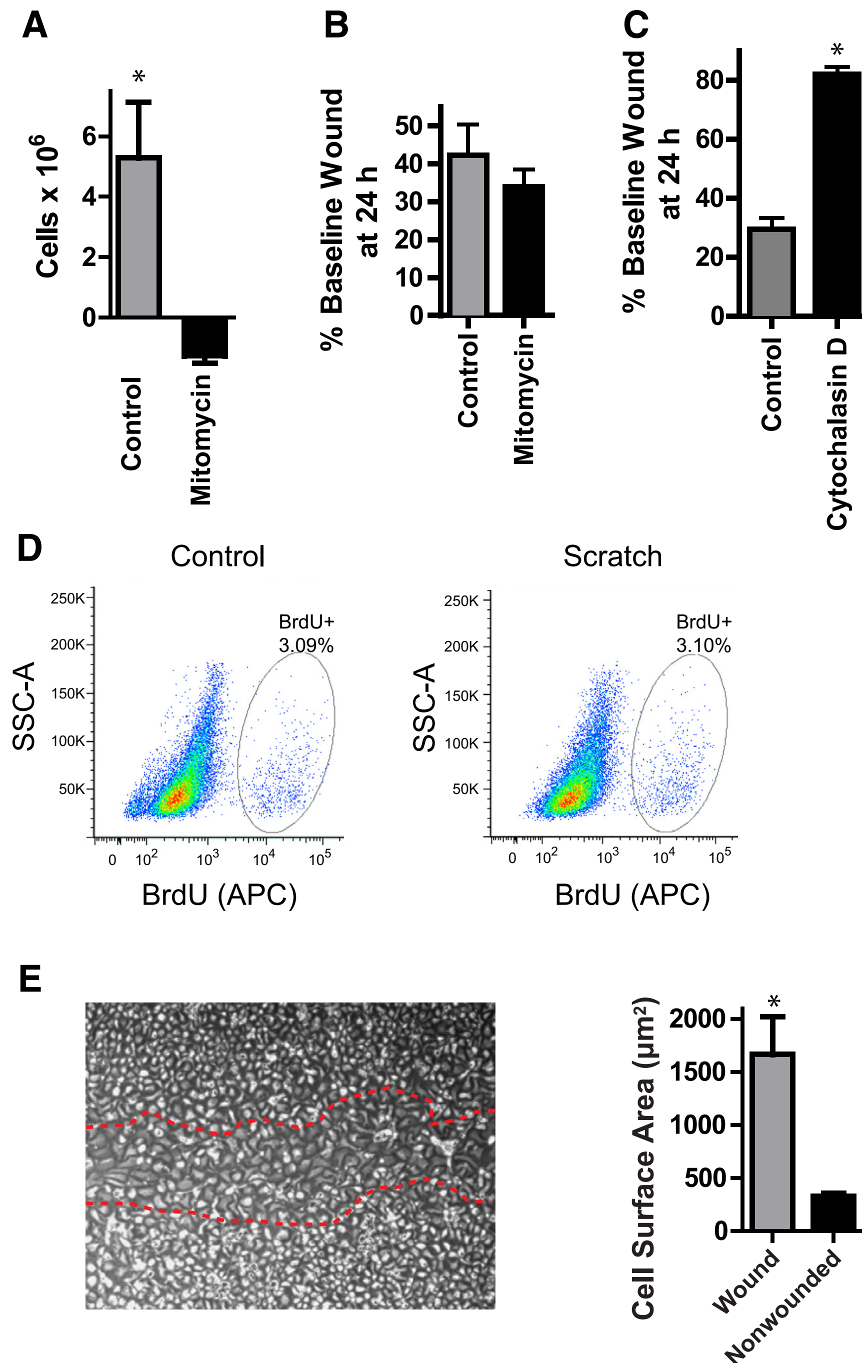

Figure 3 Scratch wounds repair via epithelial cell spreading. **A:** MLE-12 cells were grown to 30% confluence. Some wells were trypsinized and counted at t = 0; others were treated with 1 µg/mL mitomycin. Cells were counted 24 hours later. The difference in cell number between t = 0 and t = 24 hours is shown. **B** and **C:** MLE-12 cells were subjected to scratch wounding, followed by treatment with 1 µg/mL mitomycin (**B**) or 0.1 µg/mL cytochalasin D (**C**). Images were acquired at t = 0 and 24 hours, and the percentage of initial wound width remaining was calculated. **D:** Rat ATII cells were subjected to scratch wounding and cultured in the presence of BrdU. At 24 hours, cells were trypsinized, stained with an anti-BrdU antibody, and analyzed by flow cytometry. **E:** Rat ATII cells were subjected to scratch wounding. At 48 hours, the surface area of cells within the wound (**red dotted lines**) or >1 mm away from the wound was measured. Data are expressed as means ± SEM (**A**–**C** and **E**). $^*P < 0.05$. APC, allophycocyanin; ATII, alveolar type II; BrdU, bromodeoxyuridine; SSC, side scatter.

(Figure 7, A and B). SDF1 protein was also up-regulated after lung injury (Figure 7C and Supplemental Figure S9A). In addition, SDF1 was up-regulated in human ARDS lung tissue (Figure 7D). To determine whether SDF1/CXCR4 signaling may be involved in epithelial repair after lung injury, mice were treated with the CXCR4 antagonist AMD3100 daily, starting at day 3 after LPS, that is, after injury was established. CXCR4 blockade resulted in delayed resolution of permeability (Figure 7E) without any effect on inflammation (Supplemental Figure S9B). Of note, one mouse in the AMD3100 group died at day 5 after LPS, but no mice in the control group died.

Discussion

After ATI cell loss during lung injury, ATII cells reepithelialize the denuded basement membrane by proliferation and spreading. In this study, we have demonstrated that HIF signaling contributes to both critical steps of repair of the alveolar epithelium after lung injury. HIF signaling was shown to be activated in ATII cells during

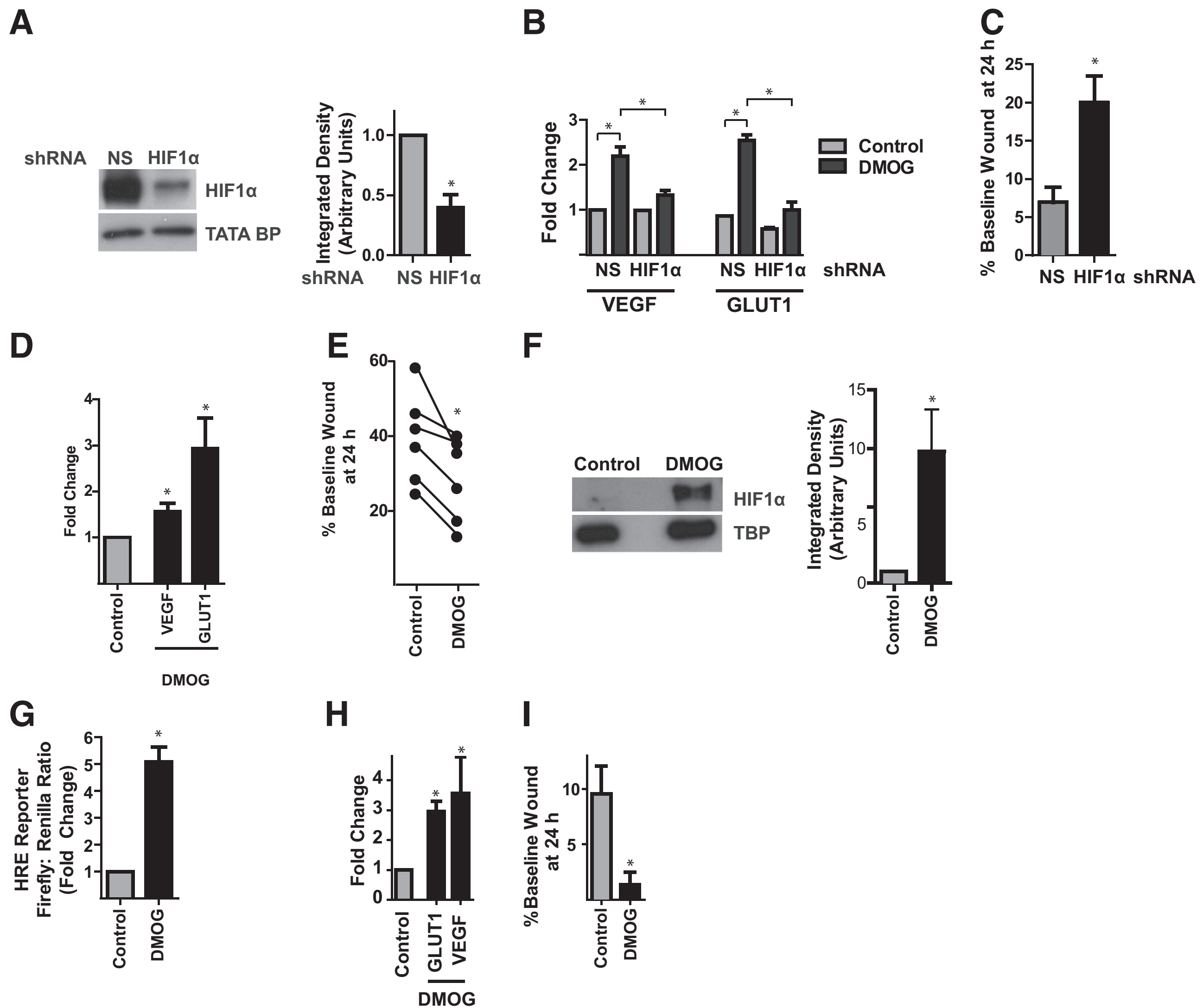

Figure 4 HIF promotes ATII cell spreading during epithelial repair. **A–C:** MLE-12 cells were transduced with a lentiviral construct expressing a NS or HIF1α shRNA. **A:** Cells were exposed to 1% oxygen for 24 hours, followed by immunoblotting of nuclear extracts for HIF1α. **B:** Cells were stimulated with 1 mmol/L DMOG and qPCR was performed. **C:** Cells were grown to confluency and subjected to scratch wounding. The wound was imaged at t = 0 and 24 hours, and wound width was measured. **D:** Cultured rat ATII cells were stimulated with DMOG 1 mmol/L for 48 hours. RNA was harvested and qPCR was performed. **E:** Rat ATII cells were grown to confluence, subjected to scratch wounding, and cultured in the presence or absence of 0.5 to 1 mmol/L DMOG. **F:** MLE-12 cells cultured under normoxic conditions were treated with 1 mmol/L DMOG for 16 hours, followed by immunoblotting of nuclear extracts for HIF1α. **G:** MLE-12 cells were cotransfected with the HRE Firefly luciferase reporter plasmid and the Renilla luciferase reporter plasmid and treated with 1 mmol/L DMOG for 24 hours. Luciferase activity was determined. **H:** MLE-12 cells were treated with 1 mmol/L DMOG for 6 hours; qPCR was performed. **I:** MLE-12 cells were grown to confluence, subjected to scratch wounding, and cultured in the presence or absence of 1 mmol/L DMOG. Data are expressed as means ± SEM (**A–D** and **F–I**). $n = 6$ independent experiments (**E**). $^*P < 0.05$. ATII, alveolar type II; DMOG, dimethyloxalylglycine; GLUT1, glucose transporter 1; HIF, hypoxia-inducible factor; HRE, hypoxia response element; NS, nonsilencing; qPCR, real-time quantitative PCR; VEGF, vascular endothelial growth factor.

the repair phase after lung injury, and ATII cell–specific *HIF1α* KO mice exhibited a defect in ATII cell proliferation. The HIF target gene *VEGF* promoted ATII cell proliferation, both in cultured ATII cells as well as during repair after lung injury *in vivo*. HIF1α also promoted ATII cell spreading. *CXCR4* and its ligand *SDF1* were found to be HIF target genes in ATII cells, were up-regulated in lung injury, and promoted ATII cell spreading *in vitro* and the recovery of barrier function *in vivo*. By using state-of-the-art ATII cell–specific KO mice to dissect mechanisms of ATII cell proliferation, our study builds on previous work by us and others assessing mechanisms of ATII cell proliferation.[49–52] In addition, we suggest the novel concept that a chemokine-receptor pathway, previously implicated in hematopoietic cell migration,[26,27] is activated in structural cells, ATII epithelial cells, during lung injury and drives ATII cell spreading and the restoration of epithelial barrier integrity. In summary, our data suggest that after lung injury, HIF is activated in ATII cells and promotes ATII cell

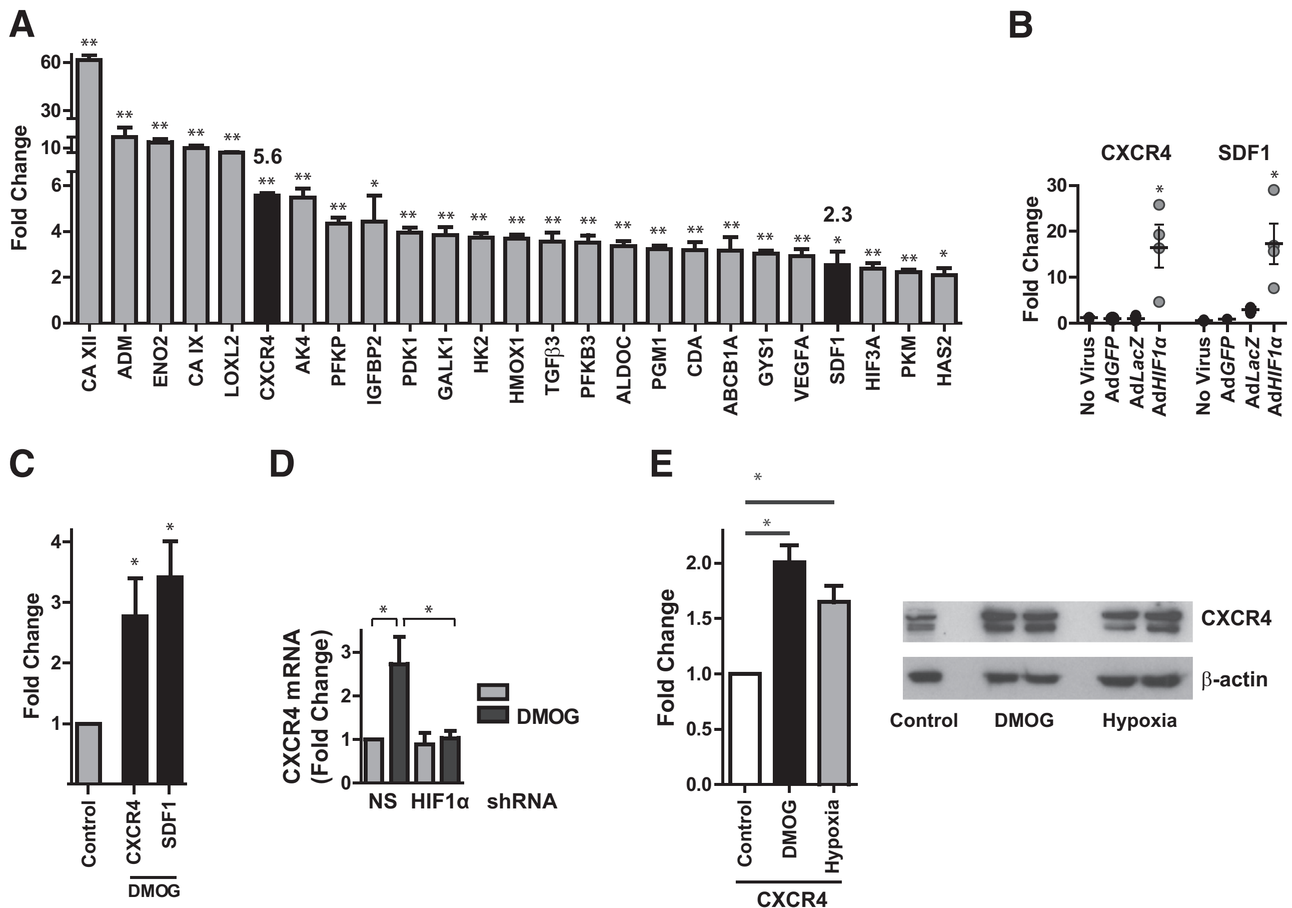

Figure 5 *CXCR4* and *SDF1* are HIF target genes in ATII cells. **A** and **B:** Rat ATII cells were transduced with an adenovirus containing a constitutively active mutant HIF1α construct (Ad*HIF1α*) or *GFP* (Ad*GFP*) or *LacZ* (Ad*LacZ*). A genome-wide microarray (**A**) or qPCR (**B**) demonstrating mRNA expression of selected genes was performed. Fold-change of CXCR4 (5.6) and SDF1 (2.3) are indicated. **C:** Cultured rat ATII cells were treated with 1 mmol/L DMOG for 48 hours, RNA was harvested, and qPCR was performed. **D:** MLE-12 cells transduced with lentivirus expressing HIF1α or NS shRNA were treated with 1 mmol/L DMOG for 6 hours, and qPCR was performed. **E:** MLE-12 cells were treated with DMOG 1 mmol/L or cultured in 1% oxygen for 16 hours, and qPCR or Western blot analysis for CXCR4 was performed. Data are expressed as means ± SEM (**A and C–E**). $^*P < 0.05$, $^{**}P < 0.01$. ATII, alveolar type II; DMOG, dimethyloxalylglycine; HIF, hypoxia-inducible factor; NS, nonsilencing; qPCR, real-time quantitative PCR; SDF, stromal cell-derived factor.

proliferation by VEGF and ATII cell spreading via SDF1/CXCR4 (Supplemental Figure S10).

The finding that HIF promotes ATII cell proliferation during repair after lung injury is consistent with the previously reported role of HIF in cell proliferation during tumorigenesis and angiogenesis.[12,13] Although some literature has suggested a role for VEGF in alveolar epithelial cell proliferation,[53] to our knowledge, our study is the first to demonstrate this effect in cultured primary ATII cells or during repair in an animal model of lung injury. Because HIF-dependent *VEGF* expression has been implicated in alveologenesis,[20,21] this study substantiates the notion that developmental pathways may be reactivated to promote tissue repair after injury. Still, the mechanisms by which HIF1α promotes ATII cell proliferation are likely multifactorial, possibly also involving up-regulation of other growth factors as well as glucose transporters and glycolytic enzymes that drive glycolysis, which in turn results in the synthesis of macromolecules (eg, nucleic acids, lipids, proteins) required for cell replication.[54] Indeed, we observed up-regulation of several growth factors and glucose transporters/glycolytic enzymes by HIF1α in ATII cells (Figure 5). Another intriguing possibility is that HIF-dependent VEGF production by the alveolar epithelium triggers endothelial cell proliferation during repair after vascular injury as an example of endothelial–epithelial crosstalk during coordinated regeneration of multiple cell types in the alveolus.

Our results are also consistent with the role of HIF in cell motility during tumorigenesis[13] as well as during wound repair in keratinocytes,[14,15] a mechanism by which occlusive dressings induce local hypoxia to accelerate closure of surgical wounds.[55] Moreover, HIF signaling has been implicated in barrier function at other mucosal surfaces.[16,17,56] Our data support the notion that during lung injury, there is HIF-dependent up-regulation of both the chemokine SDF1 and its receptor CXCR4 and that SDF1/CXCR4 signaling promotes ATII cell spreading and

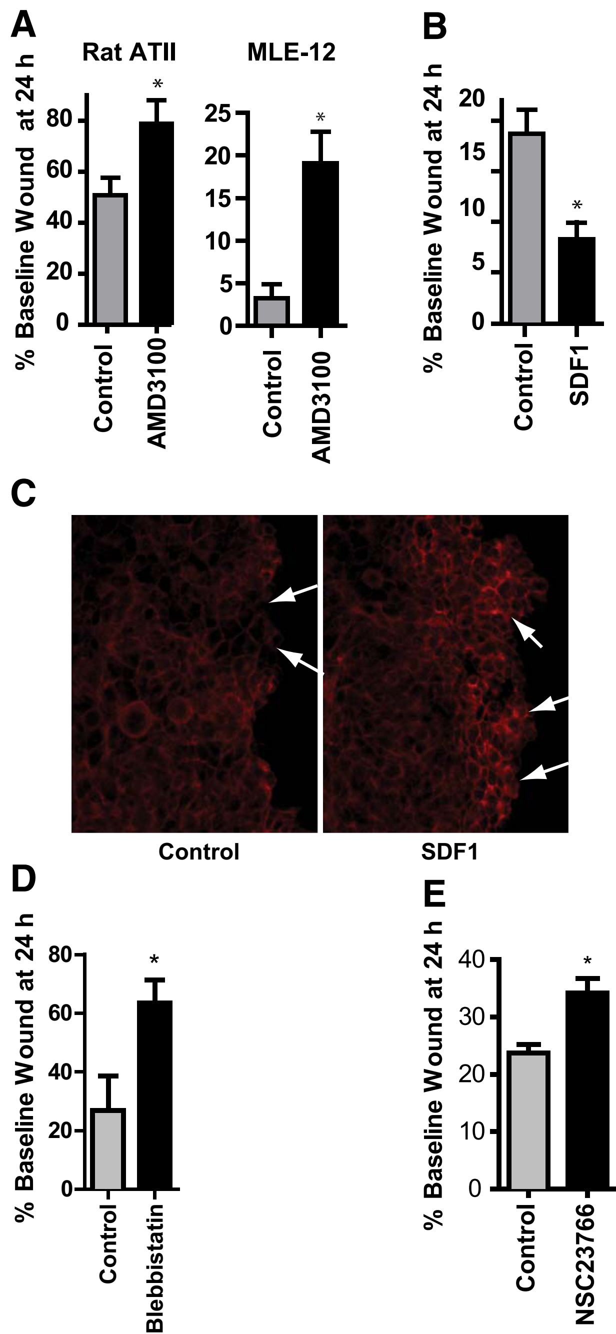

Figure 6 SDF1/CXCR4 signaling promotes ATII cell spreading. **A–E:** Rat ATII (**A**) or MLE-12 (**A–E**) cells were grown to confluence and subjected to scratch wounding. Immediately after the wound was made, 5 μg/mL AMD3100 (**A**), 500 ng/mL rSDF1 (**B** and **C**), 200 μmol/L blebbistatin (**D**), or 100 μmol/L NSC23766 (**E**) was added. **A**, **B**, **D** and **E:** Images were acquired, and the percentage of initial wound width remaining at 24 hours was calculated. **C:** Cells were fixed 5 minutes later and stained for phalloidin. **Arrows** indicate actin polymerization at leading wound edge. Data are expressed as means ± SEM (**A**, **B**, **D**, and **E**). *$P < 0.05$. ATII, alveolar type II; SDF, stromal cell-derived factor.

barrier restitution. The observed role for the SDF1/CXCR4 axis in cell spreading and barrier function is consistent with previous literature.[29,57] Note that, although cell spreading, as measured by an increase in surface area covered by individual cells, contributes to wound closure in the scratch wound assay, we have not excluded the possibility that cell migration might also participate. Although previous reports have revealed SDF1/CXCR4 activation during lung injury,[30,31] our study extends these findings by revealing a novel functional effect of this pathway. The effect of CXCR4 blockade on the resolution of permeability after lung injury, taken together with the direct role of SDF1/CXCR4 in ATII cell spreading demonstrated *in vitro*, suggests that CXCR4 blockade might inhibit the restitution of barrier integrity by the inhibition of cell spreading. Although the CXCR4 inhibitor AMD3100 may also have effects on hematopoietic cells, we observed no difference in inflammatory cell influx. The expression of SDF1 in the lungs of ARDS patients underscores the relevance of this signaling pathway to the human disease. Although our results implicate HIF-dependent autocrine SDF1/CXCR4 signaling, we do not exclude the possibility of additional sources of SDF1, such as macrophages or platelets.[58] Notably, in the immunostaining of mouse lung (Figure 7, C and D and Supplemental Figure S9A), SDF1 appears to line the alveolar septa, suggesting that it has been secreted. It is not possible to discern from these images what the cellular source of the SDF1 is, although ATII cells clearly produce SDF1 *in vitro*. In addition to its role in spreading, SDF1 may also promote alveolar regeneration by indirect mechanisms, such as inducing other cell types to produce epithelial growth factors.[58] Finally, other HIF-dependent genes may contribute to cell spreading in other settings[14,15,59,60]; whether these pathways contribute to ATII cell spreading during repair after lung injury should be investigated.

Although severe, prolonged hypoxia and supraphysiologic activation of HIF can have deleterious effects, including alveolar epithelial apoptosis and lung injury,[61–63] the alveolar epithelium tolerates moderate hypoxia well.[61–64] We suggest that in inflammatory lung injury, physiologic activation of HIF signaling promotes repair, a notion that is consistent with its overall role in adaptation to hypoxic stress (eg, by angiogenesis, erythropoiesis, and glycolysis). In addition to dose-dependent effects, HIF may have pleiotropic effects depending on the specific target genes expressed. An intriguing possibility is that HIF induces expression of proapoptotic genes in susceptible cells while promoting repair in neighboring surviving cells by upregulation of different target genes (eg, *VEGF*, *CXCR4*, and *SDF1*).

Future studies should examine the mechanism by which HIF is activated in ATII cells during lung injury. Possibilities include hypoxic stabilization of HIF, perhaps because of focal oxygen consumption by inflammatory cells or microorganisms or decreased gas exchange because of airspace and interstitial edema, microvascular thrombosis, or atelectasis. In addition, various stimuli, including reactive oxygen/nitrogen species,[65] LPS,[66] succinate,[67] and growth factors and cytokines,[68] can induce HIF

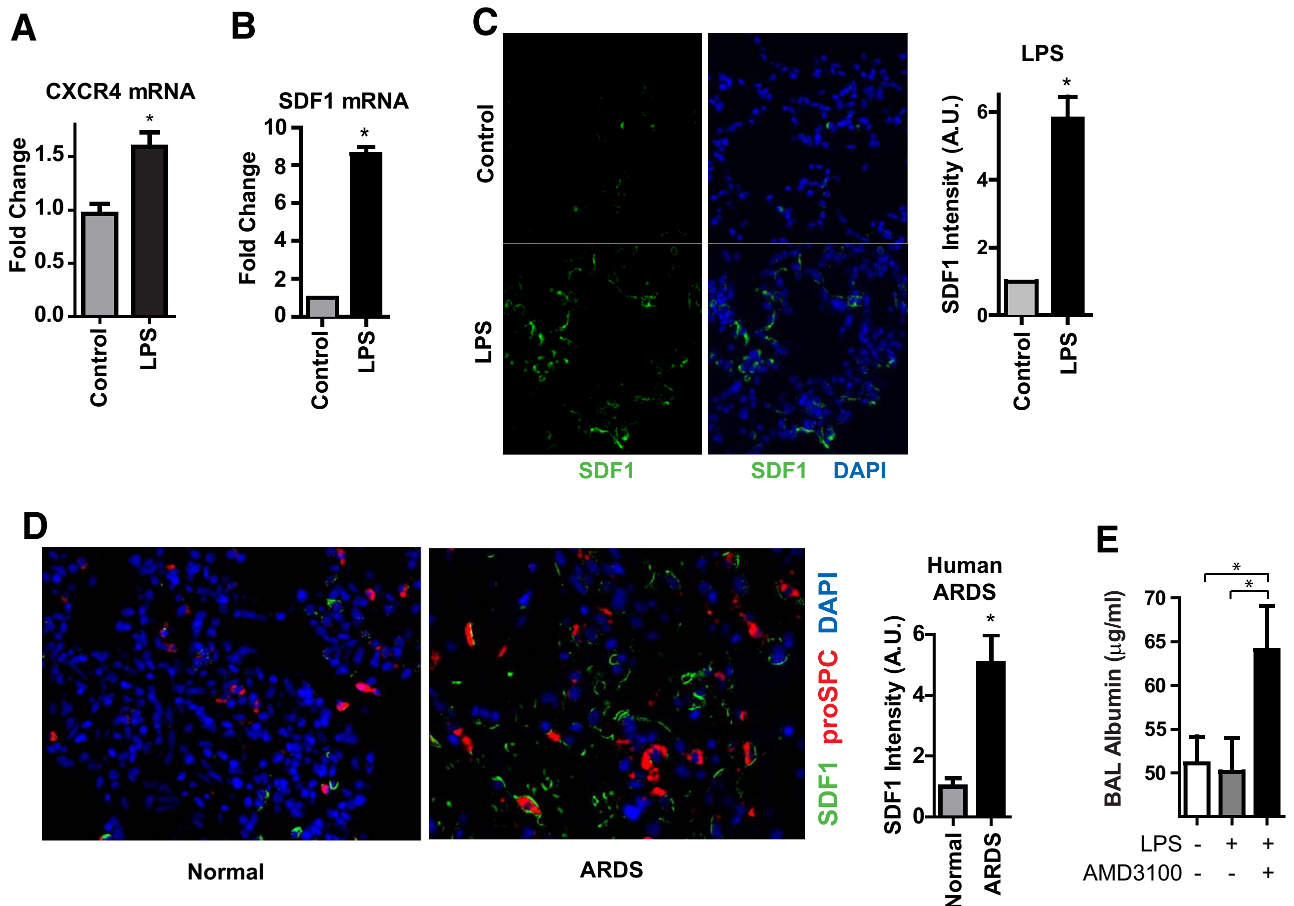

Figure 7 CXCR4 and SDF1 are up-regulated in animal models of lung injury and ARDS patients. **A**–**C** and **E:** Mice were treated with i.t. LPS. **A** and **B:** At day 3, RNA was harvested from the whole lung homogenate, and qPCR was performed. **C:** Lung sections were stained for SDF1. **D:** Lung sections from normal human lungs rejected for lung transplantation or autopsy specimens from ARDS patients were stained for SDF1 and proSPC. **C** and **D:** SDF1 intensity per HPF was quantified by ImageJ software version 1.48 (NIH, Bethesda, MD; *http://imagej.nih.gov/ij*). **E:** Beginning at day 3 after LPS, mice were administered AMD3100 i.p. daily and euthanized at day 7. BAL albumin was measured by ELISA. Data are expressed as means ± SEM. $n \geq 5$ humans or mice per group. $^*P < 0.05$. ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assay; HPF, high power field; LPS, lipopolysaccharide; proSPC, prosurfactant protein C; qPCR, real-time quantitative PCR; SDF, stromal cell-derived factor.

stabilization or synthesis under normoxic conditions. Regardless of the mechanism driving HIF activation, it is likely that the surviving ATII cells located in the most injured areas, that is, the areas in most need of repair, are precisely those cells in which HIF is most highly activated. Interestingly, there is a small degree of active HIF signaling in the naive lung, including in ATII cells (Figure 1, B and C). The function of HIF1 in ATII cell homoeostasis remains to be determined, but maintenance of normal lung structure and barrier function does not appear to require HIF signaling (Supplemental Figure S4, B and C). We acknowledge that our conclusions are partially based on *in vitro* data that support but do not prove our hypothesis that this signaling cascade occurs *in vivo*.

ATII cell–specific KO of *HIF1α* partially attenuates ATII cell proliferation. Other pathways, which have been identified by us and others, also contribute to ATII cell proliferation.[51,52,69–72] The mechanism by which these pathways may act cooperatively with HIF1α to regulate repair should be the subject of future study. The observation that VEGF blockade more potently inhibits ATII cell proliferation than *HIF1α* KO suggests that additional mechanisms may regulate VEGF. Future studies could also address the potential role of HIF2α, which regulates alveologenesis by VEGF,[21] in VEGF expression and ATII cell proliferation during repair after lung injury. Similarly, knockdown of HIF1α only partially attenuates ATII cell spreading; other pathways are known to contribute.[45,73–75] Finally, additional studies are warranted to determine which transcriptional cofactors might be required for HIF-dependent gene expression in ATII cells in the setting of lung injury. We speculate that β-catenin and p300, transcriptional cofactors that we have previously linked to ATII cell proliferation and spreading during repair,[49,76] may bind to and enhance the transcriptional potency and specificity of HIF at the HRE, as has been demonstrated in other settings.[77,78]

Conclusions

We demonstrate here that HIF signaling promotes ATII cell proliferation and spreading, the two critical stages necessary to restore normal epithelial structure and function after lung injury. We speculate that an improved understanding of the molecular signals that promote ATII cell proliferation and spreading could ultimately be translated into novel therapeutic interventions to enhance lung repair in ARDS. The HIF pathway may be one target for such interventions.

Acknowledgments

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2017.04.012*.

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