Forkhead box m1 mRNA expression of lung of preterm and term rabbits

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Abstract. Previous studies on forkhead box m1 (Foxm1) of mice demonstrated the correlation between this gene and lung maturation. However, no study has been conducted on human Foxm1 with regard to lung maturation. The aim of this study was to compare the mRNA expression of surfactant protein (SP)-A, -B, -C and Foxm1 gene of preterm rabbits to that of full-term ones and to determine the association between Foxm1 and lung maturation. New Zealand white rabbits were grouped according to gestational age. Cesarean sections were carried out after rabbits were divided into two groups of 30-31 days of gestation (term group, n=18) and 26-27 days of gestation (preterm group, n=18). mRNA expression levels of SP-A, -B, -C and Foxm1 were compared by using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). The relative ratios of SP-A, -B and -C mRNA expression levels of the preterm to term groups were 0.380, 0.563 and 0.448:1, respectively, on qRT-PCR. By contrast, Foxm1 expression was increased in the preterm group and its relative expression ratio to the term group was 2.166:1 for RT-PCR and qRT-PCR, which was double that of the Foxm1 gene in the term group. Moreover, a significant correlation between the expressions of these genes was found. Foxm1 is considered to be an important gene required for the lung maturation of preterm rabbits in correlation with SP genes.

Introduction

Forkhead box m1 (Foxm1) is involved in various cell proliferations and it disappears in differentiated cells (1-4). Studies have focused on the function of Foxm1 in relation to lung development in species such as mice and rats. Kalin *et al* (5) reported that conditionally Foxm1 deleted mice had no changes in lung growth, branching morphogenesis, or epithelial proliferation in lung maturation of mice, but it caused a respiratory failure after birth. Foxm1 deficiency resulted in the reduction of the size of peripheral saccules, the number of type I pulmonary epithelial cells and mRNA expression of T1-α and aquaporin 5 in quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis in mice (5). Foxm1 was found to regulate the expression of surfactant protein (SP)-A, -B, -C and -D in lung maturation (5).

Although the relationship between Foxm1 and lung maturation in mice was previously reported, no study has focused on the functions of Foxm1 in lung maturation of human or rabbit. Since rabbit lungs are known to have coterminous characteristics in the functional and structural aspects to those of humans (6,7), the effects of Foxm1 on the lung development of humans could be studies more closely by using rabbit models.

This study was conducted to identify the Foxm1 expression of preterm rabbits and to compare the expression levels of SP-A, -B, -C and Foxm1 mRNA between the lung tissues from preterm and term rabbits with different lung developmental stages.

Materials and methods

Animal protocol. The Animal Experimentation Committee in Kyung Hee University at Gangdong approved the protocol (KHNMC-IACUC-10-03). Eleven pregnant New Zealand white rabbits delivered their newborn rabbits by cesarean section according to gestational age on 25.8±0.7 days, which corresponded to the canalicular stage of lung development (preterm group) and 30.2±0.4 days of gestation, which represented the alveolar stage (term group). The weights at birth of the two groups were 32.9±7 and 50.3±9.6 g. The lung tissues of other newborn rabbits were dissected and frozen quickly in liquid nitrogen for total RNA extraction (n=18 of preterm group and n=18 of term group).

RNA isolation. Total RNA was extracted from the rabbits' fetal lung tissues of the preterm and term groups using the

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TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction. For mRNA analysis, RT-PCR for Foxm1 was performed according to the manufacturer's instructions. PCR was carried out using EconoTaq® PLUS GREEN 2X Master mix (Lucigen Corporation, WI, USA) with Bioer (GeneQ) PCR machine (Bioer Technology Co., Ltd., Hangzhou, China). All applications were run according to the manufacturer's instructions. PCR products were applied to electrophoresis on an agarose gel (1.5%) and stained with ethidium bromide. PCR products were then quantified using the software program 'ImageJ', which measures gel density and normalized against the expression of housekeeping gene (18S) mRNA.

Quantitative real-time reverse transcription-polymerase chain reaction. qRT-PCR analysis was performed for the quantification of mRNA expression of SP-A, -B, -C and Foxm1 of rabbit lungs using a Chromo4 real-time PCR (Applied Bio-Rad, Foster City, CA, USA) according to the manufacturer's instructions. Samples were amplified with AccuPower® 2X Greenstar qPCR Master mix (Bioneer, Daejeon, Korea) and combined with inventoried SYBR®-Green I gene expression assay. Reactions were analyzed in triplicate and mRNA expression levels were normalized to 18S. Relative quantification of mRNA expression was performed using the $2^{-\Delta\Delta CT}$ method.

The primer design of Foxm1 gene of rabbits. Foxm1 primers were designed due to the lack of sequences of Foxm1 of rabbits. Sequence alignments of primers were generated by comparing human Foxm1 sequences (GenBank; accession no. NM_202002.1) to whole sequences of chromosome no. 8 of rabbit (GenBank; accession no. NW_003159267.1) using the software program of primer 3, version 3.0 site (http://biotools.umassed.edu/bioapps/primer3_www.cgi; accessed October 11, 2011). The size of the PCR product was 327 bp and the annealing temperature was 50.5˚C.

Cloning and sequencing for Foxm1 mRNA of rabbit. The PCR products of Foxm1 of rabbit lung tissues were purified with a gel extraction kit (LaboPass™ Gel; Cosmo Genetech, Seoul, Korea) according to the manufacturer's instructions. Cloning was carried out using a TA Cloning® kit (Invitrogen Life Technologies) without restrictive enzymes, according to the manufacturer's instructions. Nucleotide sequences were aligned using BigDye® Terminator v3.1 Cycle Sequence kit (Applied Biosystems, Inc., Foster City, CA, USA).

Immunohistochemical staining. Lung tissues of obtained preterm and term rabbits were fixed overnight with 10% buffered formalin and then embedded into paraffin blocks. The tissue blocks were sectioned into 3-4 μm sections. Slides were incubated with primary antibody against Foxm1 (ab55006, mouse monoclonal antibody; Abcam, Cambridge, MA, USA) at a dilution of 1:400. The slides were stained with liquid diaminobenzidine tetrahydrochloride (DAB+), a high-sensitivity substrate-chromogen system (K3468; DakoCytomation, Glostrup, Denmark). Counterstaining was performed with Meyer's haematoxylin. The images on the slides were visualized using an Olympus BX40 light microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

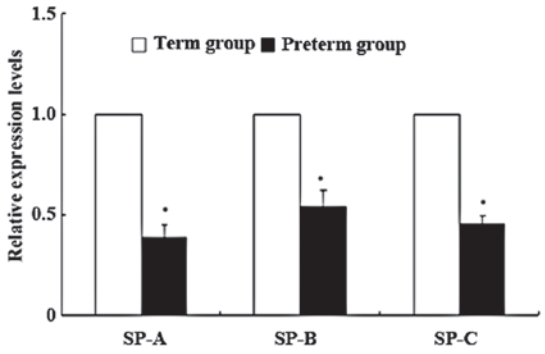

Figure 1. Relative expression levels of surfactant protein (SP)-A, -B and -C mRNA. Relative expression levels of SP-A, -B and -C mRNA were measured by quantitative real-time reverse transcription-polymerase chain reaction for quantification of mRNA expression. The expression of SP-A, -B and -C was decreased 0.380, 0.563 and 0.448 times more in the preterm group than in the term group. The gene expression is presented with a scale of fold change calculated by $2^{-\Delta\Delta CT}$. *P<0.05.

Statistical analysis. Data analyses were performed using Statistical Package for Social Sciences (SPSS, version 17). Comparisons of mRNA expression levels between preterm and term groups were performed by the Student's t-test. The Pearson's correlation coefficient was used to confirm statistical correlations of the mRNA expression. Data are presented as means ± SD and P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of SP-A, -B and -C mRNA using qRT-PCR. When the relative ratio of the mRNA expression levels of SP-A, -B and -C in the term group was fixed at 1, the expression levels were clearly decreased to 0.386, 0.539 and 0.450, respectively, in the preterm group (P<0.01, Fig. 1). There were no differences in the mRNA expression levels of SP-A, -B and -C in relation to birth order or locations of lung tissues of fetal rabbits in the same mother.

Comparisons of Foxm1 expression levels of preterm and term rabbit lung models using RT-PCR and qRT-PCR. Gel density of RT-PCR products was measured to quantify the expression level of Foxm1 in each group (Fig. 2). Using RT-PCR, when the ratio of the term to preterm group was 1:2.08, the Foxm1 mRNA expression level of the preterm group was increased to 2.1 times more than that of the term group. The ratio of multiple changes in the threshold cycle (CT) of the two groups was 1:2.166 in qRT-PCR. Therefore, the Foxm1 mRNA expression level was increased by more than double in the preterm group (Fig. 3) and this indicated that both qRT-PCR and RT-PCR had similar results.

After performing RT-PCR, the products were examined by gel refinement, cloning and gene sequencing analysis (Cosmo Genetech, Co., Ltd., Seoul, Korea). Based on that result, homological confirmation and gene sequence analysis

Table I. Pearson's correlation coefficient between surfactant protein (SP)-A, -B, -C and forkhead box m1 (Foxm1) mRNA expression in real-time reverse transcription-polymerase chain reaction.

Item	SP-A total (term/preterm)	SP-B total (term/preterm)	SP-C total (term/preterm)	Foxm1 total (term/preterm)
SP-A	1.00 (1.00/1.00)	-	-	-
SP-B	0.89[a] (0.81[a]/0.76[a])	1.00 (1.00/1.00)	-	-
SP-C	0.85[a] (0.77[a]/0.78[a])	0.87[a] (0.92[a]/0.55[a])	1.00 (1.00/1.00)	-
Foxm1	-0.46[a] (-0.20/-0.48[a])	-0.35 (-0.20/-0.44[a])	-0.34 (0.16/-0.23)	1.00 (1.00/1.00)

[a]P<0.05.

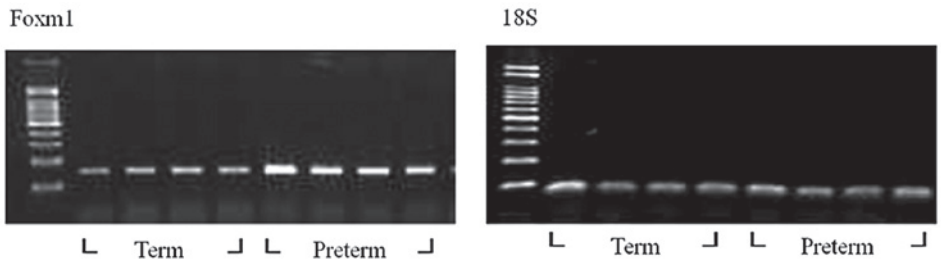

Figure 2. Reverse transcription-polymerase chain reaction (RT-PCR) products of forkhead box m1 (Foxm1) and housekeeping gene (18S). Increased PCR production of Foxm1 in the preterm group is shown and that Foxm1 mRNA expression of the preterm group was increased 2.1 times over that of the term group.

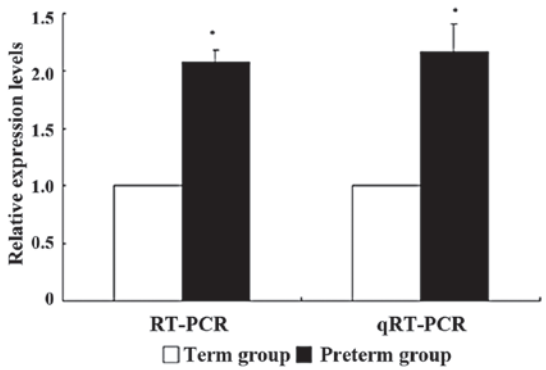

Figure 3. Relative expression of forkhead box m1 (Foxm1) to housekeeping gene (18S) mRNA. The reverse transcription-polymerase chain reaction (RT-PCR) product is increased about 2.080 times in the preterm group compared with term group. The expression of Foxm1 mRNA measured by quantitative real-time RT-PCR (qRT-PCR) was increased by 2.166 times in the preterm group than in term group. The gene expression is presented with a scale of fold change calculated by $2^{-\Delta\Delta CT}$. *P<0.05.

were performed using the BLAST program. Our reported sequence matched 99% from that of another study for Foxm1 gene of rabbits and we ascertained that those gene sequences matched 89% with the human Foxm1 gene sequence. The GenBank accession no. of our Foxm1 sequence of rabbits is JN817622.

We confirmed that there were statistical correlations of mRNA expression between SP-A, -B, -C and Foxm1 through Pearson's correlation coefficient. Statistically, a strong positive correlation was determined among SP-A, -B and -C, but Foxm1 had a negative correlation with SP-A regardless of the group. However, when the results were separated into preterm and term groups, the results were different. There were no correlations between Foxm1 and SP-A, -B and -C mRNA expression levels in the term group, while there were negative correlations between Foxm1 and SP-A and -B statistically in the preterm group (Table I).

Immunohistochemical staining of Foxm1 of lung tissue from rabbits. Decreased sacculation, aerations and mesenchymal thickening were observed in the preterm rabbits as compared to the term rabbit lungs. The cells, which were stained with Foxm1 antibody, were expressed in the most immature epithelial and endothelial cells of the lung tissues of the preterm rabbits. General expression of Foxm1 staining cells was decreased in the term rabbit lung tissues (Fig. 4).

Discussion

Studying animal models to investigate the effect of gene expressions in the lung maturation of humans is useful due to their accessibility. However, each species of animal has different lung maturation stages (8-10) and it is difficult to predict the lung maturation of humans using animal models. Although rabbits with Foxm1 are suspected to be associated with lung maturation, few studies have been conducted regarding this association and most studies have used rat or mouse models. Rabbits are commonly used to study the human respiratory system and are a commonly used research model. Moreover, alveolar maturation stages and SP production processes of rabbits are known to be very similar to those of humans (6,9). In addition, reciprocal chromosome painting using human chromosomes showed complete homology in

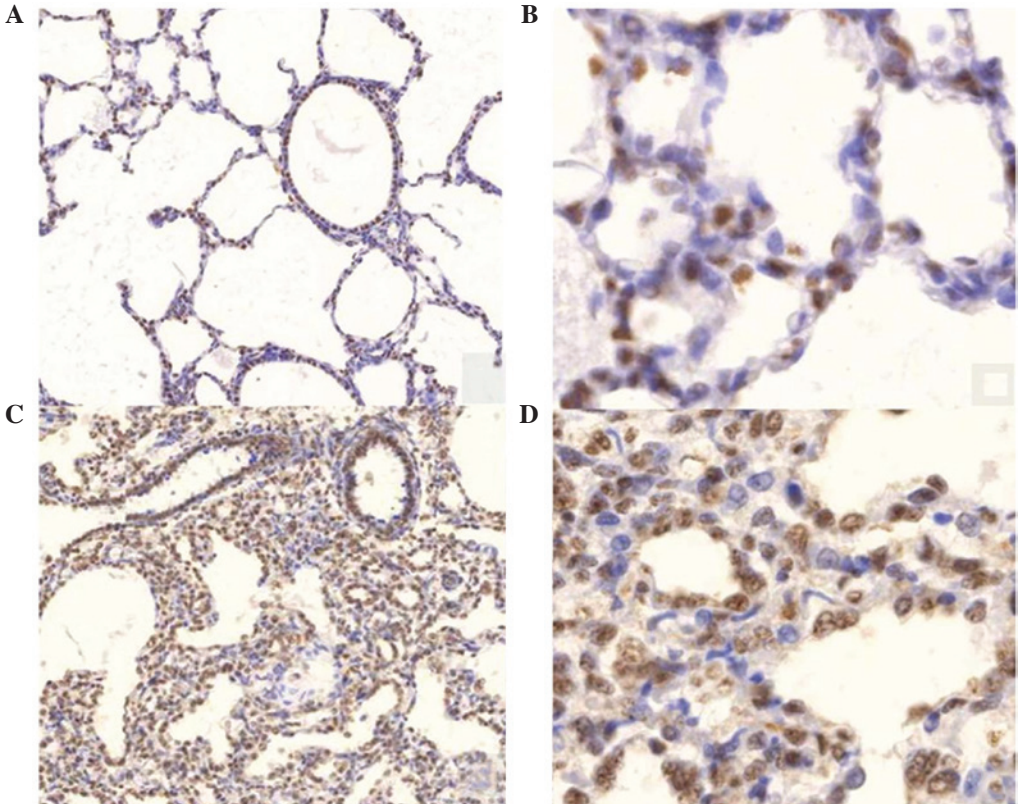

Figure 4. Immunohistochemical staining of lung specimens of fetal rabbits. Immunohistochemical expression of forkhead box m1 (Foxm1) in the preterm rabbit is evident and generally increased in epithelial and endothelial cells of lung tissues compared to the term rabbit. The general expression of Foxm1 in mature lung tissue is decreased compared to immature lung tissue (A, magnification x10; B, magnification x100). Representative immunohistochemical staining showed diffuse immunoreactivity to Foxm1 on lung tissue of a preterm rabbit (C, magnification x40; D, magnification x100).

more than three chromosomes (7). Based on these observations, a rabbit seems to be a more appropriate model compared with other classical animal models for studying human lung development and various gene expressions during intrauterine and early neonatal life (11-14).

To the best of our knowledge, this is the first study to report the Foxm1 expression level of rabbit lung; the gene sequence of Foxm1 of rabbits has also not been reported. For the present study, we designed rabbit Foxm1 primers using human Foxm1 gene information to obtain mRNA products by RT-PCR and qRT-PCR from rabbit lung tissues. Furthermore, partial cloning was conducted for the first time using RT-PCR products and we reported the Foxm1 sequence. The Foxm1 sequence presented in this study is likely to be useful for studies on the function of Foxm1 in lung maturation.

In a study using a mouse model, Foxm1 expression was found to be markedly decreased until birth and then to be slightly recovered after birth (15). The changes of Foxm1 expression level according to the gestational period might be a result of cell proliferation and mitosis. In undifferentiated respiratory cells, Foxm1 is involved in cell proliferation by directly activating various gene expressions that control the cell cycle and induce lung maturation as well (15). By deletion of Foxm1 gene, the lung maturation was suppressed as a respiratory failure developed by poor lung maturation, although morphologic property was not affected (4). Similarly, we found that the Foxm1 expression level of the preterm group was double compared to that of the term group in RT-PCR and qRT-PCR, with previous studies reporting similar findings (15). In the present study, lungs of the preterm group were found to have respiratory cell proliferation and SP secretion in correlation with Foxm1 expression until the lung maturation was achieved.

The expression levels of SP-A, -B and -C of the lung tissues of preterm groups were significantly different with term groups which were measured by qRT-PCR and it agrees with some previous studies. They reported that clearly increased mRNA expression levels were found on 26th day of gestational age compared with 28th or 30th day with mice models (16).

Furthermore, a significant statistical correlation between SP-A, -B, -C and Foxm1 mRNA expression levels was found in the preterm group. In this preterm group, the Foxm1 mRNA expression level was found to be increased, whereas SP-A and -B mRNA expression levels were found to be decreased, showing a negative correlation between the two factors. SP genes are known to be associated with lung maturation. However, Foxm1 might be associated with lung maturation in a negative manner. By contrast, there was no such correlation between the two variables for the term group, suggesting Foxm1 mRNA expression was markedly reduced as the lung was already mature in this group. Therefore, Foxm1 may be involved in lung maturation in a negative manner.

However, several limitations exist in the present study. First, further research on the serial changes of Foxm1 expression according to the gestational period is required to obtain more detailed information on the roles of Foxm1 on lung maturation. In addition, the exact time periods for when Foxm1 expression level stops increasing or when it starts

decreasing should be identified. Second, more detailed investigation on the lung maturation is required using a numerical method despite the expressions of SP genes being analyzed in the present study. Third, measuring the protein expression levels of Foxm1 and SPs is necessary to clarify the correlation between these molecules.

In conclusion, in the present study, we designed a partial sequence of Foxm1 gene of a rabbit and carefully defined this gene as an important factor for the maturation of lung in preterm rabbits. In addition, Foxm1 mRNA expression of preterm rabbits was double that of term rabbits. Therefore, future studies should be conducted to confirm Foxm1 is important in the prediction of lung maturation in premature infants or prognosis of preterm infants with respiratory distress syndrome.

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