Deterioration of alveolar development in mice with both HIF-3α knockout and HIF-2α knockdown

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Abstract

Objective: Earlier studies from our group using hypoxia-inducible factor 3α knockout mice showed impairments in lung remodeling and lung endothelial cells. Another research from our group demonstrated that impaired expression of hypoxia-inducible factor 2α induced compensatory expression of hypoxia-inducible factor 1α in hypoxia-inducible factor 2α knockdown mice. The present study uncovers more insights by extending the investigation, utilizing mice with both hypoxia-inducible factor 3α knockout and hypoxia-inducible factor 2α knockdown.

Results: No mice with both hypoxia-inducible factor 3α knockout and hypoxia-inducible factor 2α knockdown died immediately after birth. The mice with both hypoxia-inducible factor 3α knockout and hypoxia-inducible factor 2α knockdown exhibited impaired alveolar sacs and lung alveolar structure and decreased endothelial cell numbers. Analysis of relative mRNA expression revealed depressed expressions of hypoxia-inducible factor 1α, vascular cell adhesion molecule 1, vascular endothelial cadherin, angiopoietin 2, Tie-2, and vascular endothelial growth factor in the lungs of mice with both hypoxia-inducible factor 3α knockout and hypoxia-inducible factor 2α knockdown compared to that in wild-type mice. Further analysis is needed to elucidate the impaired development occurred in the lung endothelial cells.

Keywords: HIF-3α, HIF-2α, Double-mutant mice, Lung, Alveolar

Introduction

Hypoxia-inducible factors (HIFs) are heterodimers that consist of three oxygen-sensitive α-subunits (HIF-1α, HIF-2α, and HIF-3α) and a β-subunit, the aryl hydrocarbon receptor nuclear translocator (ARNT). HIFs act as regulators of the molecular hypoxic response [1, 2]; in a study examining normal alveolarization in fostered newborn rats, HIFs promoted alveolar development and regeneration by preventing and repairing oxygen-induced alveolar damage [3]. Nonetheless, HIF-1α inhibition using antisense knockdown in vitro during early lung development decreased vascular development and epithelial branching morphogenesis in lung explants [4]. In contrast, the conditional overexpression of HIF-1α in embryonic lung epithelium also impaired branching morphogenesis and lung maturation and affected vascular lung abnormalities, including hemorrhages and increased lymphangiogenesis [5]. Collectively, these data suggest that interference in the alveolar epithelium by oxygen pressure changes, including hypoxia, can affect alveolar homeostasis, leading to epithelial injuries and diseases such as lung fibrosis [6–9]. A previous study from our group that used HIF-3α knockout (−/−) mice showed impaired lung remodeling exhibited by the walls of the secondary septa in subdivided alveoli, and immunostaining of alveolar endothelial cells presented an increase in defective space in the interalveolar septa and hyperplasia of endothelial cells during the maturation of alveolar formation in these knockout mice [10]. Additionally, another study from our group revealed that these HIF-3α −/− mice showed impairments in lung endothelial cells presented by slow growth and a decreased number of tubes formed by endothelial cells [11]. Furthermore, a
different but related study from our group demonstrated that impaired expression of HIF-2α in HIF-2α knock- 
down (kd/kd) mice induced compensatory expression of 
HIF-1α [12]. The present study uncovers more insights by 
extending the investigation from those previously stated 
findings of our group utilizing HIF-3α −/− and HIF-2α 
dkd/kd (double-mutant) mice. In this study, male and 
female HIF-3α −/− and HIF-2α knockdown heterozy-
gote (kd/+ ) mice were interbred, resulting in the double-
mutant mice previously mentioned.

Main text
Materials and methods
Mice
All of the experiments performed were approved by the 
ethics committee of the University of Tsukuba. All wild-
type (WT) and mutant mouse lines were of the C57BL/6J 
genetic background. HIF-2α kd/kd mice were gener-
ated as previously reported [13]. HIF-3α −/− mice were 
obtained as previously published [11]. Mating of 12 pairs 
of HIF-3α −/− and HIF-2α kd/+ mice for a breeding 
period of 1 year generated the double-mutant mice. The 
genome DNA was extract from tail of neonatal pup. The 
genotype of mouse was determined by polymerase chain 
reaction (PCR) as described previously [11, 13].

Isolation and culture of cells
The WT, HIF-2α kd/kd, HIF-3α −/−, and double-mutant 
mice were sacrificed by given the overdose treatment of 
anesthetic reagent (isoflurane; WAKO, Japan), and lung 
tissue from these mice were harvested as previously men-
tioned [14]. Lungs from WT, HIF-2α kd/kd, HIF-3α −/−, 
and double-mutant mice were dissected at postnatal day 
(P) 0 for hematoxylin and eosin staining and at postna-
tal week 6 for cell culture. Collagenase digestion (Nitta 
Gelatin, Osaka, Japan) was used to prepare the lung cell 
suspensions. Afterward, these cells were cultured in high 
glucose Dulbecco’s modified Eagle’s medium (DMEM; 
GIBCO) supplemented with 10% fetal bovine serum 
(FBS), 0.1 mmol/L nonessential amino acids, 2 mmol/L 
L-glutamine, penicillin–streptomycin, and 10−4 mol/L 
β-mercaptoethanol (HAVA medium) [15] and main-
tained without any addition of growth supplements.

Immunohistochemistry and section staining
The lung tissue samples from WT, HIF-3α −/−, and dou-
ble-mutant mice were fixed with 4% paraformaldehyde 
combined with phosphate-buffered saline at 4 °C over-
night and embedded in OCT compound (Sakura Finetek, 
Tokyo, Japan). Sections (5 μm) were then prepared for 
immunohistochemical and hematoxylin and eosin stain-
ing. Serial cryostat sections were incubated with CD31 
(1:1000; clone: MEC 13.3; BD Biosciences, San Diego, 
CA, USA) antibody. The sections, after being washed, 
were incubated with an HRP-conjugated secondary 
antibody (1:2000; Vector Laboratories, Burlingame, 
CA, USA). Different sections were incubated with anti-
HIF-1α, anti-HIF-2α, anti-vascular cell adhesion mole-
cule 1 (VCAM-1), and anti-vascular endothelial cadherin 
(VE-cadherin) antibodies and stained using MOM™ kit 
(Vector Laboratories) referring to the manufacturer’s 
instructions.

Quantitative reverse transcription polymerase chain reaction 
(qPCR)
Total RNA was obtained from samples (n = 3) with the 
use of the extraction reagent (Sepasol-RNA I Super G; 
Nakalai Tesque, Kyoto, Japan). cDNA was then synthe-
sized by reverse transcription (Reve’Tra Ace; TOYOBO, 
Osaka, Japan). The expression level was analyzed by 
using the 7500 Fast Real-Time PCR machine (Applied 
Biosystems, Carlsbad, CA, USA) with SYBR-green (Life 
Technologies, Carlsbad, CA, USA). Experiments were 
performed in triplicate, and the resulting data were ana-
yzed by the delta CT method.

Results
No double-mutant mice died immediately after birth
Interbreeding between male and female HIF-3α −/− 
and HIF-2α kd/+ mice resulted in 198 offspring within 
1 week. Of the total number of offspring, 5.1% were sur-
prisingly double-mutant mice, with very few pups still 
alive at birth, 34.3% were HIF-3α −/− and HIF-2α +/+ , 
and 60.6% were HIF-3α −/− and HIF-2α kd/+. Almost 
none of the double-mutant mice were alive at birth.

The double-mutant mice had impaired alveolar sacs and lung 
alveolar structure and decreased endothelial cell numbers
Hematoxylin and eosin staining of the lung in WT, 
HIF-3α −/−, and double-mutant mice was performed 
at P0 (Fig. 1). We found that alveolar sacs were almost 
imperceptible in the lungs of double-mutant mice, and 
the appearance of the blood vessels in the lungs of these 
mice was different than that in the lungs of HIF-3α −/− 
and WT mice. There is no phenotype data for HIF-2α 
kd/kd mice at present. Moreover, immunohistochemis-
try analysis of sections from neonatal WT and double-
mutant mice at 2–3 days of age (Fig. 2) by CD31 staining 
showed impaired lung vessel structure of the neonatal 
double-mutant mice accompanied with the decreased 
of endothelial cell numbers. HIF-1α and HIF-2α staining of 
the neonatal double-mutant mice revealed the decreased 
expressions of both HIFs and endothelial cell numbers. 
VCAM-1 and VE-cadherin staining of the neonatal dou-
ble-mutant mice showed their expressions and also the 
decreased of endothelial cell numbers.
The double-mutant mouse lung exhibited depressed expressions of HIF-1α, VCAM-1, VE-cadherin, Ang-2, Tie-2, and VEGF

We analyzed the relative mRNA expression levels of potentially related genes HIF-1α, HIF-2α, VCAM-1, VE-cadherin, angiopoietin 1 (Ang-1) and 2 (Ang-2), Tie-2, vascular endothelial growth factor (VEGF), and Flk-1. The analysis utilized the whole lung tissue from neonatal WT, HIF-2α kd/kd, HIF-3α−/−, and double-mutant mice at 2–3 days of age (Fig. 3). Surprisingly, we found that HIF-1α, VCAM-1, VE-cadherin, Ang-2, Tie-2, and VEGF expressions were reduced in the lung of the neonatal HIF-3α−/− mice and even more depressed in the lung of the neonatal double-mutant mice. We also found that HIF-2α and Flk-1 expressions were similarly reduced in the lung of the neonatal double-mutant mice. Additionally, Ang-1 was unexpectedly expressed excessively in the lung of the neonatal double-mutant mice.

Discussion

The present study has elucidated that no double-mutant mice died immediately after birth. This new finding is slightly opposed to the common knowledge that HIF deficiency is immediately postnatally lethal. The reason that none of the double-mutant mice died postnatally remains unclear. Furthermore, this study is the first report that the alveolar sacs of the double-mutant mice are impaired. The current result has broadened the understanding from our previous study that revealed incomplete alveolar spaces in HIF-3α−/− mice [10]. Immunohistochemistry results have shown that the decreased of endothelial cell numbers, which impair proliferative and angiogenic activities appeared to contribute to impaired lung alveolar structure of the neonatal double-mutant mice. In addition, the lung endothelial cells isolated from the neonatal double-mutant mice showed the impaired proliferative ability (data not shown), suggesting the functional impairment of these cells by both HIF-3α−/− and HIF-2α kd/kd. The decreased of HIF-1α and HIF-2α expressions causing of lacking to overcome the happening of oxygen homeostasis disruption also deteriorate such lung alveolar structure. Such pathological features may provide further insight into the molecular mechanism of alveolar development especially

Fig. 1 The double-mutant mice had impaired alveolar sacs. The lung of WT mice, HIF-3α−/− mice, and double-mutant mice at P0 was examined based on hematoxylin and eosin staining. H3, hypoxia-inducible factor 3α; H2, hypoxia-inducible factor 2α; ::, intercrossed with; −/−, knockout; kd/kd, knockdown; WT, wild-type

Fig. 2 The double-mutant mice had impaired lung alveolar structure and decreased endothelial cell numbers. Immunohistochemistry analysis performed to sections from neonatal WT and double-mutant mice at 2–3 days of age using CD31, HIF-1α, HIF-2α, VE-cadherin, and VCAM-1 staining. HIF-1α, hypoxia-inducible factor 1α; HIF-2α, hypoxia-inducible factor 2α; H3, hypoxia-inducible factor 3α; H2, hypoxia-inducible factor 2α; ::, intercrossed with; −/−, knockout; kd/kd, knockdown; VE-cadherin, vascular endothelial cadherin; VCAM-1, vascular cell adhesion molecule 1; WT, wild-type
### WT vs. H3-/-; H2kd/kd

| Marker       | WT         | H3-/-; H2kd/kd |
|--------------|------------|----------------|
| CD31         | ![CD31 WT](image1) | ![CD31 H3-/-; H2kd/kd](image2) |
| HIF-1α       | ![HIF-1α WT](image3) | ![HIF-1α H3-/-; H2kd/kd](image4) |
| HIF-2α       | ![HIF-2α WT](image5) | ![HIF-2α H3-/-; H2kd/kd](image6) |
| VE-cadherin  | ![VE-cadherin WT](image7) | ![VE-cadherin H3-/-; H2kd/kd](image8) |
| VCAM-1       | ![VCAM-1 WT](image9) | ![VCAM-1 H3-/-; H2kd/kd](image10) |
for further investigation at the embryonic stage. We next acknowledged that our current results on the gene expressions in the lung of the neonatal HIF-3α−/− mice are partly in line as well as in contrary to the results showed on our former study utilizing the adult HIF-3α−/− mice [11]. The contrary results in the current study are mostly correlated to the angiogenic gene regulations due to the depressed mRNA levels of HIF-1α, HIF-2α, VCAM-1, VE-cadherin, Ang-2, Tie-2, HIF-2α, VEGF, and Flk-1 in the lung of the neonatal HIF-3α−/− mice. The mRNA levels of HIF-1α, VCAM-1, VE-cadherin, Ang-2, Tie-2, and VEGF are even more depressed in the lung of the neonatal double-mutant mice. Our understanding of these new findings may be further understood in light
of knowledge that the VEGF/Flik-1 and angiopoietin/Tie-2 signaling pathways are vital for the maintenance of endothelial cell homeostasis [16–18], which then can explain impaired alveolar sacs and lung alveolar structure conditions. Ang-1 that is interestingly highly expressed even though HIF-1α is inversely expressed in the lung of the neonatal double-mutant mice, which against the previous report concluding HIF-1α explicitly targets Ang-1 [19] may indicate other regulations involved. Overall, these findings should be investigated further to firmly elucidate the impaired development occurred in the lung endothelial cells.

Limitations

The limited number of double-mutant mice that survive postnatally may hinder the acquisition of sufficient samples for the isolation and identification of the lung endothelial cells.

Abbreviations

ATII: alveolar type 2; Ang-1: angiopoietin 1; Ang-2: angiopoietin 2; ARNT: aryl hydrocarbon receptor nuclear translocator; cDNA: complementary deoxyribonucleic acid; DMEM: Dulbecco’s modified Eagle’s medium; FBS: fetal bovine serum; HAVIA: high-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 0.1 mM/mL nonessential amino acids, 2 mM/L L-glutamine, penicillin-streptomycin, and 10−6 mol/L β-mercaptoethanol; HIF: hypoxia-inducible factors; HIF-1α: hypoxia-inducible factor 1α; HIF-2α: hypoxia-inducible factor 2α; HIF-3α: hypoxia-inducible factor 3α; kd/kd: knockdown heterozygotes; mRNA: messenger ribonucleic acid; n: sample number, OCT: optimum cutting temperature; P: postnatal day; PCR: polymerase chain reaction; qPCR: quantitative real-time polymerase chain reaction; RNA: ribonucleic acid, VCAM-1: vascular cell adhesion molecule 1; VE-cadherin: vascular endothelial cadherin; VEGF: vascular endothelial growth factor; WT: wild-type, −/−: knockout, +/-: homozygotes.

Authors’ contributions

FZA and TY contributed equally to this work. FZA conceptualized the study; analyzed and interpreted all immunohistochemistry and section staining and qPCR data, and was a major contributor in writing the manuscript. TY designed the study on the lung by using double-mutant mice; performed breeding of all the mice, isolated all lung tissue samples, and performed and analyzed all immunohistochemistry and section staining and qPCR; and critically revised the manuscript. OO designed the study on the lung by using double-mutant mice; performed and qPCR data, and was a major contributor in writing the manuscript. TY analyzed and interpreted all immunohistochemistry and section staining. OW designed the study on the lung by using double-mutant mice and critically revised the manuscript. All authors read and approved the final manuscript.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

This published article includes all data generated or analyzed during this study.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The studies with all of the experiments and protocols were submitted to and approved by the ethics committee of the University of Tsukuba.

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