Hypoxia-Induced Mitogenic Factor Has Antiapoptotic Action and Is Upregulated in the Developing Lung

Coexpression with Hypoxia-Inducible Factor-2α

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Hypoxia-induced mitogenic factor (HIMF), also called FIZZ1 or RELMα, was a newly found cytokine. Hypoxia caused robust HIMF induction in the lung, and HIMF has potent pulmonary vasoconstrictive, proliferative, and angiogenic properties. To investigate the role of HIMF in lung development, we determined its spatial and temporal expression. From embryonic day (E)16 to postnatal day (P)28, HIMF was strongly expressed in the cytoplasm of bronchial epithelial cells, type II cells, endothelial cells, and primitive mesenchymal cells. Treatment with HIMF resulted in a significant reduction of apoptosis in cultured embryonic lung, thus revealing a previously unknown function of HIMF. Because *HIMF* gene is upregulated by hypoxia and contains a hypoxia-inducible transcription factor (HIF) binding site, we subsequently investigated whether HIMF was coexpressed with HIF-2α or HIF-1α. HIF-1α expression was temporally distinct from HIMF expression. In contrast, HIF-2α was present in endothelial cells, bronchial epithelial cells, and type II cells from E18 to P28. Thus, HIMF and HIF-2α were temporally and spatially coexpressed in the developing lung. These results indicate a role for HIMF in lung development, possibly under the control of HIF-2, and suggest that HIMF regulates apoptosis and may participate in lung alveolarization and maturation.

Recently, we discovered the preeminent role of a hypoxia-induced mitogenic factor (HIMF) and member of the resistin family of proteins (1, 2), in the functional and structural response to chronic hypoxia in the adult murine lung (3). Apart from adipose tissue and spleen, HIMF was exclusively expressed in the lung, where it was detected in bronchial epithelial cells, type II alveolar cells, and endothelial cells. HIMF increased pulmonary arterial pressure and vascular resistance more potently than either endothelin-1 or angiotensin II and had potent proliferative and angiogenic properties. Because the role of HIMF in lung development has not been investigated in the past, we designed this study to explore the temporal and spatial expression of HIMF in embryonic mouse lung development.

Histologically, lung development is divided into four chronological stages in the mouse: (*i*) pseudoglandular stage (embryonic day [E]9.5–E16.5), the bronchial and respiratory tree develops and an undifferentiated primordial system forms; (*ii*) canalicular stage (E16.5–E17.5), terminal sacs and vascularization develops; (*iii*) terminal sac stage (E17.5 to postnatal day [P]5), the number of terminal sacs and vascularization increase and type I and II cells differentiate; and (*iv*) alveolar stage (P5–P30), terminal sacs develop into mature alveolar ducts and alveoli (4).

Angiogenesis, proliferation, and apoptosis are integral parts of adaptation and plasticity, for example in the response to hypoxia (5). In embryonic lung, apoptosis is involved in lung remodeling and maturation in the saccular and alveolar stages (6). Prenatally, mesenchymal cells have been shown to undergo apoptosis, presumably as a mechanism to thin the septa and establish an adequate pulmonary alveolar–capillary interface (7). Because the effect of HIMF on apoptosis is not known, we investigated the presence of apoptosis in lung explant cultures treated with HIMF. Apoptotic cells in the lung were identified *in situ* using terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL).

HIMF had been shown to be upregulated by chronic hypoxia *in vivo*. Consistent with this observation was the finding, that the 3′ region of the gene contains a binding site for the hypoxia-inducible transcription factor (HIF)-1 and HIF-2 (3). Oxygen regulated gene expression as well as hypoxia signaling via HIFs has been shown to play an important role in embryonic development (8, 9).

To elucidate the role of HIMF in embryonic lung development, we examined the temporal and spatial expression of HIMF during different stages of lung development and in relation to the expression of the hypoxia-inducible transcription factors HIF-1α and HIF-2α. We found that HIMF is specifically upregulated in the saccular and alveolar stages in bronchial epithelial cells and type II alveolar cells. While searching for the presence of HIFs we observed that HIF-1α expression was temporally clearly distinct from HIMF expression. In contrast, HIF-2α was temporally and spatially coexpressed with HIMF. In addition, functional data provided evidence that HIMF has antiapoptotic properties *ex vivo*.

Materials and Methods

Animals

Timed pregnant C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were killed by halothane overdose; the abdominal cavity and uterus were opened, and embryos were harvested for study. Lungs from mice aged P1–P40 were obtained similarly. All experiments were approved by the Animal Care and Use Committee of Johns Hopkins University.

Northern Blot

Embryonic, neonatal and adult lung total RNA was extracted with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's instructions. Fifteen micrograms of total RNA from E14, E15, E16, E17, E20, P1, P3, and adult lung was separated in 1% agarose gel. After RNA was transferred to nylon membrane overnight, the hybridization was performed at 45°C with ^{32}P-labeled HIMF cDNA. The membrane was washed with 2 × saline sodium citrate (SSC), 0.1% sodium dodecylsulfate (SDS) at 45°C followed by 0.2 × SSC, 0.1% SDS at 55°C. The membrane was exposed to a phosphoimager screen,

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Abbreviations: diaminobenzidine, DAB; hypoxia-inducible transcription factor, HIF; hypoxia-induced mitogenic factor, HIMF; horseradish peroxidase, HRP; phosphate-buffered saline, PBS; sodium dodecylsulfate, SDS; saline sodium citrate, SSC.

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and the signal was detected with a Storm Phosphoimager (Molecular Dynamics, Sunnyvale, CA). Ethidium bromide–stained 18 s and 28 s RNA was used as loading control.

Western Blot

The embryonic and postnatal mouse lung tissue collection and homogenization were performed as described before (10). In brief, protein extracts were separated on a 4–20% polyacrylamide precast gel (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. Immunodetection was performed using the following antibodies: HIMF antibody (1:1,000, produced as before [3]), HIF-1α (1:500, Novus Biologicals, Littleton, CO), HIF-2α (1:1,000; Novus), and α-tubulin (1:1,000; Santa Cruz, Santa Cruz, CA), which was used as loading control. Incubation with primary antibody was followed by incubation with a 1:3,000 dilution of goat anti-rabbit or goat anti-mouse horseradish peroxidase (HRP)-labeled antibody (Bio-Rad) and visualization with ECL (Amersham Biosciences, Buckinghamshire, UK).

In Situ Hybridization

Synthetic fragments of sense and anti-sense oligonucleotides for HIMF were used as probes. The sequences were: sense 5′-gactctctcttgcactagtgtca-3; antisense: 5′-ttgacactagtgcaagagagagtc-3′. Both of oligoprobes were labeled with 6-Fam. Tissues were treated as described below in HISTOLOGY. After deparaffinization, the slides were treated with 1ug/ml proteinase K in 100 mM tris, pH 8.0, and 50 mM EDTA for 15 min and were fixed in 2% paraformaldehyde for 5 min at room temperature. Sense and antisense probes (500 ng/ml) were added separately in the hybridization buffer (50% formamide, 4 × SSC, 100 μg/ml salmon sperm DNA, and 1 × Denhardt's solution) and were incubated overnight at 45°C. After being washed with 2 × SSC, 0.1% SDS at 45°C and 0.2 × SSC, 0.1% SDS at 55°C, the slides were examined under an Olympus florescent microscope (Olympus, Tokyo, Japan).

TUNEL Labeling

The TUNEL method was performed according to the manufacturer's instructions (Roche, Indianapolis, IN). Briefly, after 24 h incubation with or without recombinant HIMF, the E13.5 and E16 embryonic lungs form different pregnancies ($n = 3$) were snap frozen and 5-μm sections were cut. The tissue sections were fixed immediately in 4% paraformaldehyde for 20 min and washed with phosphate-buffered saline (PBS) and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Each of the sample slides received 50 μl of TUNEL reaction mixture and was incubated for 60 min at 37°C. After washing with PBS, the sections were analyzed under an Olympus fluorescent microscope. Negative control slides were performed without TUNEL mixture. Ethidium bromide (0.5 μg/ml, red) in PBS was used for nuclear staining. TUNEL positive apoptotic cells were quantified per high-power field (×40) and the difference to vehicle treated controls analyzed with the paired *t* test.

Histology

Embryonic and postnatal lungs, each from a different pregnancy ($n = 3$) or litter, respectively, were examined histologically. Because HIF-1α is known to be degraded in intact cells within minutes after exposure to oxygen, embryos and lungs were immediately fixed with 4% buffered formalin (pH 7.4), processed routinely, and embedded in paraffin using an automatic tissue processor (Leica TP 1020; Leica, Heidelberg, Germany). Embedded tissues were serially sectioned at 2 μm on a Micron microtome (HM 440E; Micron, Neuss, Germany) and stained with hematoxylin-eosin, Masson's trichrome, and van Gieson's elastica stain. The sections were examined using an Olympus photomicroscope.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissues. Two-micrometer sections were deparaffinized with xylene and progressively rehydrated in decreasing percentages of ethanol. Antigen sites were retrieved by heating the sections on slides in 0.01 mol/liter sodium citrate in a microwave oven and cooling for 30 min to room temperature. Endogenous peroxidases were degraded by immersion of the sections in 0.3% hydrogen peroxide in methanol. Sections were blocked with blocking solution (CSA-Kit K1500; Dakocytomation, Carpenteria, CA) and incubated either with anti-HIMF antiserum (1:100; Alpha Diagnostic Int., San Antonio, TX) or anti-alkaline phosphatase antiserum (1:50; Abcam Inc., Cambridge, MA) at 4°C overnight or with anti–HIF-2α antiserum (1:1,000; Novus Biologicals) for 15 min at room temperature. The primary antibody was linked (anti-rabbit or anti-goat link antiserum, respectively) to a signal amplification system (CSA for HRP amplification, Envision for alkaline phosphatase amplification; Dakocytomation). Double stain block (Dakocytomation) was applied between two primary antibodies. HRP-labeled antibodies were developed with 3,3′-diaminobenzidine (DAB), alkaline phosphatase–labeled antibodies were developed with Fast Red.

Anti–HIF-1α monoclonal antiserum (1:100; Abcam Inc.) or anti-ARNT monoclonal antiserum (1:250; Abcam Inc.) were incubated for 15 min at room temperature. For visualization a signal amplification system was deployed (CSA, K1500; Dakocytomation) using DAB as chromogen.

Ex Vivo Embryonic Lung Culture

E13.5 and E16 lung cultures were prepared as described (11). Briefly, after maternal death, sterile laparotomy was performed, and the uterus was removed with embryos intact and placed in ice-cold Hanks' balanced salt solution. Under a dissecting microscope, using microdissection forceps, the fetuses were removed from the uterus and the lungs dissected free of surrounding structures. Embryonic lungs were then placed in 50-mm culture dishes on GVWP membranes (Millipore, Bedford, MA) at the air–liquid interface in serum-free and hormone-free (BGJb) medium (Gibco BRL, Grand Island, NY) containing 0.2 mg ascorbic acid, 50 μg streptomycin, and 50 units penicillin per milliliter of culture medium. The lungs were cultured for 24 h as control or with added HIMF (100 nM final concentration). Recombinant HIMF was produced as described (3). Untreated control lungs received no additional treatment.

Results

Gene Expression in Embryonic Mouse Lung

Although we had previously reported the prominent role of HIMF in adult mouse lung (3), it was not known whether HIMF was expressed in the developing lung. Therefore, Northern blot analysis of embryonic lungs from mice E14 to P3 and of adult lung was performed (Figure 1). We found a robust *HIMF* expression from E16 through P3 and only a low level expression at E14 and E15. Because the mouse pregnancy has a duration of 20 d, one can conclude that *HIMF* is upregulated in the last trimester of the pregnancy and in the perinatal period, possibly indicating an important role of HIMF in embryonic and perinatal lung maturation and lung function.

Next we wanted to elucidate the localization of the cells expressing *HIMF* within the lung. Using *in situ* hybridization we

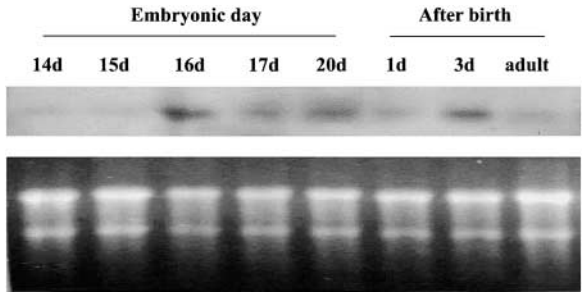

Figure 1. Expression of HIMF mRNA in the developing lung. Fifteen micrograms of total RNA from lungs isolated and pooled from litters at E14 to adult was analyzed as described in MATERIALS AND METHODS. The blot was hybridized with mouse *HIMF*. The *lower panel* shows the 28S and 18S rRNA as a control for loading. *HIMF* was expressed in late gestation from E16 onwards spanning into the perinatal period. In the pseudoglandular stage (E14 and E15) as well as at 4 mo (adult) *HIMF* expression was low.

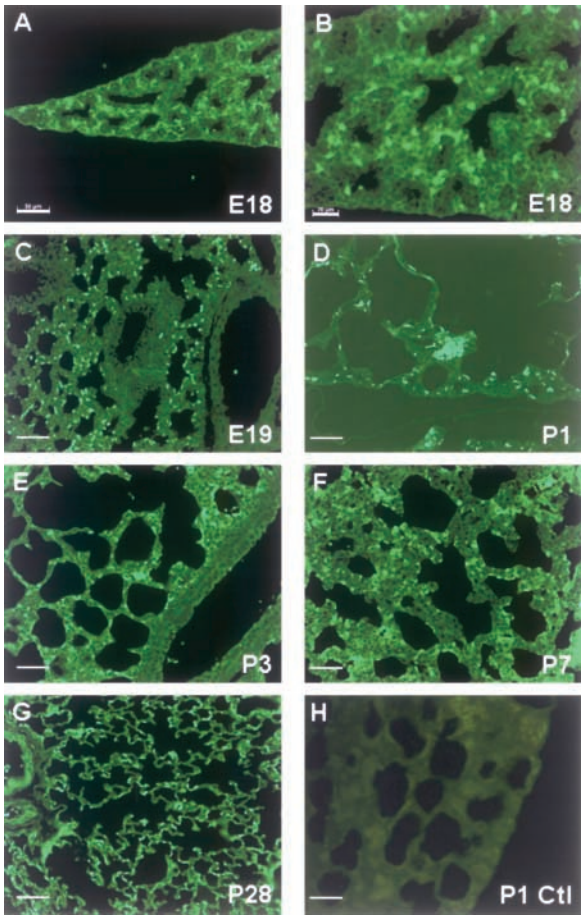

Figure 2. mRNA-*in situ* hybridization of HIMF expression in mouse lung. Tissue sections were probed with 6-Fam–labeled antisense oligodeoxynucleotide probes for fluorescence detection. Expression of HIMF mRNA was consistently found from E18 to P28. *HIMF* mRNA expression in embryonic lung (*A–C*) was localized to mesenchymal, interstitial cells (*B*). In postnatal lung (*D–G*) *HIMF* mRNA expression was frequently observed in cells lining the alveolar space (*E*, *G*) as well as some interstitial cells. Sense control (*H*) is depicted in P1 lung.

found *HIMF* mRNA expressed in alveolar cells (Figures 2B, 2C, and 2G) and interstitial cells (Figures 2D and 2E) during the saccular and alveolar stages of lung development. In contrast, a strong expression of *HIMF* mRNA was noted in bronchiolar epithelial cells of adult lung (data not shown).

Expression of HIMF, HIF-1α, and HIF-2α in Embryonic Lung Extracts

To show the presence of the *HIMF* gene product in the developing lung, a Western blot analysis was performed. HIMF expression started at E16, was readily detectable at E17 and strongly expressed through late pregnancy until the perinatal period at P3 (Figure 3A). No HIMF was detected at E14 or E15. Probing the lung protein extract for the presence of HIF-1α revealed high concentrations of HIF-1α at E14 but very low levels during the latter stages. In contrast, HIF-2α was readily detectable from E14 through E20 and postnatally until P3 (Figure 3A). In addition, later stages of postnatal lung development (Figure 3B) were immunoprobed, normalized to tubulin and quantified revealing a significant ($P < 0.005$) upregulation of HIMF protein from throughout the saccular and alveolar stages (P1–P40, Figure 3C).

Immunohistochemical Localization of HIMF and Coexpression with HIF-2α

Next we were interested in demonstrating which cells express HIMF in the developing lung. A strong HIMF expression was found in the saccular stage of lung development at E18 in late gestation (Figure 4). Here HIMF was detected ubiquitously in the epithelial cells of conducting airways (Figure 5A) and in cells lining the primitive alveoli and some cells of the interalveolar mesenchyme (Figures 5B and 5C). Endothelial cell of the intrapulmonary vessels were stained positive for HIMF as well (data not shown). In contrast, HIMF expression could not be detected at the pseudoglandular stage (data not shown). The expression of HIMF remained at high levels until P3 (Figure 5E), where HIMF was found in bronchiolar epithelial, alveolar epithelial, and endothelial cells. The dual immunohistochemistry depicted in Figure 5 revealed that throughout the saccular and aleolar stages HIMF was colocalized with HIF-2. At P7 the nuclear accumulation of HIF-2α was particularly prominent (Figure 5F). In the late alveolar stages HIMF–HIF-2α colocalization was readily detectable in bronchial epithelial cells (Figures 5H and 5K) and in type II alveolar cells (Figures 5I and 5J).

Weak Expression of HIF-1α in the Cannalicular and Saccular Stages

To further explore the possibility of a regulatory effect of HIFs on the expression of HIMF, we investigated whether HIF-1α, and ARNT were expressed temporally synchronized with HIMF.

In pseudoglandular lung tissue at E14 (Figure 6A), HIF-1α was detected mainly in bronchial epithelial cells. In contrast, in the canalicular (Figure 6B) and terminal sac (Figures 6C and 6D) stages of lung development HIF-1α was found at very low levels, incidentally suggesting that at these stages of lung development the tissue is not hypoxic. ARNT, the dimerization partner of HIF-1α and HIF-2α, was expressed in all lungs investigated (Figures 6E and 6F, and data not shown) without significant differences in signal strength between the developmental stages. Interestingly, HIF-1α was readily detectable in the nuclei of cardiomyocytes at E12 (Figure 6G), which is consistent with the known importance of HIF-1α for the development of the cardiovascular system.

Effect of HIMF on Apoptosis in Lung Explants

During the last trimester of pregnancy, organ growth and maturation are dominant developmental processes, of which programmed cell death has been recognized to be an important feature. Therefore, the effect of HIMF on apoptosis in the developing lung was investigated in E13.5 and E16 lung explant organ cultures (Figure 7A). Treatment with 100 nM recombinant HIMF significantly reduced the number of TUNEL-positive, apoptotic cells ($P < 0.005$, Figure 7B). These findings indicate that HIMF might play an important role in the regulation of apoptotic activities in the lung.

Discussion

The anatomic development of the lung can be regarded as a continuous process from the advent of the laryngotracheal groove until adulthood. Although recently considerable insight had been gained into budding morphogenesis and early pulmonary development (4, 12), the regulators of normal lung development during late gestation are not known, and the mediators that interfere with lung development are not well understood (13).

In this study we investigated the expression of HIMF in the developing mouse lung. We discovered that HIMF is present in

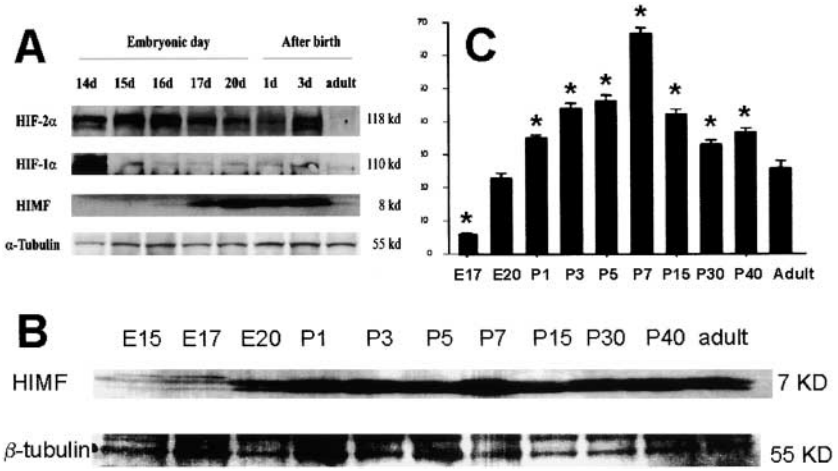

Figure 3. Developmental Western blot for HIMF, HIF-1α, HIF-2α, and α-tubulin 25 μg of total protein from lungs isolated and pooled from different litters ($n = 3$) were analyzed. HIMF was strongly upregulated in the saccular (*A*) and alveolar (*C*) stages of lung development. Expression of HIMF was very low at E14-E16. HIF-1α concentration was high in E14 lung but remained low from E15 through late gestation until P3 (*A*). In contrast, HIF-2α was readily detected in E14 to P3 lung but not in adult lung (*A*). Densitometric quantitation of HIMF expression normalized to α-tubulin (*B*) revealed a significant ($*P < 0.005$) increase of HIMF protein in the alveolar stage compared with HIMF levels in adult lung (mean ± SEM, paired *t* test).

the developing lung and that pulmonary expression of HIMF is upregulated specifically in the saccular and alveolar stages. In the pseudoglandular stage HIMF was not found to be expressed, whereas in the saccular and alveolar stages HIMF was readily detectable and continued to be markedly upregulated until P40. Cells staining positive for HIMF were epithelial cells of the conducting airways, mesenchymal cells, and type II alveolar cells. In addition, results of dual immunohistochemistry showed that HIMF and HIF-2α were colocalized.

The 8-kD protein HIMF, also known as FIZZ1 (Found in Inflammatory Zone [1]) and Relm-α (Resistin-Like Molecule [14]), was originally discovered in lung and adipose tissues in mouse and human. In adult mouse lung, low-level HIMF mRNA and protein expression has been reported in bronchial epithelial cells and peribronchial stroma (15). Upon allergic pulmonary inflammation as well as in chronic hypoxia, HIMF expression markedly increased in bronchial epithelium, type II alveolar cells, and pulmonary vasculature (1, 3). In addition, HIMF is upregulated in macrophages in chronic type II inflammation and thus has been considered a specific marker of alternatively activated macrophages (16).

Few data exist as to the function of HIMF. Analyzing glucose metabolism, Moore and coworkers demonstrated that HIMF expression was markedly reduced in diabetic mice, suggesting that HIMF might be involved in adipocyte homeostasis (17).

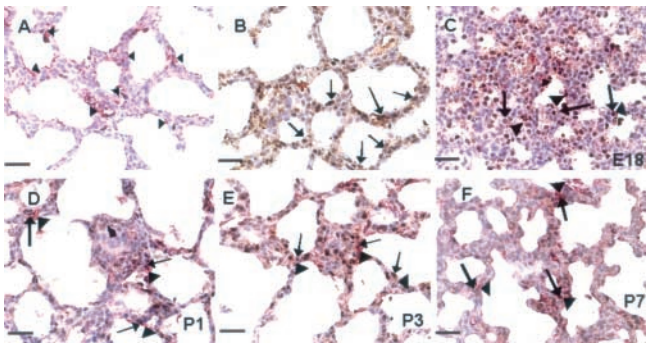

Figure 4. HIMF protein is expressed in type II alveolar cells *in vivo*. Standard and double immunohistochemistry (DIHC) for alkaline phosphatase (Fast Red) and HIMF (DAB). The localization of alkaline phosphatase as a marker for type II alveolar cells (*A*, *red color*) and HIMF (*B*, *dark brown*) was shown as separate immunohistochemistries (*A*, *B*) as well as the localization of HIMF in type II alveolar cells demonstrated using DIHC for alkaline phosphatase and HIMF (*C–F*). DIHC clearly demonstrates the expression of HIMF in type II alveolar cells, where HIMF was mainly localized in the cytoplasm. (*C*) E18, (*D*) P1, (*E*) P3, (*F*) P7. All photos at magnification ×400, scale 20 μm. *Arrows* indicate HIMF expression, *arrowheads* indicate alkaline phosphatase expression.

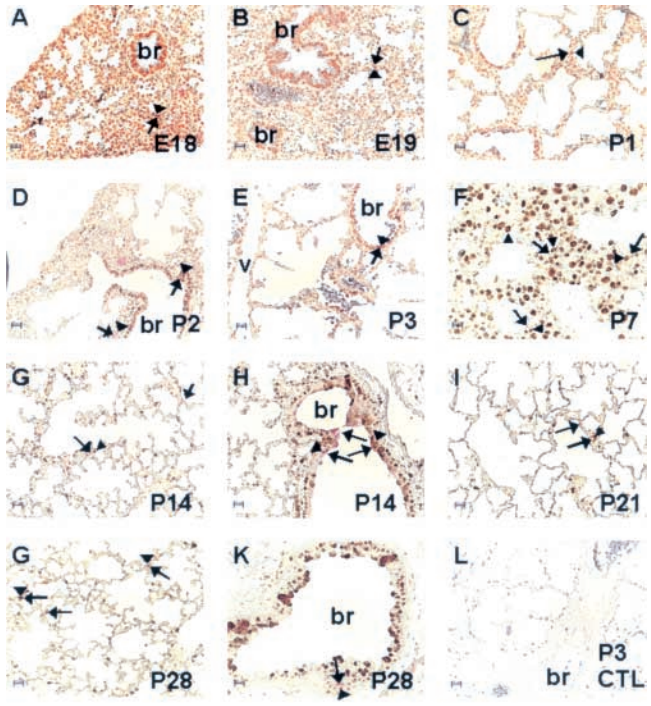

Figure 5. In vivo simultaneous detection of HIMF and HIF-2α protein in the developing lung. In the saccular stage (*A*, *B*), HIMF (*arrows*) was strongly expressed in immature alveolar, interstitial, and epithelial cells with HIF-2α localized to the cell nucleus. In postnatal lung days P1–P3, HIMF protein was clearly detectable (*arrows in C–E*) but less prominent compared with E18–E19 lungs. On postnatal day P7 (*F*), HIF-2α (*arrowheads*) was prominently expressed in the cell nucleus of interstitial cells. During the late alveolar stage (P14–P28) bronchiolar epithelial cells coexpressed cytoplasmatic HIMF (*arrows*; *H*, *K*) and nuclear HIF-2α (*arrowheads*; *H*, *K*). Negative control without primary antibodies was from P3 lungs (*L*). br, bronchiolus; v, vein. All panels at magnification ×200, scale 20 μm. *Arrows* indicate HIMF expression, *arrowheads* indicate HIF-2α expression.

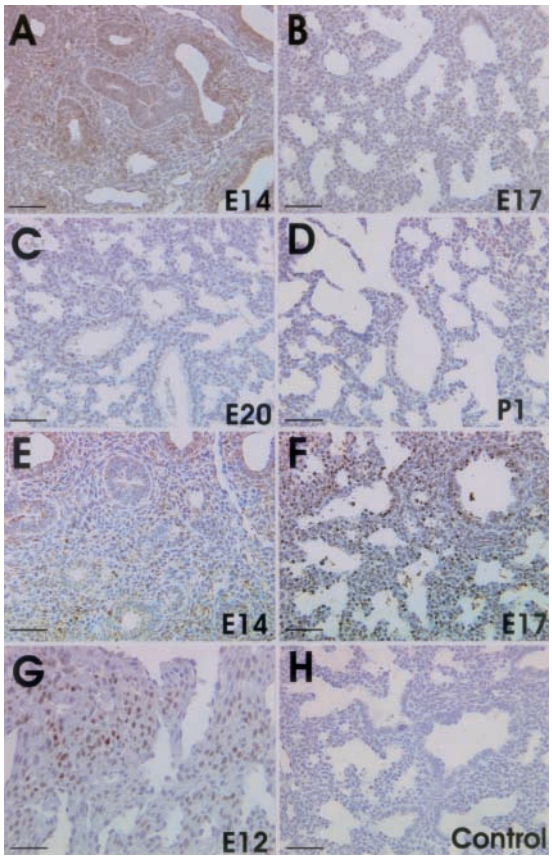

Figure 6. Expression of HIF-1α and its dimerization partner ARNT (HIF-1β) in the developing lung and heart. In pseudoglandular lung tissue (*A*) at E14 HIF-1α was detected mainly in epithelial cells. But in the canalicular (*B*) and terminal sac (*C*, *D*) stages of lung development HIF-1α was found at very low levels, incidentally suggesting that at these developmental stages lung tissue is not hypoxic. ARNT was ubiquitously expressed in lung tissue independent of developmental stage. As an example, ARNT expression at E14 (*E*) and E17 (*F*) are shown. HIF-1α was readily detectable in the nuclei of cardiomyocytes at E12 (*G*) thus highlighting the importance of HIF-1α for the development of the cardiovascular system. (*H*) Isotype negative control. All photos at magnification ×200, scale 50 μm.

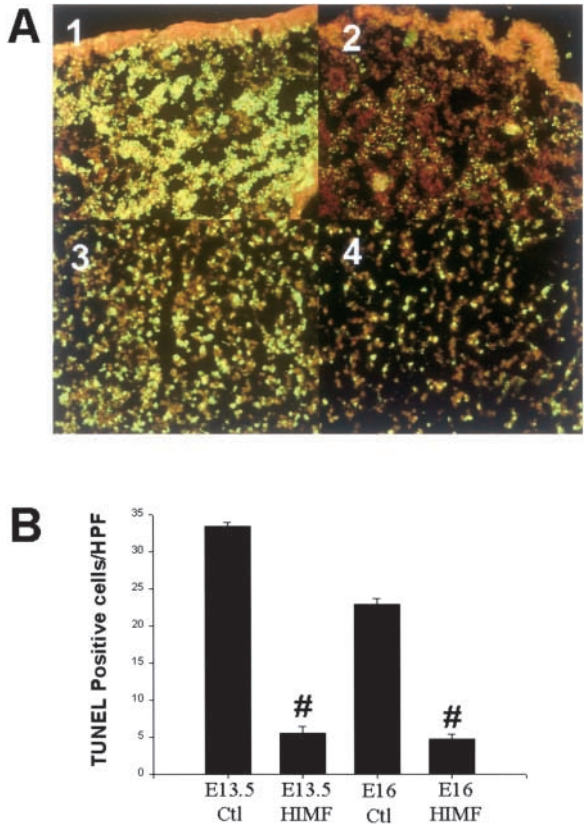

Figure 7. HIMF reduced apoptosis in embryo lung explant organ culture. (*A*) Embryonic lung explants treated with HIMF. TUNEL assay was performed to detect apoptosis and ethidium bromide (0.5 μg/ml, *red*) in PBS was used for nuclear staining. Lung explants from different pregnancies ($n = 3$) treated with HIMF (100 nM) for 24 h (E13.5, photo #2, and E16, photo #4) were found to have reduced numbers of TUNEL-positive cells compared with vehicle-treated control lung explants (photo #1: E13.5; photo #3: E16). (*B*) TUNEL-positive apoptotic cells were quantified per high-power field (×40, $n = 3$). HIMF treatment significantly reduced the number of apoptotic cells ($^{\#}P < 0.005$, paired *t* test).

The administration of HIMF to preadipocytes inhibited their differentiation into adipocytes (18). Stutz and colleagues provided evidence of functional binding sites for STAT6 and CCAAT/Enhancer-binding protein (C/EBP) in the HIMF promotor (19). We recently showed that HIMF has potent proliferative, angiogenic, and pulmonary vasoconstrictive effects (3). In rat pulmonary microvascular smooth muscle cells, AKT and phosphatidylinositol 3-kinase participate in HIMF downstream signaling. But, up till now the receptor of HIMF has remained elusive, contributing to our incomplete understanding of the HIMF signaling pathway.

Programmed cell death has an important function in the cellular remodeling of the developing lung (7), in particular during late gestation, when HIMF expression is at the highest level. Analysis of HIMF-treated lung explants revealed that HIMF significantly decreased apoptotic activity and increased lung cell density. In preterm infants, lung injury from infection or respirator therapy contributes to the development of bronchopulmonary dysplasia (20), which is morphologically characterized by a premature arrest of alveolarization and lung development (21). Because HIMF has potent proliferative, angiogenic, and pulmonary antiapoptotic properties, it is tempting to speculate that the growth-modulating properties of HIMF might overcome the developmental arrest of bronchopulmonary dysplasia.

Oxygen is an important physiologic mediator of embryonic and fetal development. *In vitro* studies demonstrate that proper embryonic development is dependent on physiologically low oxygen tensions (3–5%) (22). A motif analysis of the genomic *HIMF* sequence revealed an HIF-binding site (HBS) in the 3′ untranslated region. Since HIMF has been shown to be induced by hypoxia *in vivo*, it is reasonable to assume that the HBS in the *HIMF* gene is functionally responsive to HIFs (3).

HIF-1, a heterodimer composed of a regulated α-subunit and a constitutively expressed β-subunit (23), orchestrates the expression of genes contributing to the adaptation to reduced cellular oxygen availability and impaired energy state (24, 25). HIF-1α expression in the embryo is primarily found in neural, mesenchymal, and vascular tissues (8, 26). In agreement with the crucial role of HIF-1α for the early embryonic cardiovascular development, we found a strong HIF-1α expression in the heart at E12. In contrast, we observed a low expression of HIF-1α protein in the canalicular, saccular, and alveolar stages, thus confirming the findings of Jain and coworkers, who demonstrated low levels of HIF-1α mRNA in the developing lung (27). A possible explanation could be the absence of hypoxia of the

lung during embryonic development. Using the hypoxia marker pimonidazole, Lee and colleagues established that HIF-1α and vascular endothelial growth factor expression were spatiotemporally colocalized with hypoxic regions in embryos (28). They observed marked hypoxia in the developing brain and heart, but they were unable to detect hypoxic regions in the lungs (28). Compernolle and associates reported a pimonidazole adducts–positive signal exclusively in type II alveolar cells that was not present in directly neighboring cells (29). Still, these findings altogether indicate that in the developing lung there is no hypoxia present sufficiently severe to induce widespread accumulation of HIF-1α.

Recently, a second hypoxia-inducible transcription factor termed HIF-2α has been described (30–33). In human embryos aged 3–6 wk (34) and in the developing mouse embryo (27), mRNA expression of the two HIFs was investigated previously, and both studies described a high expression level of HIF-2α mRNA in the developing lung. Of note, comparing expression levels of *HIF-1*α and *HIF-2*α in 11 human cell lines, the highest levels of *HIF-2*α mRNA have been found in fetal lung fibroblasts (35). Our data expand this knowledge and we described for the first time the detailed pulmonary expression pattern of HIF-2α protein.

Compernolle and coworkers recently reported an HIF-2α knockout of which 50% of the HIF-2α$^{-/-}$ embryos died *in utero* from cardiac failure at E13.5. Of note, the remaining HIF-2α$^{-/-}$ embryos were born at term but died within 2–3 h after birth from fatal respiratory distress syndrome (29). Though therapeutic administration of inhaled vascular endothelial growth factor to preterm mice alleviated the respiratory pathology, the putative HIF target gene HIMF might have contributed to the observed phenotype. Unfortunately, an HIMF knockout mouse model is not available at present.

In summary, we showed that HIMF is expressed in the developing lung in the saccular and alveolar stages. HIMF protein colocalizes spatially and temporally with HIF-2α but not HIF-1α. While HIF-2α itself might play an important role in lung maturation, it is tempting to speculate that HIMF might be a downstream mediator of HIF-2α in embryonic lung development and maturation. The determination of the functional role of HIMF in embryonic lung warrants further investigation, ultimately contributing to a better understanding of diseases like bronchopulmonary dysplasia, characterized by a premature arrest of lung maturation.

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