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Title page
Original Paper

Effect of insulin-like-growth factor and its receptors regarding lung development in fetal mice

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4 figures,
1 table

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Abstract

In congenital diaphragmatic hernia (CDH), both mortality and morbidity are mainly caused by pulmonary hypoplasia and persistent pulmonary hypertension. Insulin-like growth factors (IGFs) are one of the growth factors that may play an important role in the fetal lung development. Elucidating the roles of these growth factors regarding fetal lung development would thus provide new insight regarding the optimal therapy for CDH patients. The aim of this study is to investigate the role of IGFs in the fetal lung development. The fetal lungs were dissected from embryonic day (E) 11.5 to E18.5 mice. The mRNA expression of IGFs and its receptors was analyzed by real-time RT PCR. In addition, the lungs dissected from the E17.5 mice were divided into the following three groups; lungs cultured only in the serum-free medium (group I: n=5), lungs cultured in medium containing either IGF-I (group II: n=5), or IGF-II (group III: n=5). All cultures incubated for 48 hours were investigated by immunohistochemistry, using the antibodies of thyroid transcription factor (TTF)-1, prosurfactant protein (proSp)-C, alpha smooth muscle actin (α-SMA), and anti-proliferating cell nuclear antigen (PCNA).

The mRNA expression level of both IGF- I and IGF-II was higher during the period from E11.5 to E16.5 than that of later stage. In contrast, the mRNA expression of both IGF-I receptor (IGF-IR) and IGF-II receptor（IGF-IIR）was observed to be higher during the period from E17.5 to E18.5 than that at any other stage. The number of positive cells for TTF-1, proSp-C and α-SMA increased more in both groups II and III than in group I.

Based on our findings, IGFs are therefore suggested to induce alveolar and vascular maturation in the late stages of fetal lung development. In addition, the effect of IGFs

on fetal lung maturation is thought to be related to the increase of both IGF-IR and IGF-IIR in the fetal lung. Therefore, the administration of IGFs to the fetal CDH lung may thus be able to effectively improve the symptoms of hypoplastic lung.

INDEX WORDS: insulin-like growth factor, lung, fetus, development,
congenital diaphragmatic hernia

Introduction

Congenital diaphragmatic hernia (CDH) remains to be one of the challenging diseases for neonatal surgeons. According to the current established postnatal therapy, such as inhaled nitric oxide therapy, high-frequency oscillatory ventilation, and delayed surgery, the overall survival rate has now significantly improved to about 70% at some facilities.[1,2] Nevertheless, CDH infants with severe pulmonary hypoplasia and associated persistent pulmonary hypertension still tend to have a high mortality and morbidity rates[3,4]. To improve the outcome in CDH patients with severe pulmonary hypoplasia, fetal treatment in order to accelerate the maturation process of hypoplastic lungs is thought to be needed.

In general, growth factors are considered to play an important role during the process of the pulmonary organogenesis *in vivo and in vitro*[5]. Regarding such growth factors, insulin-like-growth factors (IGFs) expressed throughout the perinatal period and they play an important role in lung development[6-16]. The IGF-I mRNA expression is found in mesenchymal cells and IGF- II is expressed in the lung epithelium during the fetal period[6]. Such IGFs bind with their receptors and regulate both cell proliferation

and differentiation. In both human CDH patients and the experimental model of nitrofen-induced CDH in rodents, IGFs have been identified in the late stage of the lung development and the antenatal glucocorticoid-therapy has been suggested to induce the maturation of such lungs via the IGF system[7,8]. In addition, there is increasing evidence suggesting that the IGF system itself also plays a pivotal role in the development and differentiation of fetal lung[6-16]. Recent studies have shown that the targeted deletion of the IGF-I or IGF-II mice resulted in delayed lung maturation[10-12]. Sixty percent of IGF-I knock-out mice died because of respiratory failure and IGF-II knocked out mice also demonstrated delayed lung development at the end of the gestation[10-12]. However, very little data are available regarding the chronological changes of IGFs and IGFRs expressions. Furthermore, the direct effect of IGFs has not yet been investigated regarding lung maturation during the late fetal period.

We herein simultaneously analyzed the mRNA expression of the IGFs and IGFRs by real-time reverse transcription polymerase chain reaction (RT PCR). In addition, the effect of IGFs on developmental fetal lungs was also investigated using an organ culturing system.

Materials and methods

Experimental animals

ICR mice were purchased from a commercial breeder, SLC (Shizuoka, Japan). The day that the vaginal plug was confirmed was designated as an embryonic day (E) 0.5. Fetuses were obtained during the periods from E 11.5 to 18.5 (term 18.5-19.5) mice.

Fetal lungs were dissected out from the thoracic cavity, snap-frozen and stored at -70 °C until RNA extraction. In addition, the lungs dissected from the E17.5 mice were incubated for 48 hours in various mediums using an organ culture system. All animal experiments were conducted in compliance with the "Guidelines for the care and use of laboratory animals" established by our university.

Total RNA extraction and real-time RT PCR

Total RNA was extracted from 5 lungs each day using TRIZOL® reagent (Invitrogen, USA) according to the manufacturer's instruction. First-strand cDNA was synthesized using the Superscript III First-strand Synthesis Supermix (Invitrogen, USA) according to the manufacturer's instructions. This was designed to convert 1μg of total RNA into 20μl of first strand cDNA using oligo dT_{20} primer. To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification while using primers for the constitutively expressed gene, glyceroldehyde-3-phosphate dehydrogenase (GAPDH) and normalized. All PCR reactions were performed using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals, Germany)[17]. The primer sequences, annealing temperature and cycling numbers for GAPDH, IGF-I, IGF-II, IGF-IR and IGF-IIR were listed in Table 1. The cycling conditions were initial denaturation at 95°C for 10 minutes, followed by specific cycling numbers at 95°C for 15 sec, dependent annealing temperature for 5 sec, and 72°C for 5sec.

Organ culture

Lung tissue specimens were dissected from E17.5 ICR mice. The isolated lungs

were placed on polyethylene terephthalate filters (Falcon® 3502 cell-culture insert: Becton Dickinson and Company, Franklin Lakes, USA: 8μm pore size) containing following three groups of serum-free medium in each well: group I (n=5)；the serum-free medium which contained chemically defined BGJb medium (Gibco BRL, Grand Island, NY: containing a total of 2 ml medium in each well) with penicillin-streptomycin (Gibco BRL, Grand Island, NY: 100U and 100mg/ml), L-ascorbic acid (NACALAI TESQUE, Inc., Kyoto, Japan: 150μg/ml) and transferrin (SIGMA, ST. Louis, USA: 50μg/ml), group II (n=5)； 500ng/ml of recombinant mouse IGF-I (TECHNE Cor. Minneapolis, USA) was supplemented with the medium in group I, group III (n=5); 500ng/ml of recombinant mouse IGF-II （TECHNE Cor., Minneapolis, USA）was supplemented with the medium in group I. The cultures were incubated in 95% air/5% CO_2 at 37°C for 48 hours

Immunohistochemistry

The lung tissue specimens were stored in 70% buffered ethanol after 48 hours' incubation in three groups of organ culture. Thereafter, they were embedded in paraffin. Paraffin sections (5μm) were dehydrated in xylene for 10 minutes, rehydrated through alcohol, and incubated for 20 minutes in methanol with 3% H_2O_2 to block endogenous peroxidase. All specimens were then pretreated in 10mM citrate buffer (pH 6.0) for 15 minutes using a microwave. The sections were then incubated with 10% normal goat serum for 30 min in a moist chamber and then were incubated overnight at 4°C with the primary antibody. The lung specimens were stained with monoclonal rabbit antibody to TTF-1(anti-TTF-1 mouse monoclonal antibody, Abcam. Ltd., diluted in 1:45) to detect the nuclei of lung airway epithelial cells. Pro SP-C (anti-pro SP-C mouse polyclonal

antibody, CHEMICON International, Inc., diluted in 1:1000) was stained to clarify the expression patterns of the epithelial cells including differentiated alveolar type II cells. In addition, α-SMA (anti-α-SMA rabbit polyclonal antibody, Abcam. Ltd., diluted in 1:200) was also stained to determine the degree of vascular development in the fetal lung. In addition, proliferating cell nuclear antigen (PCNA) (anti PCNA mouse monoclonal antibody, Dakocytomation.Co.Ltd, diluted in 1:100) was used to identify PCNA positive cells as a marker of cell proliferation.

Next, the sections were then washed in phosphate-buffered saline (PBS) and incubated for 10 min at room temperature with biotinylated goat anti-rabbit immunoglobulin G (IgG) antisera. Then they were washed in PBS and incubated for 10 min at room temperature with peroxidase-conjugated streptavidin. Immunohistochemical signals were visualized using avidine-biotine-peroxidase complex, and 3,3'-diaminobenzidine substrate. Slides were counterstained with hematoxylin and examined and photographed by standard microscopy.

Statistical analysis

The data were analyzed using Student's *t* test. The results are expressed as the mean ±standard deviation (SD). A value of $P<0.05$ was considered to be statistically significant.

Results

mRNA expression of IGFs and IGFRs

The mRNA expression of IGF-I in both E13.5-E14.5 and E15.5-E16.5 were

significantly higher than E11.5-E12.5 and E17.5-E18.5 (Fig.1A). The expression of IGF-II mRNA in E17.5-E18.5 was significantly lower than that at any other fetal stages (Fig.1B). At the same time, the mRNA expression of the IGF-IR in E13.5-E14.5 was significantly higher than E11.5-12.5 (Fig.1C). Further, the expression of IGF-IR mRNA in E17.5-E18.5 was significantly higher than that in E15.5-E16.5 (Fig.1C). The mRNA expression pattern of IGF-IIR was almost the same in that of IGF-IR. The expression of IGF-IIR mRNA in E13.5-E14.5 was significantly higher than that in E11.5-12.5 (Fig.1D). In addition, the expression of IGF-IIR mRNA in E17.5-E18.5 was significantly higher than that in E15.5-E16.5 (Fig.1D).

Immunohistochemical Analysis of the effect of IGFs

TTF-1 protein was localized to the nuclei of the main bronchial and respiratory epithelium during the fetal period (Fig.2). The number of the TTF-1 positive cells in groups II and III (Fig.2B and Fig.2C) were clearly higher than that in group I (Fig.2A).

ProSp-C protein was located in the cytoplasm of epithelium cells in distal lung buds (Fig.3). The number of proSp-C positive cells in group II and III (Fig.3B and Fig.3C) had increased in the distal lung epithelium, in comparison to that in group I (Fig.3A). α-SMA staining revealed protein to be located in the muscle layers of both artery and trachea and bronchus in group I (Fig.4A). In contrast, α-SMA positive cells were detected in myofibroblasts in both group II and group III in addition to the muscle layer of artery and both trachea and bronchus (Fig.4B and Fig.4C).

PCNA positive cells were mainly detected in both the respiratory cells and mesenchymal cells in all groups. The number of PCNA positive cells was much higher in groups II and III than those in group I. (data not shown).

Discussion

The high mortality rate in CDH is caused by hypoplastic lung and the associated persistant pulmonary hypertension[3,4]. Hypoplastic CDH lung is less mature than normal lungs which is thought to be due to an arrested development during the canalicular or saccular stage of the lung development[18,19]. A reduction in bronchial divisions is observed in hypoplastic lungs, thus resulting in a secondary reduction in number of respiratory bronchi and alveoli[20]. The alveoli present are also immature with thickened intraalveolar septa, thus leading to a reduced capillary-air interface, which is essential for gas exchange[21,22]. To improve this state of lung immaturity, recent studies have focused on the molecular alterations related to growth factors in the fetal lungs of both human CDH and animal CDH models[8,9,22].

IGF-I and IGF-II are both major growth factors. IGFs have 62% sequences of all which are identical to those of proinsulin, and are produced by almost all cells in the body[5]. Both IGF-I and IGF-II modulate proliferation and differentiation during embryogenesis through either paracrine or autocrine pathways, thus mediating the rapid lung growth during the fetal period[13,14]. Some studies have shown that IGFs play an important role in the late stage of the lung development[10-12,14,15]. The deletion of either IGFs or IGFRs in transgenic mice causes either pulmonary hypoplasia or delayed lung maturation which thus induces perinatal respiratory failure and perinatal death[10-12,14-16]. In the targeted disruption of IGF-I, the phenotype characteristically demonstrates a lower airway volume, retardant epithelial growth and a failure of capillary remodeling. Therefore, about 60% of IGF-I knockout mice die because of respiratory failure during the perinatal period[11,12]. In addition, IGF-II knockout mice demonstrate an immature

lung with a thicker alveolar septae and poorly organized alveoli, in comparison to the lungs of the wild type mouse[12-15]. Mice, which carry a null mutation of IGF-IR, tend to die after birth due to respiratory failure induced by muscle hypoplasia[13-15]. IGF-IIR knockout mice are also born 30% heavier than wild types and die during the neonatal period due to the several organ malformations including lung abnormalities[16]. Taking such evidence of the IGF system regarding lung development into consideration, elucidating the mechanism of the IGF system in lung development is thus expected to improve the outcome of CDH patients with severe pulmonary hypoplasia.

In this study, the mRNA expression of both these growth factors and its receptors was investigated in the fetal lung development. The results showed that mRNA expression of both IGF-I and IGF-II were predominant in E13.5 to E16.5, in comparison to the late stage (from E17.5 to E18.5) of the lung development. Previous reports suggest that IGF- I plays an important role as a growth factor in the late stages of fetal lung development, while IGF–II plays a fundamental role in both embryonic and fetal lung development[14,15]. Both the IGF-I and IGF-II bind to IGF-IR, thus resulting in the stimulation of several signaling pathways related to the lung development[10-15]. Similarly, the IGF-IIR is thought to bind to both IGF-I and IGF-II[15]. The chronological observations of this study showed that the number of both IGF-IR and IGF-IIR gradually increased over time during the period from E13.5 to E18.5. Based on the previous studies and our results, the production of both IGF-I and IGF-II proteins may occur at an earlier stage than the formation of both IGF-IR and IGF-IIR in the cell membrane during the fetal period, and it may also combine with both the IGF-IR and IGF-IIR in the late stage, thus leading to the alveolar formation.

In this study, the fetal lung, in the medium including either IGF-I or IGF-II, grew

and differentiated in comparison to that in the serum-free medium. TTF-1 is localized to the nuclei of the main bronchial and respiratory epithelium, especially in the distal alveolar epithelium cells lining the conducting airways[23]. At the same time, TTF-1 is thought to mediate the expression of Sp-C [23]. ProSp-C was distributed in the cytoplasm of epithelium cells in distal lung buds which encoding the Sp-C. Some reports have shown proSp-C to be closely related to the distribution of type II epithelial cells, and the intensity of staining increases over time during the fetal period[23]. Our findings also showed the expression of both TTF-1 and proSp-C to increase in the distal alveolar epithelium in both groups II and III. Therefore, IGFs may bind to their receptors in the fetal late stage thus inducing an increased expression of TTF-1, and leading to an increased proSp-C expression. These results may suggest that the IGFs can therefore induce the differentiation of distal respiratory cells during the fetal period.

α-SMA is located in the smooth muscle of both artery and airwaytract[24]. In our study, IGFs stimulated the expression of α-SMA. The positive cells were myofibroblasts in the stroma. This result may suggest that IGFs promote vasculogenesis in the late stage of lung development. A recent study showed that immunotargeting, using an anti-IGF-IR neutralizing antibody in human fetal lung explants, leads to a severe immature vascularization of the pulmonary vessels[18]. In addition, the increased number of PCNA positive cells cultured either IGF-I and IGF-II, indicated that IGFs stimulate all types of cell proliferation in the late stages of lung development.

In conclusion, our data suggest that IGFs play a pivotal role in the late stage of lung development through IGFRs when the numbers of IGFs' receptor have been increased. Fetal treatment of such IGFs systems, which can induce both lung maturation and vascularization, may therefore be able to potentially improve the outcome of severe

CDH patients by improving the state of pulmonary hypoplasia and immaturity of the lung during the fetal period.

Acknowledgment

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Figure legend

Table 1. The GeneBank accession numbers and nucleotide sequences of the PCR primers used to real-time RT PCR

Fiugre 1. The mRNA expression of IGFs and IGFRs in the lungs of mice during the fetal periods from E11.5 to E18.5. All figure indicated that two embryonic days were combined to each one bar of E11.5-E12.5, E13.5-E14.5, E15.5-E16.5, E17.5-E18.5.

Figure 2. TTF-1 immunohistochemical staining in the organ cultured lungs.
A: group I. B: group II. C: group III
Scale bar = 100 μm

Figure 3. ProSp-C immunohistochemical staining in the organ cultured lungs.
A: group I. B: group II. C: group III.
Scale bar = 100 μm

Figure 4. α-SMA immunohistochemical staining in the organ cultured lungs.
A: group I. B: group II. C: group III.
Scale bar = 100 μm

TABLE 1. GeneBank Accession Numbers and Nucleotide Sequences of the PCR Primers Used to real-time PCR

Gene	Accession number	Forward primer	Reverse primer	Annealing temperature	Product size
IGF-Ⅰ	NM_010512	5’-tggatgctcttcagttcgtg-3’	5’-gtcttgggcatgtcagtgtg-3’	59	220
IGF-Ⅱ	NM_010514	5’-ccttcgccttgtgctgcat-3’	5’-acggttggcacggcttgaa-3’	59	134
IGF-ⅠR	NM_010513	5’-ctttcaatcccaagctgt-3’	5’-tgaaacggagaacatcac-3’	59	141
IGF-ⅡR	NM_010515	5’-gtgtcctctgggtgtggact-3’	5’- ctcctccttgctgacctttg-3’	59	241
GAPDH	NM_1037921	5’-ggagcgagacccctctaacatc-3’	5’-ctcctggttcacacccatcac-3’	60	181

Table 1. The GeneBank accession numbers and nucleotide sequences of the PCR primers used to real-time RT PCR

Figure 1

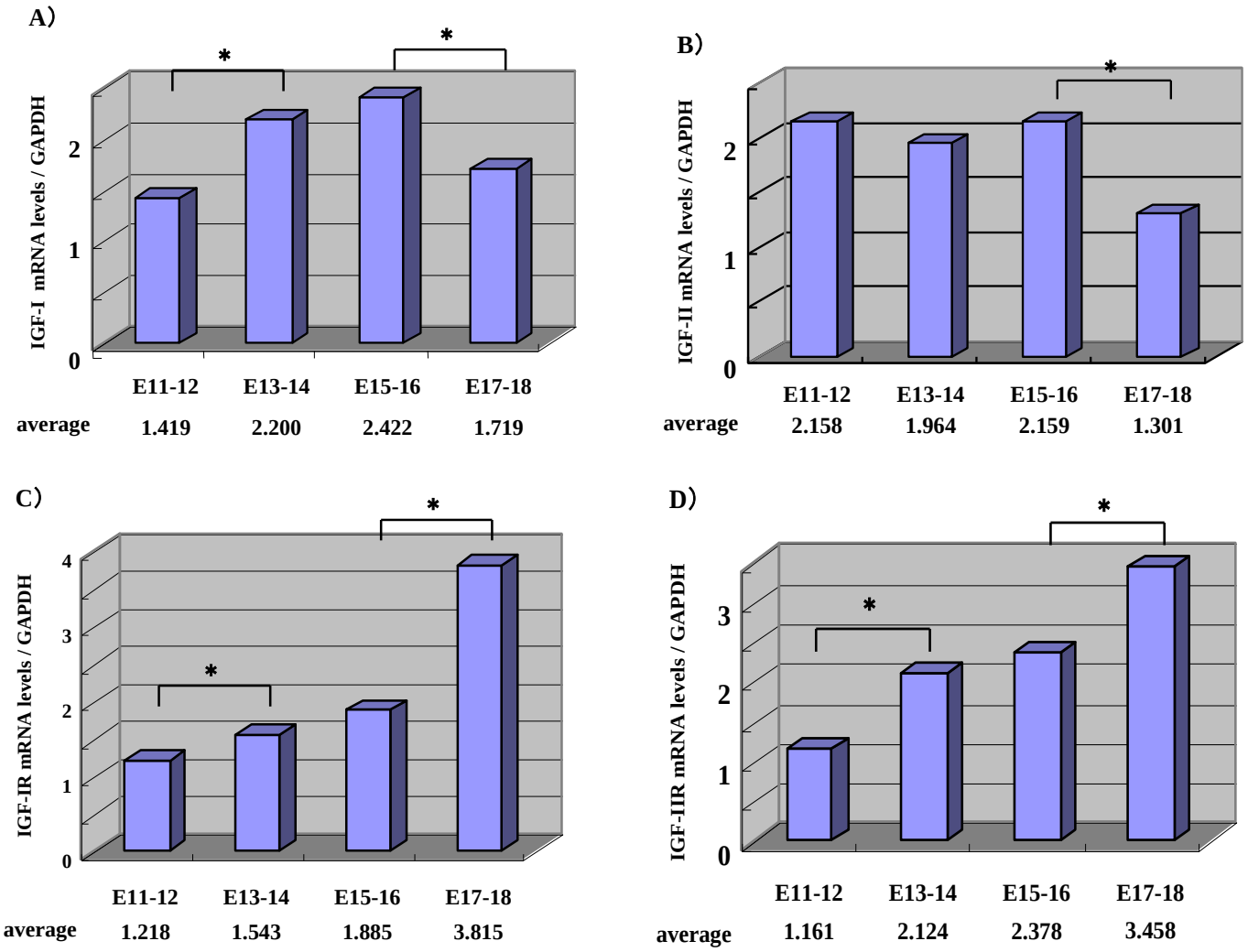

Figure 1. The mRNA expression of IGFs and IGFRs in the lungs of mice during the fetal periods from E11.5 to E18.5. All figure indicated that two embryonic days were combined to each one bar of E11.5-E12.5, E13.5-E14.5, E15.5-E16.5, E17.5-E18.5.

＊; A value of $P<0.05$ was considered to be statistically significant.

Figure 2

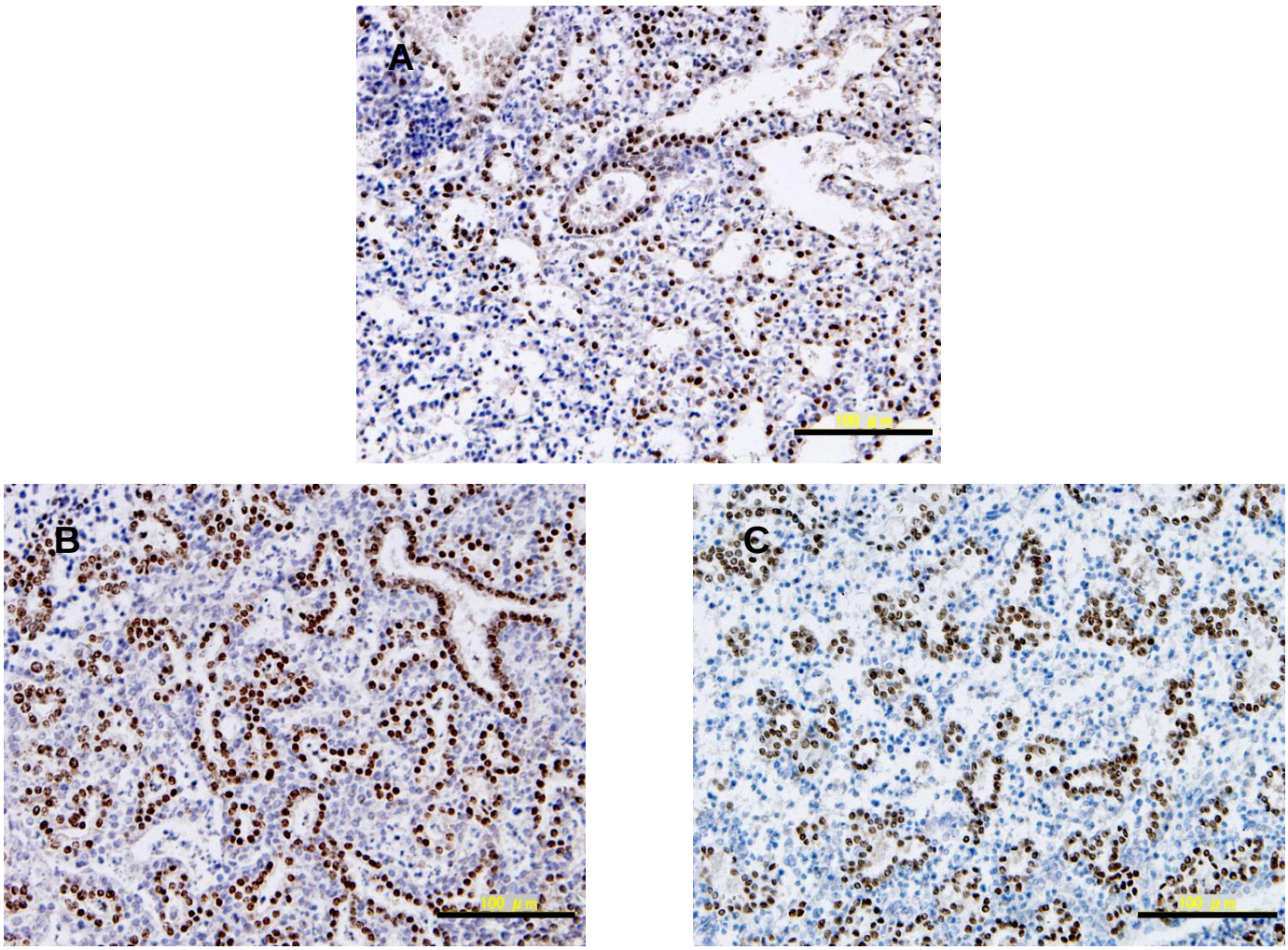

Figure 2. TTF-1 immunohistochemical staining in the organ cultured lungs.

A: group I. B: group II. C: group III Scale bar = 100 μm

Figure 3

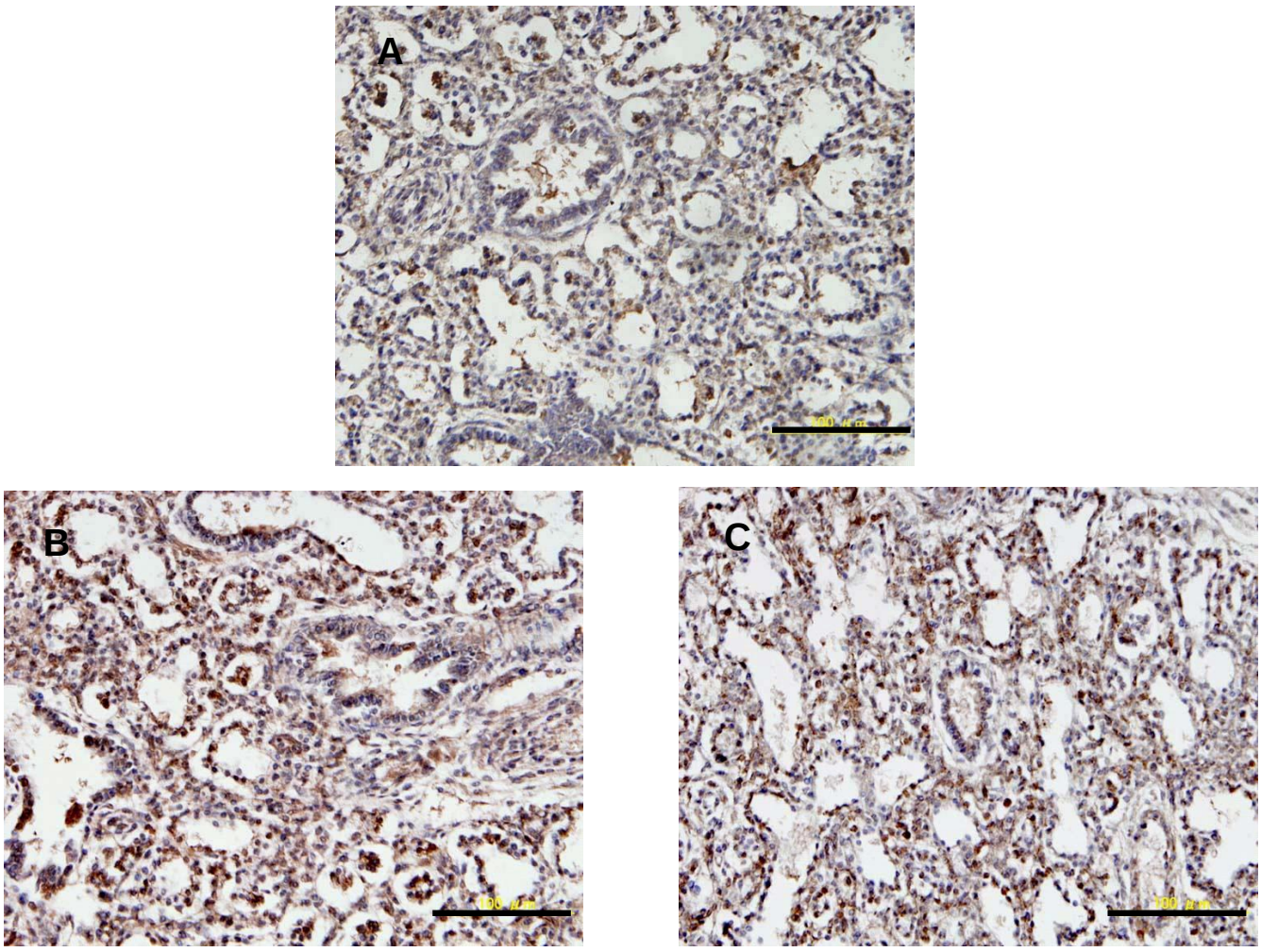

Figure 3. ProSp-C immunohistochemical staining in the organ cultured lungs.

A: group I. B: group II. C: group III. Scale bar = 100 μm

Figure 4

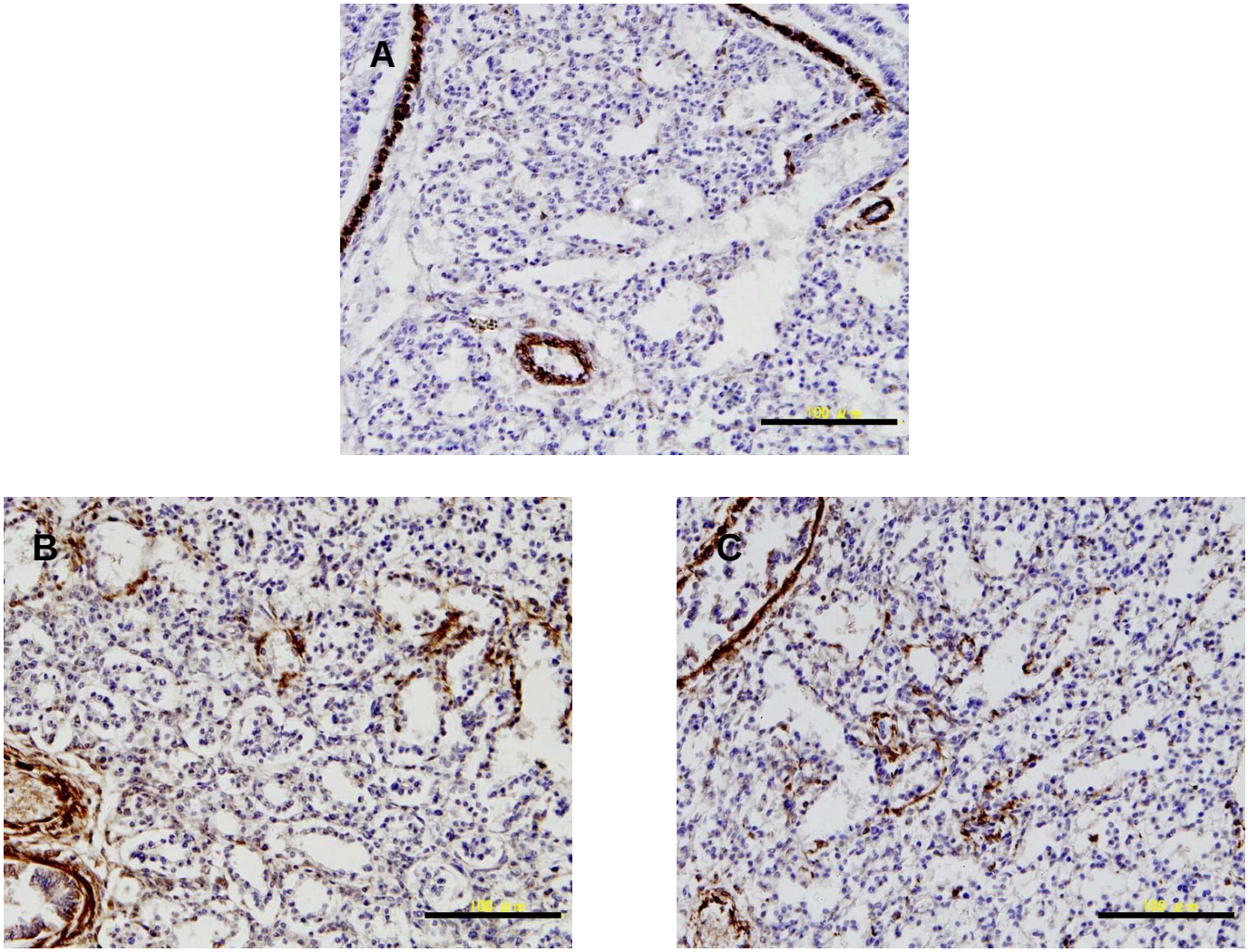

Figure 4. α-SMA immunohistochemical staining in the organ cultured lungs.

A: group I. B: group II. C: group III. Scale bar = 100 μm