

Ets-1 participates in and facilitates developmental expression of hypoxia-induced mitogenic factor in mouse lung

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1. ABSTRACT

Hypoxia-induced mitogenic factor (HIMF) possesses mitogenic, vasoconstrictive, angiogenic, and antiapoptotic effects. While HIMF is known to be expressed in developmental mouse lung, its gene expression regulation during this period is completely unknown. Genomic sequencing of HIMF gene has shown that there is an Ets-1 binding site in its 5'-promoter-region. To test the hypothesis that Ets-1 protein expressed in developing mouse lung may participate in the process of HIMF gene expression regulation via direct involvement or facilitation, we characterized the proximal promoter of HIMF gene (409-bp long fragment that includes the -347-bp promoter region from transcription start site), and investigated HIMF and Ets-1 expression with western blot and immunohistochemistry, electrophoretic mobility shift assay (EMSA) for Ets-1, HIMF promoter luciferase reporter gene assays, and chromatin-immunoprecipitation

(CHIP). Western blots revealed that both Ets-1 and HIMF proteins were developmentally expressed in the lung. Immunohistochemical localization revealed Ets-1 signals in the nucleus of HIMF-expressing airway epithelial cells and alveolar type II cells, whereas HIMF was localized in cytoplasm. Presence of Ets-1 protein within E16, E20, and adult lung nuclear extract was demonstrated by EMSA. Co-transfection of Ets-1 expression plasmid with HIMF promoter construct increased luciferase activity in NIH3T3 cells, but mutation or deletion of Ets-1 site eliminated HIMF promoter luciferase activity. CHIP with anti-Ets-1 antibody revealed Ets-1 binding to HIMF promoter region in E20 and P1 but not in E15 lung. We conclude that Ets-1 participates in the process of HIMF gene expression and Ets-1-mediated HIMF expression may play an important role in lung development and maturation.

2. INTRODUCTION

Developmental expression and regulation of genes in the lung play critical roles for its growth and maturation (1-5). Many genes from transcription factors to growth factors participate in the coordinated lung morphogenesis, branching, and maturation (2, 6-9). Temporal and spatial expression of these proteins directs proliferation and differentiation of lung progenitor cells, and determines the morphology and function of the lung (2, 10-12). Disruption of the order of gene expression during the lung developmental period will unquestionably result in malformed lung and impede its air-exchange function (5-7, 10, 13). Hypoxia-induced mitogenic factor (HIMF) [Genbank accession number AF516926] is a protein found in the lungs of a mouse model of hypoxia-induced pulmonary hypertension (14). This protein, also called Found in inflammatory zone 1 (FIZZ-1) from an inflammatory lung model (15) or Resistin-like molecule-alpha (RELMalpha) from adipose tissues (16, 17), belongs to a new family of cysteine-rich secreted proteins. The secreted mature form of HIMF protein is 89 amino-acid residues in length, with a motif consisting of 10 cysteine residues with a unique spacing of C-X₁₁-C-X₈-C-X-C-X₃-C-X₁₀-C-X-C-X-C-X₉-CC (C is for Cysteine and X is for any amino acid residue) at the C terminus. However, its function in the pulmonary system is still not completely understood. Previous study has demonstrated that HIMF possesses mitogenic, vasoconstrictive, and angiogenic properties (14). HIMF not only enhances pulmonary microvascular smooth muscle cell proliferation, it can also induce elevation of pulmonary arterial pressure. Its vasoconstrictive ability is more potent than angiotensin II, endothelin-1, and serotonin. In addition, recent studies from our laboratory demonstrated that HIMF also modulates VEGF and surfactant protein B and C expression (18, 19), indicating HIMF may play important roles in pulmonary vascular development and lung maturation. Moreover, we have also shown that HIMF expression is developmentally regulated in embryonic mouse lungs and it has antiapoptotic function in cultured embryonic lung explants (20). From embryonic day (E) 16 to postnatal day (P) 28, HIMF protein has been strongly expressed in bronchial epithelial cells and alveolar type II cells. Treatment with HIMF resulted in a significant reduction of apoptosis in cultured embryonic lung. HIMF is co-localized with hypoxia-inducible factor-2alpha (HIF-2alpha) in the developing airway epithelial cells and alveolar type II cells, indicating HIMF expression may be involved with HIF-2alpha. In addition, genomic sequencing of the HIMF gene, especially its 5'-upstream promoter region has revealed an array of transcription factor (TF) binding sites, such as Ets-1, GATA, PPAR-gamma, and C/EBP, indicating the possible role of these TFs in the regulation of HIMF gene expression during lung development.

Ets proteins comprise a family of transcription factors that share a unique DNA binding domain, the Ets domain (21-23). The name "Ets" stems from a sequence that was detected in an avian erythroblastosis virus, E26, where it formed a transforming gene together with *Deltagag* and *c-myb* (24, 25). The newly discovered

sequence was called E26 transforming specific sequence or Ets. Ets domain proteins belong to the superfamily of winged helix-turn-helix (wHTH) DNA-binding proteins which includes also hepatocyte nuclear factor HNF-3gamma, heat shock factor HSF and catabolite activator protein CAP (23). The consensus sequence for Ets-1 is PuCC/a-GGAA/T-GCPy as determined by several rounds of selection and amplification of Ets-1 binding sites (26). Studies have demonstrated that Ets-1 is widely expressed during murine embryo development and is associated with mesodermal cells involved in morphogenetic processes such as organ formation (27). In addition, Ets-1 has been involved in vascular development and angiogenesis (28, 29), and it participates in transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1) in coordination with hypoxia-inducible factor-2alpha (HIF-2alpha) (30). Kola and colleagues have reported that Ets-1 expression in embryos is in a temporal and tissue-specific manner in mice. Ets-1 expression is first detected by Northern blots on embryonic day 8 (E8) in total embryonic RNA. Thereafter, the levels of Ets-1 mRNA appear to increase in the embryo such that on E15, Ets-1 is expressed at high levels in all of the individual organs. However, on E16, a dramatic shift in the pattern of Ets-1 expression occurs. Ets-1 is no longer ubiquitously expressed at high levels. Rather, its high level of expression is maintained in some organs, especially in the lung, indicating Ets-1 may play important roles in the expression regulation of lung specific genes. A recent study reported by Kathuria and coworkers (31) has shown that Ets-proteins regulate caveolin-1 gene expression in lung type I epithelial cells, rather than in endothelial cells. This strongly suggests that Ets-1 participates in alveolar development and maturation. To elucidate the role of Ets-1 in HIMF gene expression during lung development, in this study we examined Ets-1 expression and its relation with HIMF gene expression.

3. MATERIALS AND METHODS

3.1. Reagents and animals

All experiments performed with animals were approved by the Animal Care and Use Committee of Saint Louis University. Timed pregnant C57/Bl6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were euthanized by halothane overdose. For the collection of the embryonic lungs, laparotomy was performed, the embryos were harvested and the lungs were excised for nuclear protein extraction, western blot, immunohistochemical, and chromatin-immunoprecipitation (CHIP) analyses. Ets-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA.), which is an affinity purified rabbit antibody raised against a peptide mapping at the C-terminus of Ets-1 of human origin. It specifically interacts with Ets-1 and does not cross-react with other members of the Ets family proteins, like Ets-2, Erg-1, Erg-2, Elk, PU.1, and PEA3. Luciferase reporter gene system was purchased from Promega (Madison, WI).

3.2. Cell culture

NIH3T3 and mouse lung epithelial-12 (MLE-12) cells were obtained from ATCC (Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium

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(DMEM, Invitrogen, Carlsbad, CA; for NIH3T3 cells), or RPMI-1640 (for MLE-12 cells) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 microgram/ml streptomycin (Sigma, St. Louis, MO).

3.3. Western blots for Ets-1 and HIMF in developing lung

Timed pregnant, neonatal, and adult mice were sacrificed and the E15 and E17 lungs were excised by manual dissection with the aid of a dissecting microscope. Lung samples were collected from embryonic day 15 (E15), E17, E20, postnatal day 1 (P1), P3, P5, P7, P15, P30, P40 and adult (10 weeks old) mice. Total protein was determined from individual (E20 and P1 to adult) or pooled (E15 and E17) whole lungs as previously described (20). The protein concentration was determined using Bradford method. Fifty micrograms of total lung proteins from each sample were subjected to electrophoresis. In brief, protein extracts were separated on a 4-20% SDS-polyacrylamide pre-cast gel (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. Immunodetection was performed using the following antibodies: HIMF (1:1000, Alpha Diagnostic Int., San Antonio, TX, USA), Ets-1 (1:500), and alpha-tubulin (1:1000, Santa Cruz), which was used as loading control. Incubation with primary antibody was followed by incubation with a 1:3000 dilution of goat anti-rabbit or goat anti-mouse HRP-labeled antibody (Bio-Rad, Hercules, CA, USA) and visualized with ECL (Amersham Biosciences, Buckinghamshire, UK).

3.4. Immunohistochemical staining of Ets-1 and HIMF

Fresh frozen lung samples from E12, E15, E17, E20 embryos, P1 and adult mice were embedded in OCT (Sakura Finetek, Torrance, CA). For Ets-1 staining, five micrometer sections were cut and fixed in cold acetone for 10 min then treated with 3% H₂O₂ in methanol for 30 min to quench the endogenous peroxidase activity. After blocking with 10% goat serum in PBS, Ets-1 antibody (1:50 dilution) was applied to each section. For HIMF staining, 4% paraformaldehyde fixed, paraffin sections were used. After deparaffinization, the sections were treated with 3% H₂O₂ in methanol and blocked with 10% goat serum for 1 hour. For both Ets-1 and HIMF staining, Vector immunostaining rabbit kit (Vector, Burlingame, CA) was used and the dilution for secondary and tertiary antibodies was 1 in 200. DAB kit (Dako, Carpinteria, CA) was used for the visualization of the dark-brown positive signals.

3.5. HIMF promoter construct

A 409 base-pair (bp) fragment of DNA, including 347 bp 5'-upstream promoter region of the HIMF gene, was generated with PCR using primers (5'-CTGACTCGAGAGCAGGATCA GC TTGAATGG-3' and 5'-AGTCAAGCTTTTCCA GGACCTGGCCAGATG-3') according to the sequence from the Genbank [accession number AF516926] and mouse genomic DNA (Clontech Laboratories, Palo Alto, CA) as a template (14, 32). The PCR was performed with 0.5 micrograms of mouse genomic DNA (94 °C, 45 s; 60 °C, 30 s and 72 °C, 45 s; 30 cycles) in 50 microliters of 1 x PCR Master (Roche). The PCR fragments were purified with Qiagen PCR

purification kit (Qiagen, Valencia, CA), digested with *XhoI/HindIII* and cloned into pGL3Basic vector (Promega, Madison WI) to give pGLHIMF409 luciferase reporter construct. The construct was validated with restriction enzyme digestion and DNA sequencing. The potential transcription factor binding sites in the 347 bp 5'-upstream promoter region of HIMF gene transcription starting site were analyzed with Genomatix program MatInspector at <http://www.genomatix.de/cgi-bin/eldorado/main.pl> with Core Similarity as 1.0 and Matrix similarity higher than 0.9.

3.6. Transfection and reporter gene assay

Because there are no cell lines that constitutively express HIMF, we used freshly isolated primary mouse embryo lung cells (PMELC) for HIMF promoter activity analysis. Briefly, after isolation from E14 (no HIMF expression) and E18 embryos (with HIMF expression), the lungs were digested with 0.25% Trypsin-EDTA solution (Invitrogen) for 20 min. The cell suspension was isolated from the tissue pieces and centrifuged at 800 rpm for 8 min, then resuspended in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 microgram/ml streptomycin (Sigma, St. Louis, MO). For the detection of luciferase activity of HIMF promoter, PMELC were plated into 24-well plates and allowed the cells to grow to 60% confluency. The pGLHIMF409 construct was transfected into PMELC cells using FuGENE6 (Roche, Indianapolis, IN) following the manufacturer's instructions and pEGFP plasmid (Invitrogen) was used to monitor the transfection efficiency in the cells. The transfection mixture, containing 50 microliters of serum-free medium and the transfection reagent, was preincubated for 15 min. Plasmid DNA of pGLHIMF409 (0.5 microgram/well) and pEGFP (0.2 microgram/well) was added to the mixture and further incubated for 15 min. The standard culture medium was refreshed 1 hour before the transfection started. The transfection mixture was added dropwise to the well. Twenty four hours after transfection, the cells were examined under a fluorescent microscope to determine the transfection efficiency and then lysed with cultured cell lysis buffer (Promega). The luciferase activity was measured using Luminometer Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany). Luciferase assay system (Promega) was used for the promoter activity measurements.

3.7. Nuclear protein extract preparation

Nuclear proteins from MLE-12, NIH3T3 cells, E16, E20, and adult lung were obtained using Nuclear Extraction kit (Panomics, Redwood City, CA) according to the manufacturer's instructions. Briefly, lung tissues were homogenized in Dounce homogenizers using 15 strokes to break up cells or the tissue pieces. Then 0.5 ml of buffer A (10 mM HEPES, pH 7.9, 10 mM KCL, 10 mM EDTA, 10 mM DTT plus protease inhibitor cocktail) was added and incubated on ice for 15 min. After centrifugation at 850 x g for 10 min, the pellets were resuspended in 0.5 ml buffer A and centrifuged at 15,000 x g for 3 min. The pellets were resuspended in 100 microliter of buffer B (10 mM HEPES, pH 7.9, 0.2 M NaCl, 0.5 mM EDTA, 5% Glycerol, 1.0 mM

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DTT and protease inhibitor cocktail) and shaken for 2 hours on ice at 200 rpm. The supernatants of the samples were used as nuclear extract, following centrifugation at 15,000 x g for 5 min. The protein concentration was determined using Bradford method (33).

3.8. Co-transfection of Ets-1 expression plasmid with HIMF promoter constructs

pCMV-Sport6-Ets-1 expression vector was obtained from ATCC (Manassas, VA). Ets-1 expression was validated by transfection of HEK293 cells with pCMV-Sport6-Ets-1 and then followed by western blot analysis for Ets-1. For the co-transfection studies, NIH3T3 cells were used because preliminary experiments showed that this cell line has low luciferase activity for pGL3Basic vector and did not constitutively express HIMF. For the determination of the role of Ets-1 in HIMF promoter activity, pGLHIMF409 and pCMV-Sport6-Ets-1 were co-transfected into NIH3T3 cells with FuGENE6. Twenty four hours after transfection, the cells were lysed and luciferase activity was determined as described above. pEGFP plasmid was co-transfected in the experiments to determine the transfection efficiency.

3.9. Electrophoretic-Mobility Shift Assay (EMSA) of Ets-1

EMSA was performed using Panomics (Redwood City, CA) EMSA kit according to the manufacturer's instructions. Briefly, five micrograms of nuclear extracts from NIH3T3, MLE-12, E16, E20 and adult lung were incubated with 10 ng of Ets-1 probe (sequence: GATCTCGAGC AGGAAGTTCGA) with or without 100 ng unlabeled probe as indicated in binding buffer for 5 min. Then the samples were subjected to 5% polyacrylamide TBE gel electrophoresis for 50 min and transferred to nitrocellulose membranes, the shifted bands corresponding to the Ets-1 protein and Ets-1 probe was visualized with streptavidin-HRP, chemiluminescence system (Panomics).

3.10. Ets-1 mutation and deletion from pGLHIMF409

Site-directed mutation and deletion in the Ets-1 site were generated using GeneTailor Site-Directed Mutagenesis System (Invitrogen) following the manufacturer's instructions. The following oligonucleotide primers were used, Ets-1 mutated forward primer: 5'-gcagataaagtgggtt acatactcaagtccttga-3'; Ets-1 deleted forward primer: 5'-gcagataaagtgggttac****tcaagtccttga-3'; and Ets-1 reverse primer: 5'-aaaccactttatctgcttggaaaagtct-3' to generate constructs pGLHIMF-Ets-M and pGLHIMF-Ets-D. Briefly, after methylation of the wild type pGLHIMF409 plasmid, PCR was performed with either oligonucleotide primers of Ets-1 mutation or Ets-1 deletion with the reverse primer at 94 °C (2 min); 55 °C (30 s) and 68 °C (6 min), for 25 cycles. After 1% agarose gel electrophoresis to confirm PCR products, the DNA was transformed into DH5alpha-T1 competent cells. Plasmids were isolated and analyzed by digestion with restriction endonucleases (*XhoI/HindIII*) and DNA sequencing to validate the mutation and deletion.

3.11. CHIP with anti-Ets-1 antibody in developing lung

Chromatin-immunoprecipitation was performed according to the methods reported previously with minor

modifications (34, 35). Briefly, E15, E20, and P1 lungs were fixed in 2% formaldehyde for 15 min, followed by addition of 1M glycine, then homogenized in RIPA buffer. MLE-12 and NIH3T3 cells were fixed in 1% formaldehyde for 5 min, then treated as the tissue samples. After separation with the cytoplasmic proteins, the pellets were sonicated and the supernatant collected. Immunoprecipitation was carried out by addition of 2 micrograms of polyclonal anti-Ets-1 antibody at 4°C overnight. The controls were added with buffer only, without antibody, followed by incubation with 45 microliters of proteinA/G-agarose (Santa Cruz). After 4 hour incubation the samples were washed sequentially with TSE I, TSE II and Buffer III. Elution of Protein-DNA complexes were carried out by incubating beads in elution buffer (1% SDS, 0.1 M NaHCO₃) and heated at 65 °C overnight. The DNA fragments were purified with Qiaquick PCR Purification kit (Qiagen) in a final volume of 50 microliters of elution buffer (10 mM Tris-HCl, pH 8.5). HIMF promoter PCR was performed using the primers and the same conditions as in Section 3.5. Only 1/20 of the DNA samples were used in the input reaction. The PCR products from input, Ets-1 CHIP, and No-antibody controls were visualized by 1.5% agarose gel electrophoresis.

3.12. Data presentation and statistics

All data are represented as mean ± standard error of the mean. Comparisons between the groups were tested using ANOVA on ranks according to the Kruskal-Wallis and Dunn test. Statistical significance was set at p < 0.05. Calculations were performed with the SigmaStat 2.03 software (Jandel, Erkrath, Germany).

4. RESULTS

4.1. Western blot for Ets-1 and HIMF in developing lung

For the examination of Ets-1 and HIMF protein expression in developing mouse lungs, western blot analyses were performed. Previous study from our laboratory has demonstrated that HIMF is only expressed after E16 in the lung. We assessed parameters lungs from E15 and older animals. It showed that Ets-1 protein was highly expressed in E15 lung and slightly reduced in E17, E20, and P1. However, Ets-1 protein levels increased again from P5 through P30 then started to decrease at P40. There was only a low level expression in adult lungs (Figure 1). HIMF protein appeared at E17 and peaked at P7, then started to decrease at P30 and P40 as were Ets-1 concentrations. HIMF protein levels were also very low in adult lungs (Figure 1).

4.2. Immunohistochemical staining of Ets-1 and HIMF

For the localization of Ets-1 and HIMF producing cells, immunohistochemical detection of Ets-1 was performed in frozen sections in E12 embryos, E16, E20, P1, and adult lungs. In E12 embryo, Ets-1 positive cells were found in heart, lung, liver, and brain (data not shown). In E12 lung, Ets-1 was expressed in the nucleus of both glandular epithelial cells and mesenchymal cells (data not shown). However, with the development closer to birth,

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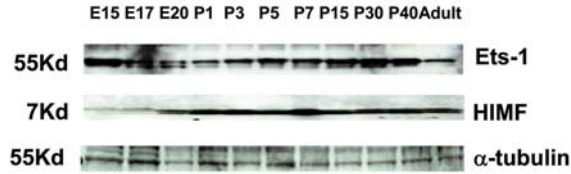


Figure 1. Western blot analyses of Ets-1 and HIMF expression in developing mouse lungs. There is a high level of expression of Ets-1 in E15 lung and then a slight decrease at E17 and E20, followed by a gradual increase during the period of alveolar stage (from P5 to P30). In the adult lung, the Ets-1 level is relatively low. HIMF protein is elevated starting from E17 and reached peak at P7, then maintained at high levels during the alveolar stage. Alpha-tubulin was taken as a loading control.

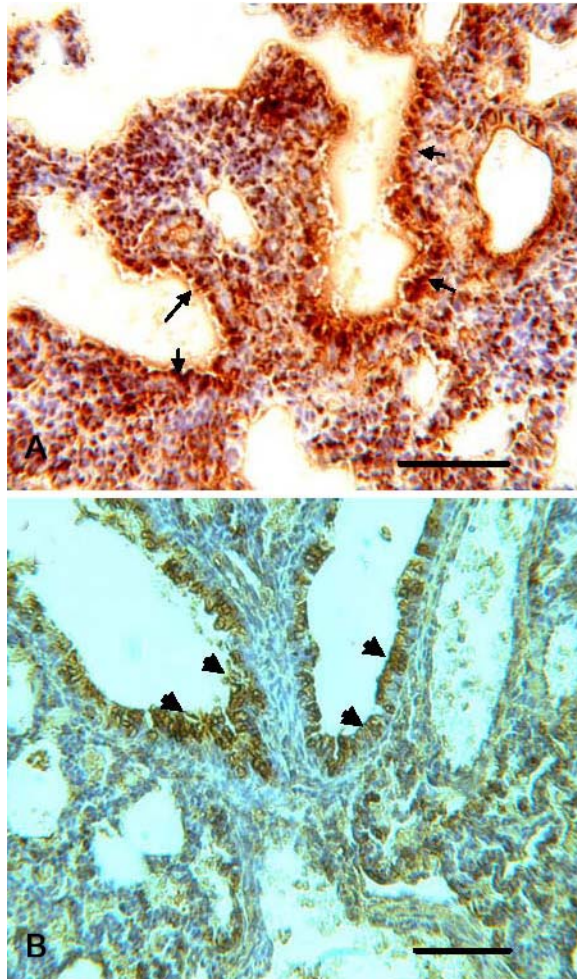


Figure 2. Immunohistochemical staining of Ets-1 (A) and HIMF (B) in E20 lung. Ets-1 protein is mainly located in the nucleus of the airway epithelium and type II cells (arrows in A) of the alveoli. However, HIMF is in the same cells, but only in the cytoplasm of the cells (arrowheads in B). Scale bar: 100 micrometer.

Ets-1 was mainly localized in the airway epithelial cells, primary type II cells of the alveoli, and some of the vascular endothelial cells (Figure 2A). HIMF immunostaining showed nearly the same pattern as Ets-1 in E20 lungs but only in the cytoplasm of the positive cells, particularly in airway epithelial cells and type II cells of the alveoli (Figure 2B).

4.3. Sequence and motif analysis of the 5'-proximal region of HIMF gene

In order to assess the promoter activity of the 5' flanking region of the HIMF gene, we cloned and analyzed the proximal promoter sequence of 409-bp fragment of HIMF gene (including -347-bp fragment sequence upstream of the transcription starting site by matching HIMF cDNA with genomic DNA sequences to determine the transcription start site), using computer-based software MatInspector. No TATA box was found in this region suggesting that HIMF expression uses a TATA-less promoter, which is likely regulated by multiple transcription factors. Moreover, several transcription factor binding sites existed in this region, including GATA, Ets-1, and C/EBP (Figure 3). These data indicated that these transcription factors have the potential to form the transcritosome and participate in the regulation of HIMF gene in the developing lung.

4.4. Luciferase reporter gene assay in the PMELC

For the determination of whether the 409-bp fragment of HIMF promoter region regulated HIMF gene expression, pGL3Basic and pGLHIMF409 were transfected into freshly isolated PMELC from E14 and E18 lung separately. Twenty four hours after transfection, the cells were examined under a fluorescent microscope to determine the transfection efficiency. The GFP positive cells from E14 and E18 PMELC transfected with pGL3Basic and pGLHIMF409 were $21.2 \pm 4.3\%$, $22.8 \pm 5.8\%$, $19.8 \pm 4.7\%$, and $20.3 \pm 5.6\%$ respectively ($p > 0.89$ among all groups). The cells then were lysed for luciferase reporter gene assay. The results showed that there was low luciferase activity in E14 PMELC transfected either with pGL3basic or pGLHIMF409 vector alone. In the E18 PMELC, there was a significant increase of luciferase activity ($p < 0.001$) compared to pGL3basic transfected E18 cells and pGLHIMF409 transfected E14 cells. These results indicated that E18 PMELC has the necessary transcription factors to promote HIMF gene expression (Figure 4).

4.5. Electrophoretic-Mobility Shift Assay (EMSA)

We next examined the effects of nuclear extracts from E16, E20 and adult lung on the Ets-1 binding and gel shift abilities using biotin-labeled and unlabeled cold Ets-1 probes. The results showed that E16, E20 and adult lung nuclear extracts contained Ets-1 protein which caused a shift of the probe. Moreover, addition of cold Ets-1 probe completely eliminated the shifting band in E20 lung nuclear extracts indicating the existence of specific Ets-1 binding protein (Figure 5).

4.6. Ets-1 mutation and deletion

To determine whether Ets-1 binding plays a key role in HIMF promoter luciferase activity, we mutated and

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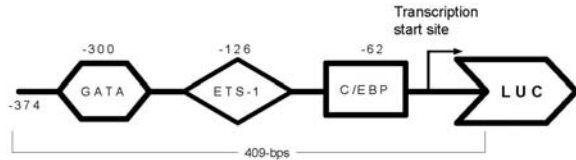


Figure 3. Sequence of transcription factor binding sites in HIMF gene 5' proximal promoter region. A 409-bp fragment including -347-bp fragment from the translation starting site was cloned into pGL3basic vector to form pGLHIMF409 construct. There are several potential transcription factor binding sites, such as GATA, Ets-1, and C/EBP. The Ets-1 site is located at the position -126-bp upstream of the transcription start site.

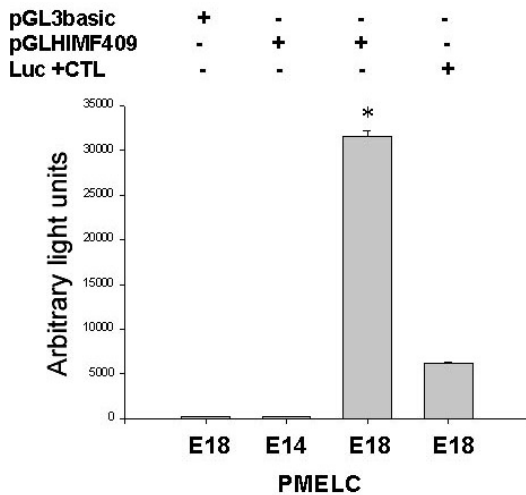


Figure 4. Luciferase activity in primary mouse embryonic lung cells (PMELC) from E14 and E18 embryos transfected with pGL3basic, pGLHIMF409, and luciferase positive control vector. There is high level of luciferase activity in E18 PMELC but not in E14 PMELC, indicating the E18 lung cells contain the proper transcription factors for HIMF expression. * $P < 0.05$ compared with the other groups. The data present 3 experiments with triplicate for each experiment.

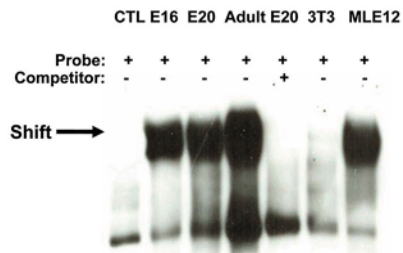


Figure 5. Electrophoretic mobility shift assay (EMSA) for Ets-1 binding proteins in E16, E20, adult lungs, NIH3T3 and MLE-12 cell nuclear extracts. There is Ets-1 protein in all the lung tissues and in MLE-12 cells, which caused the probe for gel-shift (arrow) but not in NIH3T3 cells. Unlabeled (competitor) Ets-1 probes prevented the shift detection (E20) indicating the interaction of the Ets-1 probe and the Ets-1 protein is specific. CTL is the Ets-1 probe only incubated with nuclear extraction buffer without protein.

deleted the Ets-1 binding site and generated pGLHIMF-Ets-M and pGLHIMF-Ets-D luciferase constructs and transfected them into E18 PLEMC. It showed that either mutation or deletion of the Ets-1 binding site resulted in elimination of HIMF promoter luciferase activity, indicating the importance of Ets-1 in the regulation of HIMF gene. In addition, MLE-12 cells, but not NIH3T3 cells also showed Ets-1 binding activity (Figure 6).

4.7. Co-transfection studies

To validate whether the Ets-1 binding will promote HIMF promoter activity, pGL3basic, pGLHIMF409, pGLHIMF-Ets-M and pGLHIMF-Ets-D were transfected with or without Ets-1 expression plasmid in NIH3T3 cells. It showed that mutation and deletion of Ets-1 site in the HIMF promoter construct had the same effects as we have seen in the PMELC, abolished luciferase activity. Co-transfection of Ets-1 expressing plasmid, pCMV-Sport6-Ets-1, induced significant elevations of luciferase activity only in pGLHIMF409 but not in pGLHIMF-Ets-M transfected cells, indicating Ets-1 participated in HIMF gene expression regulation (Figure 7). This result demonstrated the significance of Ets-1 in the control of HIMF promoter activity.

4.8. CHIP with anti-Ets-1 antibody in HIMF promoters

To confirm Ets-1 binding to HIMF promoter region in the developing lung, CHIP with samples from E15, E20, P1 lungs, MLE-12 and NIH3T3 cell lines was performed. The results showed that Ets-1 bound to the Ets-1 binding site in the HIMF promoter in E20 and P1 lungs but not in the E15 sample. MLE-12 cells have lower Ets-1 binding activity and NIH3T3 cells did not have any Ets-1 interaction in the HIMF promoter (Figure 8).

5. DISCUSSION

5.1. General aspects of HIMF and Ets-1

In the developing lung, branching morphogenesis involves mesenchymal-epithelial signaling that induces cellular proliferation, migration, and subsequent transcriptional activation of lung specific genes. The major function of these signaling pathways is to potentiate the activity or expression of mesenchyme- or endoderm-specific transcription factors in the lung. These, in turn, bind cooperatively to distinct promoter regions and activate target gene expression. The dynamic changes in gene expression during lung development are critical to mediate lung morphogenesis, and further maturation (2, 6). HIMF, as one of these genes, is a secreted protein, specifically expressed in the developing (20), inflammatory (15), fibrotic (36), hypoxic (14), and regenerating lungs (37).

Previous studies have shown that HIMF possesses mitogenic, vasoconstrictive, and angiogenic effects (14). Moreover, HIMF is also developmentally regulated and it has anti-apoptotic effects in the embryonic lung (20). It can stimulate lung myofibroblast differentiation (38). In addition, recent studies from our laboratory showed that there is an upregulation of HIMF gene expression in the lungs of compensatory growth after pneumonectomy, and intratracheal instillation of HIMF induced widespread cell

Ets-1 regulates HIMF in developing lung

Ets-1 wild type: gcagataaagtggttacttcctcaagtctctga
 Ets-1 mutated: gcagataaagtggttacaatacaagtctctga
 Ets-1 deleted: gcagataaagtggtttac****tcaagtctctga

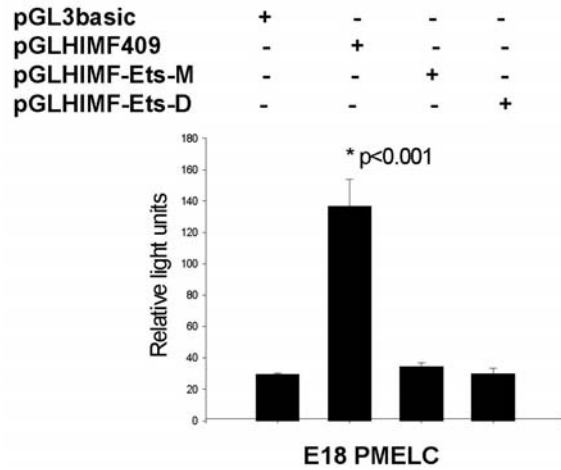


Figure 6. Luciferase activity in E18 PMELC transfected with different HIMF promoter constructs of wild type (pGLHIMF409), Ets-1 mutation (pGLHIMF-Ets-M) or Ets-1 deletion (pGLHIMF-Ets-D). Ets-1 mutation or deletion completely abolished the luciferase activity in these cells. Data represent 2 experiments and the pGL3basic activity as the baseline.

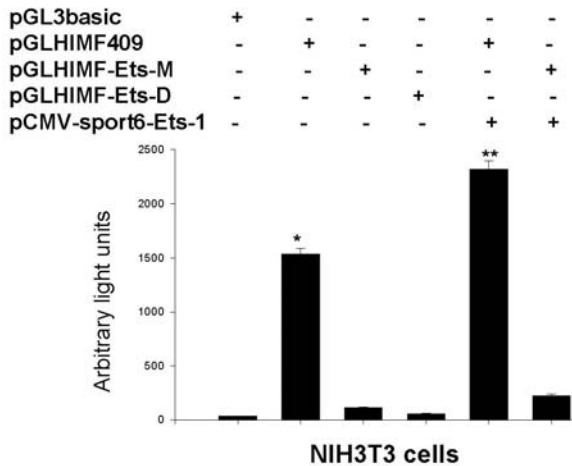


Figure 7. Luciferase activity in NIH3T3 cells transfected with different HIMF promoter constructs with wild type (pGLHIMF409), Ets-1 mutation (pGLHIMF-Ets-M) or Ets-1 deletion (pGLHIMF-Ets-D). Ets-1 mutation or deletion abolished the luciferase activity as we expected. Co-transfection of Ets-1 expression plasmid, pCMV-Sport6-Ets-1 enhanced the luciferase activity only in the wild type but not in the mutated constructs. The data represent 2 different experiments in triplicate for each experiment. * $p < 0.05$ compared with other groups; ** $p < 0.05$ compared with pGLHIMF409 group. The experiments were performed 3 times with identical results.

proliferation in airway epithelial cells, alveolar type II cells, and cells in the alveolar septa (37). Moreover, HIMF upregulates VEGF expression in lung epithelial cells and modulates surfactant protein B and C expression as well

(18, 19). It is conceivable to postulate that HIMF may be a lung specific growth factor or mediator and plays important roles in the direction of lung-specific cell differentiation and facilitates lung development and maturation. This study has focused on Ets-1 regulation of HIMF expression during the lung development. We have shown that Ets-1 protein is highly expressed in the developing lung. Most importantly, HIMF gene proximal promoter region possesses an Ets-1 binding cis-element, and co-transfection of Ets-1 expression vector strongly enhances HIMF gene promoter activity. In addition, western blots and immunohistochemical analyses have demonstrated Ets-1 and HIMF proteins co-localize to airway epithelial cells and alveolar type II cells, temporally and spatially. These data confirm the previous report that Ets-1 plays a key role in vascular and lung development (23, 27, 28). Kola and colleagues (27) examined Ets-1 and Ets-2 mRNA expression during murine embryonic development and reported that Ets-1 is differentially expressed in the yolk sacs of mouse conceptuses (E8 - E12). It is expressed at high levels on E8 - E10, at detectable levels at E11 and at low levels at E12. This pattern of expression correlates with the presence of hematopoietic stem cells in the yolk sac. Moreover, Ets-1 expression is regulated in a temporal and tissue-specific manner during mouse development in the embryo proper. Ets-1 expression is first detected at E8 and increases in the embryo such as at E15, Ets-1 is expressed at high levels in all of the individual organs, including intestine, stomach, spleen, kidney, lung, liver, heart, and thymus, indicating involvement of Ets-1 in multi-organogenesis. However, at E16 a dramatic shift in the expression pattern occurs. Ets-1 is no longer ubiquitously expressed at high levels; rather its high level of expression is confined to certain organs, especially in the lung. In other organs, such as the stomach and intestines, the Ets-1 mRNA levels are substantially reduced. In P7 neonatal mice, a further change in the pattern of Ets-1 expression continues; expression predominantly occurs in the lymphoid organs and in the brain. In adult mice, Ets-1 transcripts are detected only in the lymphoid organs and lung. Our observations corroborate with these reports. The developmental changes of Ets-1 expression pattern from multi-organ to lymphoid tissues and to the lung, and from ubiquitous mesenchymal-epithelial expression to epithelial alone indicate that Ets-1 controls not only hematopoietic/vascular developmental related gene expression, but also regulates epithelial related genes that participate in the alveolar formation and maturation in the lungs. It is worthy to note that HIMF gene expression is first observed at E16 and continues to be expressed in the later stages. Thus it is temporally correlated to Ets-1 organ-specific expression in the lung. Furthermore, CHIP with Ets-1 antibody revealed there is indeed Ets-1 binding to the HIMF promoter in E20 and P1 lungs, strongly supporting the notion that Ets-1 participates in the developmental expression of HIMF in the mouse lungs.

5.2. Distinction of HIMF promoter activity in different cells

The HIMF promoter activity in MLE-12 and NIH3T3 cells is completely different, as we have seen that there are significant distinctions between these 2 cell lines

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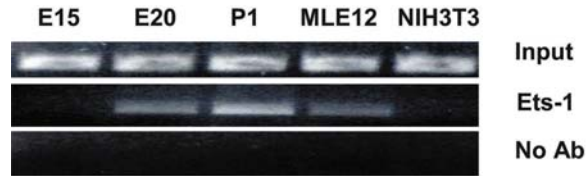


Figure 8. PCR results of HIMF promoter with Ets-1 binding. Chromatin immunoprecipitations were carried out using anti-Ets-1 antibody. Primers for the HIMF promoter were used for PCR. Representative CHIP PCR gels showed Ets-1 binding to the HIMF promoter only in E20, P1 lungs and MLE-12 cells, but not in E15 lungs and NIH3T3 cells, which show no HIMF expression. There is no PCR fragment in samples without Ets-1 antibody. The experiments were performed 3 times with identical results.

in response to pGLHIMF409 transfection. These differences can be explained by the existence of Ets-1 protein expression in these cells. EMSA from both cell line nuclear protein extracts with the Ets-1 probe has shown that there was a high level of Ets-1 protein only in MLE-12 cells. In contrast, NIH3T3 cells failed to show any gel-shift ability for Ets-1, indicating the absence of Ets-1 expression. Most interestingly, in the mutation and deletion experiments, we found that Ets-1 mutation abolished the luciferase activity in E18 PMELC (Figure 6). Furthermore, co-transfection of Ets-1 expression plasmid with the pGLHIMF409 construct strongly increased the luciferase reporter gene activity in NIH3T3 cells (Figure 7). However, the exact mechanism of the relatively high luciferase activity of pGLHIMF409 in NIH3T3 cells is not completely clear. It is possible that there may be an Ets-1 family member protein expressed in NIH3T3 cells, which is accountable for the high HIMF promoter activity. Further study is needed to clarify this question. Moreover, CHIP with anti-Ets-1 antibody confirmed Ets-1 binding in MLE-12 cells but not in NIH3T3 cells, which explains our previous observations that MLE-12 cells express HIMF mRNA (data not shown). These data strongly support the notion that Ets-1 is one of the major transcription factors in control of developmental expression of HIMF in the lung.

5.3. HIMF reporter luciferase activities

The other interesting result in this study is that the low luciferase activity in E14 PMELC after pGLHIMF409 transfection (Figure 4), as we have demonstrated that there was a high level of expression of Ets-1 protein at early stages of lung development. The possible explanation is that at E14, Ets-1 is mainly expressed in the lung mesenchyme or hematopoietic cells, but not in the glandular epithelial cells as these cells are the major sources of HIMF production in the later developing lung, or there may be a suppressor, which inhibited Ets-1 activation of HIMF gene in the early stages of embryonic development. The other possibility is that other unknown transcription factors may interact with Ets-1 in the control of HIMF expression, as previous reports have demonstrated that HIF-2 α and Ets-1 interaction regulated transcriptional activation of vascular endothelial growth factor receptor-2 expression in mouse brain capillaries (30). Detailed mechanisms of Ets-1 interaction with other

transcription factors in the regulation of HIMF gene expression warrant further investigation.

5.4. HIMF expression in the lungs

During recent years, the importance of HIMF gene expression in the lung has drawn attention from researchers. Several groups with different lung injury and inflammatory models have shown that there are upregulations of HIMF (32, 36, 39) and the HIMF gene regulation mechanism in the lung inflammation is particularly related to cytokines. Stutz and co-workers reported that FIZZ1/HIMF gene expression is regulated by IL-4 and IL-13 by a Stat₆ and C/EBP-dependent mechanism (32). They challenged the mouse with airway ovalbumin aerosol following immunization, and found that FIZZ1/HIMF was upregulated 6 hours after challenge in the lung. However, in the STAT₆-deficient mice, HIMF upregulation was abolished. Furthermore, they stimulated myeloid cell line BMnot with IL-4, which resulted in a significant upregulation of FIZZ1/HIMF mRNA. Point mutation in the STAT₆ or the C/EBP site led to loss of cytokine responsiveness. In another model of lung inflammation induced by bleomycin, Liu and colleagues (36) reported that there was a marked increase of FIZZ1/HIMF by IL-4 and IL-13 stimulation in isolated alveolar type II cells. Stimulation by IL-4/IL-13 was accompanied by increases in phosphorylated STAT₆ and JAK1 and transfection with a STAT₆ expression plasmid enhanced HIMF/FIZZ1 expression. All these data indicated that STAT₆- and C/EBP-mediated cytokines induce FIZZ1/HIMF gene expression under inflammatory conditions, which may be different from HIMF gene expression in developing lungs where Ets-1 may play important roles.

6. CONCLUSION

In this study, we investigated the relation between HIMF and Ets-1 gene expression in developing mouse lungs. We demonstrated that Ets-1 is involved in HIMF gene expression in the developing lung and Ets-1 induced HIMF gene regulation may play important roles in lung morphogenesis and maturation. We further showed that Ets-1 is a developmentally expressed transcription factor, which plays important roles in the regulation of HIMF production in mouse lung. Ets-1 is necessary, but is not sufficient to induce HIMF expression. Coordination of Ets-1 action with other transcription factors in the lung may be needed to regulate developmental HIMF production. The significance of this study is that for the first time Ets-1 has been implicated as an important developmental transcription factor that directs HIMF. As we have shown previously, HIMF increases VEGF and surfactant protein B and C expression, the Ets-1-regulated HIMF gene expression may facilitate lung development and maturation by promoting pulmonary vascular formation and enhancing surfactant protein production in mice.

7. ACKNOWLEDGEMENTS

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8. REFERENCES

1. Chinoy MR: Lung growth and development. *Front Biosci* 8: 392-415 (2003)
2. Minoo P: Transcriptional regulation of lung development: emergence of specificity. *Respir Res* 1: 109-115 (2000)
3. Cardoso WV: Molecular regulation of lung development. *Annu Rev Physiol* 63: 471-494 (2003)
4. Warburton D, Schwarz M, Tefft D, Flores-Delgado G, Anderson KD and Cardoso WV: The molecular basis of lung morphogenesis. *Mechanisms of development* 92: 55-81 (2000)
5. Massaro D and Massaro GD: Pulmonary alveoli: formation, the "call for oxygen" and other regulators. *Am J Physiol* 282: L345-L358 (2002)
6. Costa RH, Kalinichenko VV and Lim L: Transcription factors in mouse lung development and function. *Am J Physiol* 280: L823-L838 (2001)
7. Roth-Kleiner M and Post M: Genetic control of lung development. *Biol Neonate* 84: 83-88 (2003)
8. Chinoy MR, Zgleszewski SE, Cilley RE, Blewett CJ, Krummel TM, Reisher SR and Feinstein SI: Influence of epidermal growth factor and transforming growth factor Beta-1 on patterns of fetal mouse lung branching morphogenesis in organ culture. *Pediatric Pulmonology* 25: 244-256 (1998)
9. Chinoy MR, Graybill MM, Miller SA, Lang CM and Kauffman GL: Angiopoietin-1 and VEGF in vascular development and angiogenesis in hypoplastic lungs. *AJP - Lung Cellular and Molecular Physiology* 283: L60-L66 (2002)
10. Perl A-KT and Whitsett JA: Molecular mechanism controlling lung morphogenesis. *Clin Genet* 56: 14-27 (1999)
11. Prodhon P and Kinane TB: Developmental paradigms in terminal lung development. *BioEssays* 24: 1052-1059 (2002)
12. Thurlbeck WM: Postnatal growth and development of the lung. *Am Rev Respir Dis* 111: 803-844, (1975)
13. Maeda S, Suzuki S, Suzuki T, Endo M, Moriya T, Chida M, Kondo T and Sasano H: Analysis of intrapulmonary vessels and epithelial-endothelial interactions in the human developing lung. *Lab Invest* 82: 293-301 (2002)
14. Teng X, Li D, Champion HC and Johns RA: FIZZ1/RELM α - a novel hypoxia-induced mitogenic factor in lung with vasoconstrictive and angiogenic properties. *Circ Res* 92: 1065-1067 (2003)
15. Holcomb IN, Kabakoff RC, Chan B, Baker TW, Gurney A, Henzel W, Nelson C, Lowman HB, Wright BD, Skelton NJ, Frantz GD, Tumas DB, Peale FVJr, Shelton DL and Hebert CC: FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family. *EMBO J* 19: 4046-4055 (2000)
16. Steppan CM, Brown EJ, Wright CM, Bhat S, Banerjee RR, Dai CY, Enders GH, Silberg DG, Wen X and Wu GD: A family of tissue-specific resistin-like molecules. *Proc Natl Acad Sci USA* 98: 502-506 (2001)
17. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS and Lazar MA: The hormone resistin links obesity to diabetes. *Nature* 409: 307-312 (2001)
18. Tong Q, Zheng L, Dodd-o J, Langer J, Wang D and Li D: Hypoxia-Induced Mitogenic Factor Modulates Surfactant Protein B and C Expression in Mouse Lung. *Am J Respir Cell Mol Biol* 34: 28-38 (2006)
19. Tong Q, Zheng L, Lin L, Li B, Wang D, Huang C and Li D: VEGF is upregulated by hypoxia-induced mitogenic factor via the PI-3K/Akt-NF-kappaB signaling pathway. *Respiratory Research* 7: 37 (2006)
20. Wagner KF, Hellberg AK, Balenger S, Depping R, Dodd-O J, Johns RA and Li D: Hypoxia-induced mitogenic factor has antiapoptotic action and is upregulated in the developing lung: coexpression with hypoxia-inducible factor-2 α . *Am J Respir Cell Mol Biol* 31: 276-282 (2004)
21. Sharrocks AD, Brown AL and Yates PR: The Ets-domain transcription factor family. *Int J Biochem Cell Biol* 29: 1371-1387 (1997)
22. Graves Bj and Petersen JM: Specificity within the Ets family of transcription factors. *Adv Cancer Res* 75: 1-55 (1998)
23. Dittmer J: The biology of the Ets 1 proto-oncogene. *Mol Can* 2: 1-21 (2003)
24. Blair DG and Athanasiou M: Ets and retroviruses - transduction and activation of members of the Ets oncogene family in viral oncogenesis. *Oncogene* 19: 6472-6481 (2000)
25. Nunn MF, Seeborg PH, Moscovici C and Duesberg PH: Tripartite structure of the avian erythroblastosis virus E26 transforming gene. *Nature* 306: 391-395 (1983)
26. Nye JA, Petersen JM, Gunther CV, Jonsen MD and Graves BJ: Interaction of murine Ets-1 with GGA-binding sites establishes the ETS domain as a new DNA-binding motif. *Genes Dev* 6: 975-990 (1992)
27. Kola I, Brookes S, Green AR, Garber R, Tymms M, Papas TS and Seth A: The Ets1 transcription factor is widely expressed during murine embryo development and is associated with mesodermal cells involved in morphogenetic processes such as organ formation. *Proc Natl Acad Sci U S A* 90: 7588-7592 (1993)
28. Sato Y: Role of ETS family transcription factors in vascular development. *Cell Stru Fun* 26: 19-24 (2001)
29. Lavenburg KR, Ivey J, Hsu T and Muise-Helmericks RC: Coordinated functions of Akt/PKB and ETS1 in tubule formation. *FASEB J* 17: 2278-2279 (2003)
30. Elvert G, Kappel A, Heidenreich R, Englmeier U, Lanz S, Acker T, Rauter M, Plate K, Sieweke M, Breier G and Flamme I: Cooperative interaction of hypoxia-inducible factor-2 α (HIF-2 α) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). *J Biol Chem* 278: 7520-7530 (2003)
31. Kathuria H, Cao YX, Ramirez MI and Williams MC: Transcription of the caveolin-1 gene is differentially regulated in lung type I epithelial cell lines. *J Biol Chem* 279: 30028-30036 (2004)
32. Stutz AM, Pickart LA, Trifilieff A, Bauruker T, Prischl-Strassmayr E and Woisetschlager M: The Th2 cell cytokine IL-4 and IL-13 regulate found in inflammatory zone 1/resisting-like molecule a gene expression by a STAT6 and CCAAT/enhancer-binding protein dependent mechanism. *J Immunol* 170: 1789-1796 (2003)

Ets-1 regulates HIMF in developing lung

33. Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254 (1976)
34. Kuo MH and Allison WS: In vivo cross-linking and immunoprecipitation studying dynamic protein:DNA associations in a chromatin environment. *Methods* 19: 425-433 (1999)
35. Kazi AA, Jones JM and Koos RD: Chromatin Immunoprecipitation Analysis of Gene Expression in the Rat Uterus in Vivo: Estrogen-Induced Recruitment of Both Estrogen Receptor α and Hypoxia-Inducible Factor 1 to the Vascular Endothelial Growth Factor Promoter. *Molecular Endocrinology* 19: 2006-2019 (2005)
36. Liu T, Jin H, Ullenbruch M, Hu B, Hashimoto N, Moore B, McKenzie A, Lukacs NW and Phan SH: Regulation of found in inflammatory zone 1 expression in bleomycin-induced lung fibrosis: role of IL-4/IL-13 and mediation via STAT-6. *J Immunol* 173: 3425-3431 (2004)
37. Li D, Fernandez LG, Dodd-O J, Langer J, Wang D and Laubach VE: Upregulation of hypoxia-induced mitogenic factor in compensatory lung growth after pneumonectomy. *Am J Respir Cell Mol Biol* 32: 185-191 (2005)
38. Liu T, Dhanasekaran SM, Jin H, Hu B, Tomlins SA, Chinnaiyan AM and Phan SH: FIZZ1 stimulation of myofibroblast differentiation. *Am J Pathol* 164: 1315-1326 (2004)
39. Tong Q, Zheng L, Kang Q, Dodd-O J, Langer J, Li B, Wang D and Li D: Upregulation of hypoxia-induced mitogenic factor in bacterial lipopolysaccharide-induced acute lung injury. *FEBS Letters* 580: 2207-2215 (2006)

Abbreviations: Ets, E26 transforming specific sequence, HIMF, hypoxia-induced mitogenic factor, EMSA, Electrophoretic-Mobility Shift Assay, FIZZ, Found in inflammatory zone, RELM α , Resistin-like molecule- α , HIF-2 α , hypoxia-inducible factor-2 α , wHTH, winged helix-turn-helix, TF, transcription factor, C/EBP, CAATT/enhancer-binding protein, MLE-12, mouse lung epithelial-12, CHIP, chromatin-immunoprecipitation,

Key Words: Ets-1, HIMF, Lung Development, Mouse, Gene Expression

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