

Decreased Expression of Surfactant Protein Genes Is Associated with an Increased Expression of Forkhead Box M1 Gene in the Fetal Lung Tissues of Premature Rabbits

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Purpose: Recently, Forkhead box M1 (FoxM1) was reported to be correlated with lung maturation and expression of surfactant proteins (SPs) in mice models. However, no study has been conducted in rabbit lungs despite their high homology with human lungs. Thus, we attempted to investigate serial changes in the expressions of FoxM1 and SP-A/B throughout lung maturation in rabbit fetuses. **Materials and Methods:** Pregnant New Zealand White rabbits were grouped according to gestational age from 5 days before to 2 days after the day of expected full term delivery (F5, F4, F3, F2, F1, F0, P1, and P2). A total of 64 fetuses were enrolled after Cesarean sections. The expressions of mRNA and proteins of FoxM1 and SP-A/B in fetal lung tissue were tested by quantitative reverse-transcriptase real-time PCR and Western blot. Furthermore, their correlations were analyzed. **Results:** The mRNA expression of SP-A/B showed an increasing tendency positively correlated with gestational age, while the expression of FoxM1 mRNA and protein decreased from F5 to F0. A significant negative correlation was found between the expression levels of FoxM1 and SP-A/B (SP-A: $R=-0.517$, $p=0.001$; SP-B: $R=-0.615$, $p<0.001$). **Conclusion:** Preterm rabbits demonstrated high expression of FoxM1 mRNA and protein in the lungs compared to full term rabbits. Also, the expression of SP-A/B was inversely related with serial changes in FoxM1 expression. This is the first report to suggest an association between FoxM1 and expression of SP-A/B and lung maturation in preterm rabbits.

Key Words: mRNA, prematurity, protein, surfactant protein-A, surfactant protein-B, FoxM1

INTRODUCTION

Fox proteins are an extensive family of transcription factors that regulate genes associated with cellular proliferation,¹⁻⁶ differentiation,^{1,7,8} and metabolism.^{6,9-11} The expression of Forkhead box M1 (FoxM1) is induced during cellular proliferation in a variety of cell types and is extinguished in terminally differentiated cells.^{5,9-12} FoxM1 plays an important role in the development of various organs during em-

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bryogenesis¹³⁻¹⁶ and the over-expression of FoxM1 has been found to be associated with the development of tumor cells in various organs including the liver, pancreas, stomach, intestine, lung, breast, and ovaries.^{12,17}

Recently, Kalin, et al.¹⁸ reported that maturation of lung development was inhibited in transgenic mouse models in which FoxM1 was conditionally deleted in the developing pulmonary epithelium (epFoxM1^{-/-}) and severe respiratory failure was induced after birth. Also, the expressions of surfactant protein (SP)-A, -B, -C, and -D were decreased in an epFoxM1^{-/-} mouse model.¹⁸ Moreover, we reported recently that the expression of FoxM1 mRNA in preterm rabbit fetuses was increased by more twice that in full term rabbits.¹⁹

Even though FoxM1 is suspected to be related to lung maturation, not enough studies have been conducted regarding this relationship, and most studies have been conducted in rat or mouse models. Rabbits are known to undergo alveolar developmental stages and processes of SP synthesis very similar with humans,²⁰⁻²² and have been used to study the expression of SP associated genes due to their chromosomal homology to humans.^{23,24} This is the reason why rabbits are proposed as more appropriate models for studying human lung development during intrauterine and early neonatal life, compared to other classical animal models.^{21,25-27} Furthermore, a rabbit fetus at a gestational age of 27-28 days was found to have immature lungs with several pieces of evidence of respiratory distress syndrome such as decreased aeration areas.^{19,20,23,27}

Thus, we hypothesized that serial changes in FoxM1 expression would be related with the expression of SP-A/B genes and lung maturation in lung tissues of rabbit fetuses. In

the present study, the expression of FoxM1 mRNA and protein was highest and the expression of SP-A/B mRNA was lowest in the lungs of preterm rabbits compared with term rabbits. Moreover, significant correlations were observed between these genes. The FoxM1 gene seems to be an important genetic factor for lung maturation in the aspect of SP-A/B expression.

MATERIALS AND METHODS

Materials

Eleven pregnant New Zealand White rabbits were grouped according to gestational age from 5 days before to 2 days after the day of expected full term delivery (30 days) as follows: F5, F4, F3, F2, F1, F0, P1, and P2. Fetuses were harvested by Cesarean section and the total lung tissues of fetuses and adult rabbits were obtained. A total of 64 fetuses were enrolled: F5 (n=5), F4 (n=10), F3 (n=10), F2 (n=8), F1 (n=14), F0 (n=4), P1 (n=7), and P2 (n=6). The demographic findings of the subjects are listed in Table 1. All procedures of this study were approved by the Animal Research Ethics Committee of Gangdong Kyung Hee University Hospital (KHNMC-IACUC-10-03).

Lung total RNA isolation and quantitative reverse-transcriptase real-time PCR (qRT-PCR) for SP-A/B and FoxM1

Individual lung tissues from all fetuses and adult rabbits were pulverized with a mortar and pestle while frozen in liquid nitrogen. Total RNA was extracted and purified using Trizol reagent[®] (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. Briefly, centrifugation was done at 12000 rpm for 10 minutes at 4°C and the precipitates were left at room temperature for 5 minutes. Next, 0.2 mL of chloroform was added and the homogenates were mixed vigorously for 15 minutes. The homogenates were left at room temperature for 3 minutes and centrifuged at 12000 rpm for 15 minutes at 4°C. The precipitates were transferred to clean tubes and 0.5 mL of isopropanol was added. These were left at room temperature for 10 minutes and then centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatants were then removed and the RNA pellets were washed with 0.5 mL ice cold 75% ethanol. Next, these were centrifuged at 7500 rpm for 5 minutes at 4°C, and the supernatants were carefully removed. The RNA was then resuspended in 10 uL of RNase-free water.

Table 1. The Demographic Characteristics of Fetus Rabbits

Groups*	n	Gestational age (days)	Birth weight (g)
F5	5	25	26.04±1.53 (23.72-27.12)
F4	10	26	33.56±5.17 (23.43-41.4)
F3	10	27	40.42±3.70 (36.12-46.3)
F2	8	28	38.69±5.93 (32.66-48.51)
F1	14	29	48.43±7.56 (40.00-61.57)
F0	4	30	66.25±2.52 (62.85-68.87)
P1	7	NA	38.41±5.14 (32.56-47.39)
P2	6	NA	79.03±17.53 (50.00-94.26)

NA, not applicable; SD, standard deviation.

Each value is presented as mean±SD (range).

*Pregnant New Zealand White rabbits were grouped according to gestational age from 5 days before to 2 days after the day of expected full term delivery (F5, F4, F3, F2, F1, F0, P1, and P2). Cesarean sections were carried out and a total of 64 fetuses were enrolled.

RNA concentration and purity were determined spectrophotometrically by measuring fluorescence at 260 nm and 280 nm with a NanoDrop® (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Only samples with a 260/280 absorbance ratio >1.8 were used for analyses. RNA (1 µg) was reverse-transcribed to cDNA using M-MLV Reverse Transcriptase (Mbiotech Inc., Torrance, CA, USA). Gene expression was quantified using the SYBR Green PCR Master Mix Kit® (Bioneer, Daejeon, Korea) and primers (Bioneer, Daejeon, Korea) on Chromo 4® for qRT-PCR detection (Applied Bio-Rad, Foster City, CA, USA). The qRT-PCR conditions were: 95°C for 15 min; and 44 cycles at 95°C for 15 s; at 56.5°C (specific for target gene) for 20 s; and 72°C for 20 s. The annealing temperatures of each set of primers are shown in supplementary Table 1. The 18S gene was used as an endogenous control (housekeeping genes). The amount of mRNA for each sample was normalized using the geometric average of the housekeeping genes. All reactions were duplicated, and the data were analyzed by threshold cycle (CT) values of the 2^{-ΔΔCT} method. The expression levels were expressed by fold changes of expression levels with respect to term control rabbits.²⁸

The sequences of primers (18S, SP-A/B, and FoxM1) used for PCR and qRT-PCR are presented in Table 2. The sequence of primer for FoxM1 was referenced from a recent report of the authors since the FoxM1 sequence of rabbits has not yet been determined.¹⁹ The primer sequence for FoxM1 was obtained by extensive searching of databases and by comparing homology between the FoxM1 sequence of humans and the whole sequence of chromosome 8 of rabbits (Genbank, accession number: NM_202002.1 and NW_003159267.1).

Western blot analysis for FoxM1

Lungs from fetuses and adult rabbits were harvested and used to prepare protein extracts. Protein extracts were run on 8% sodium dodecyl sulfate-polyacrylamide gels and transferred to PVDF membranes (Pall Life Science, transfer membrane 0.45 µm), followed by incubation with primary

human antibodies specific for FoxM1 (1 : 1000 dilution; ab55006, Abcam®, Cambridge, UK) since rabbit antibodies are not commercially available yet. Secondary antibodies were conjugated with peroxidase-linked mouse antibody (1 : 2000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). β-actin was used as a loading control. The protein expression levels of FoxM1 were analyzed by the NIH ImageJ program, and were expressed as fold changes compared to the levels of adult rabbits.

Statistical analysis

Statistical analysis was performed using SPSS (version 18, SPSS Inc., Chicago, IL, USA) and one-way ANOVA was used to determine statistical significance. Linear regression analysis and correlation analysis were used to analyze the relations between the expression levels of each gene. *p*-values <0.05 were considered significant. All measurements were expressed as mean±standard deviation.

RESULTS

mRNA expressions of SP-A/B during lung development

The lowest SP-A/B mRNA levels were observed at F5, and they gradually increased thereafter until birth (Fig. 1). However, the changes after birth were slightly different between the mRNA expression levels of these genes. SP-A mRNA showed a slightly decreasing tendency after birth; SP-B mRNA increased at P1, but decreased at P2 (Fig. 1).

mRNA and protein expressions of FoxM1 during lung development

The highest FoxM1 mRNA level was observed at F5, and it gradually decreased thereafter until birth (Fig. 2). However, FoxM1 expression levels were slightly increased during the first day of the postnatal period (P1) and started to decrease at P2 (Fig. 2). The relative protein expression levels of FoxM1 were tested by Western blot analysis. The protein expression levels showed a gradually decreasing tendency

Table 2. Oligonucleotide PCR Primers of Surfactant Protein-A/B, 18S, and Forkhead Box M1 (FoxM1) Genes

Gene	Sequence		Annealing temperature (°C)	Fragment size (bp)
	Sense	Antisense		
Surfactant protein A	ccgctggagacttctactacct	cagttcttgcattccacttcc	57	133
Surfactant protein B	cactacttccagagccagatt	acagataccgccaccac	55	208
18S	gccgctagaggtgaaattcttg	cattcttgcaaatgctttcg	59	66
FoxM1*	aggaaagctgacttggaaac	gtgcatgttttcttccag	56.5	139

*The sequence of primers for FoxM1 was referenced from our previous report.¹⁹

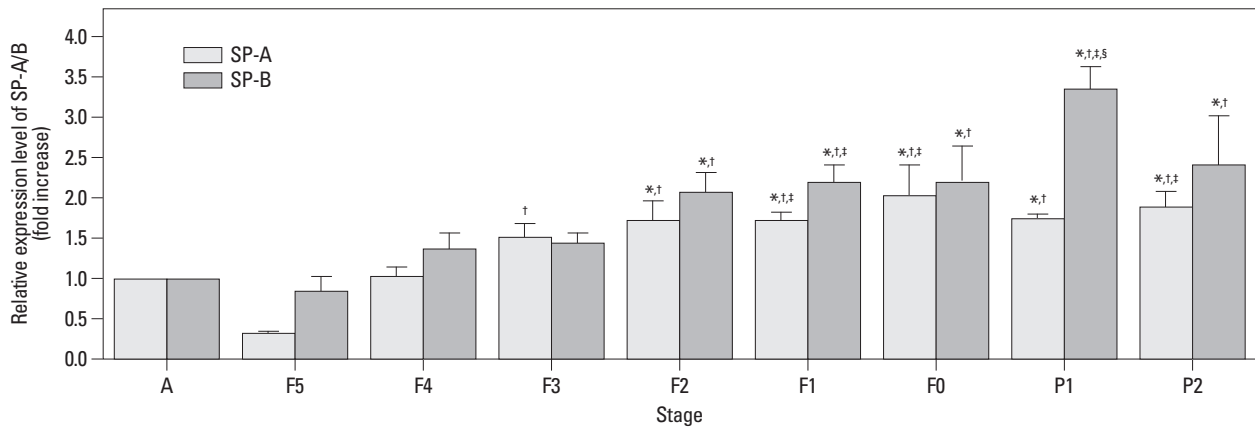


Fig. 1. Relative expression levels of surfactant protein (SP)-A and -B mRNA in rabbit lungs. Pregnant New Zealand White rabbits were grouped according to gestational age from 5 days before to 2 days after the day of expected full term delivery (F5, F4, F3, F2, F1, F0, P1, and P2). Cesarean sections were carried out and a total of 64 fetuses were enrolled. The expression levels of mRNA of SP-A and -B in fetal lung tissues were tested by quantitative reverse-transcriptase real-time PCR (qRT-PCR). qRT-PCR revealed increasing changes in endogenous SP-A and -B mRNA expression levels throughout lung development. The gene expression used a scale of fold changes compared to the mRNA level of adult rabbits, which is calculated by $2^{-\Delta\Delta CT}$. Statistical analysis was done by one-way ANOVA. A, adult rabbit. * $p < 0.05$ versus adult group. † $p < 0.05$ versus F5 or preterm group. ‡ $p < 0.05$ versus F4. § $p < 0.05$ versus F3.

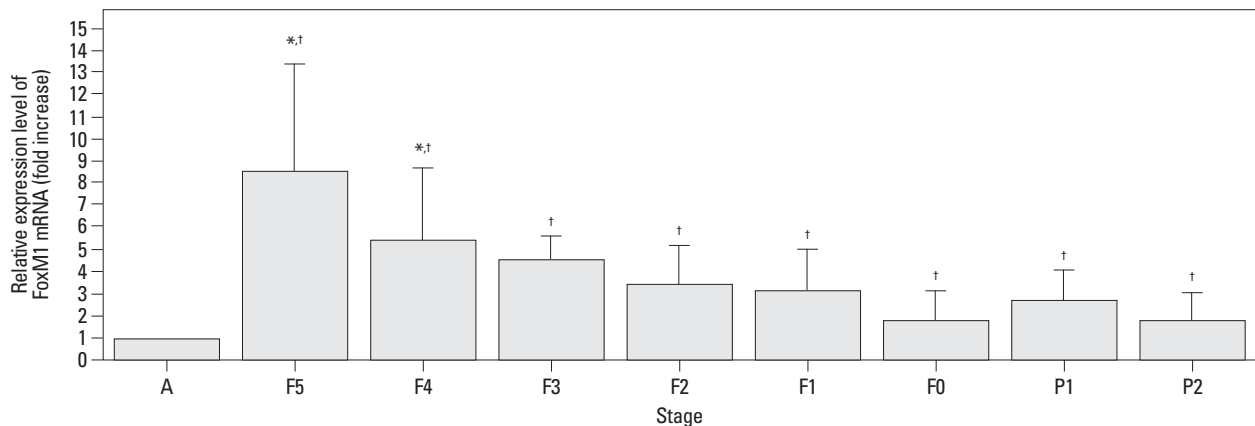


Fig. 2. Relative expression levels of Forkhead box M1 (FoxM1) mRNA in rabbit lungs. Pregnant New Zealand White rabbits were grouped according to gestational age from 5 days before to 2 days after the day of expected full term delivery (F5, F4, F3, F2, F1, F0, P1, and P2). Cesarean sections were carried out and a total of 64 fetuses were enrolled. The expression levels of FoxM1 mRNA in fetal lung tissues were tested by quantitative reverse-transcriptase real-time PCR (qRT-PCR). qRT-PCR revealed progressively decreasing changes in endogenous FoxM1 mRNA expression levels throughout lung development. The gene expression used a scale of fold changes compared to the mRNA level of adult rabbits, which is calculated by $2^{-\Delta\Delta CT}$. Statistical analysis was done by one-way ANOVA. A, adult rabbit. * $p < 0.05$ versus adult. † $p < 0.05$ versus F5.

during the lung development stages corresponding with the changes of mRNA expression (Fig. 3). However, post-natal increase was not observed. The relative protein expression levels were higher than their mRNA levels during all development stages.

Correlation analysis between expression levels of SP-A/B and the FoxM1 genes

In linear regression analysis and correlation analysis between the expression levels of mRNA and protein of the FoxM1 gene, a statistically significant positive correlation was found ($R=0.744$, $p=0.006$). The mRNA expression of SP-A and -B was negatively correlated with the mRNA expression of FoxM1 (SP-A: $R=-0.517$, $p=0.001$; SP-B: $R=-0.615$, $p <$

0.001). In addition, a statistically significant correlation was found between the mRNA expression of SP-A and SP-B ($R=0.734$, $p < 0.001$) (Table 3, Fig. 4).

DISCUSSION

No study has been conducted on the role of FoxM1 in lung development using human lung tissues. To date, most studies on genes associated with lung development have been performed with rat or mouse models.^{13,18,29,30} These studies pose some limitations in estimating lung development in human lungs since the development stages of rats and mouse lungs are different from humans.³¹⁻³³

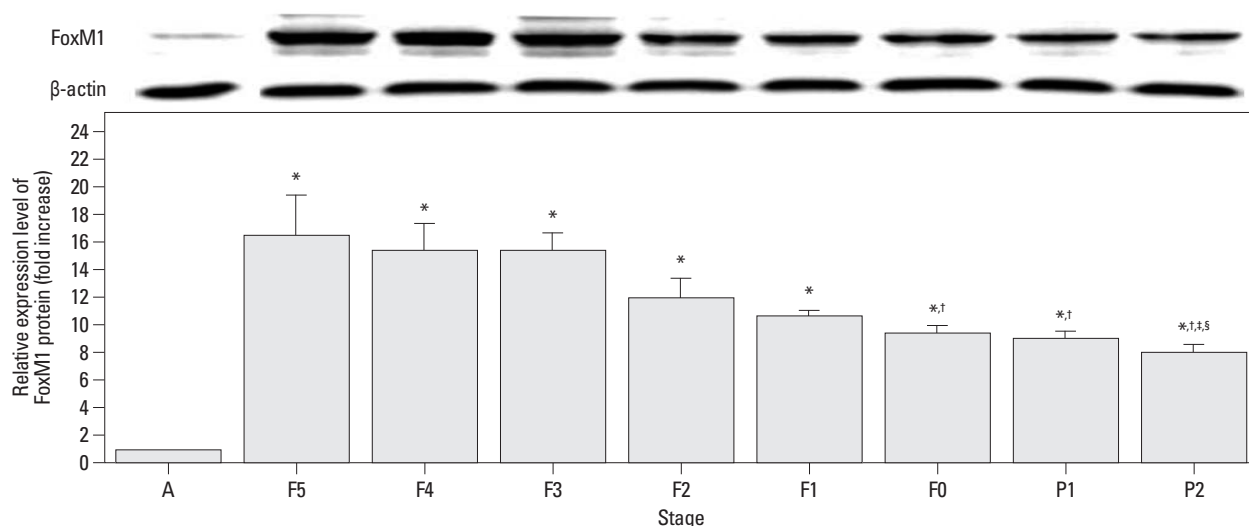


Fig. 3. Relative expression levels of Forkhead box M1 (FoxM1) protein in rabbit lungs. Pregnant New Zealand White rabbits were grouped according to gestational age from 5 days before to 2 days after the day of expected full term delivery (F5, F4, F3, F2, F1, F0, P1, and P2). Cesarean sections were carried out and a total of 64 fetuses were enrolled. The expression levels of FoxM1 proteins in fetal lung tissues were tested by Western blot. The representative bands of Western blot revealed progressively decreasing changes in endogenous FoxM1 protein band intensity throughout lung development. The protein expression used a scale of fold changes compared to the FoxM1 protein level of adult rabbits. Statistical analysis was done by one-way ANOVA. A, adult rabbit. * $p < 0.05$ versus adult. † $p < 0.05$ versus F5. ‡ $p < 0.05$ versus F4. § $p < 0.05$ versus F3.

Table 3. Correlations between Each Expression Level of mRNA and Protein of Surfactant Protein (SP)-A/B and Forkhead Box M1 (FoxM1)

Parameters	SP-A mRNA		SP-B mRNA		FoxM1 mRNA*		FoxM1 protein*	
	Correlation (r)	p value	Correlation (r)	p value	Correlation (r)	p value	Correlation (r)	p value
SP-A mRNA	-	-	0.734	<0.001	-0.517	0.001	-0.624	0.023
SP-B mRNA	0.734	<0.001	-	-	-0.615	<0.001	-0.681	0.015
FoxM1 mRNA*	-0.517	0.001	-0.615	<0.001	-	-	0.744	0.006
FoxM1 protein*	-0.624	0.023	-0.681	0.015	0.744	0.006	-	-

*These parameters were substituted with Logarithm of 10 and the Log values followed normal distribution.

Rabbits are known to have alveolar developmental stages and processes of SP synthesis that are very similar with humans.^{20-22,34} Rabbits also show chromosomal homology to humans,²³ although SP-A is encoded by two highly similar genes (SP-A1 and SP-A2) in humans and by a single copy gene in mice, rats, and rabbits.³⁵ SP-B and -C are encoded by single-copy genes that are expressed in a lung-specific manner.³⁵ In addition, the quality of the currently available rabbit genome sequencing is comparable to the rat genome sequence data.³⁶ As well, the availability of transgenic rabbit models and the larger size of rabbits compared to relatively small-sized mice are two advantages for using rabbits in studying human lung development.³⁶⁻³⁸

In the lung developmental stages of rabbit fetuses, mesodermal tissue starts forming primitive alveolar septa (canalicular stage) on the 23rd day of gestational age (which is seven days before the day of expected full term delivery; F7). On the 26th day of gestational age (F4), the cytoplasm of cuboidal cells are filled with inclusion bodies and the devel-

opment of central areas of the lungs is completed, while a thick septum is still found in the peripheral areas (saccular stage).³⁹ From the 27th day of gestational age (F3), the alveolar stage begins and the number of inclusion bodies increases rapidly in the cytoplasm. By the 30th day of gestational age (F0), inclusion bodies are found in most alveoli and the developmental stages of the lungs are finally completed.^{21,39} However, recent data pointed to the fact that the well-described mouse model does not represent crucial events in early mammalian development and stem cell biology.⁴⁰ In accordance with these observations, a rabbit model seems to be more appropriate model, compared with other animal models, in terms of studying human lung development.^{21,25-27}

FoxM1 is known to play important roles in lung development including regulation of the expression of genes essential for proliferation of mesoderm, remodeling of extracellular matrix, and pulmonary angiogenesis.¹³

Recently, Kalin, et al.¹⁸ reported that transgenic mouse

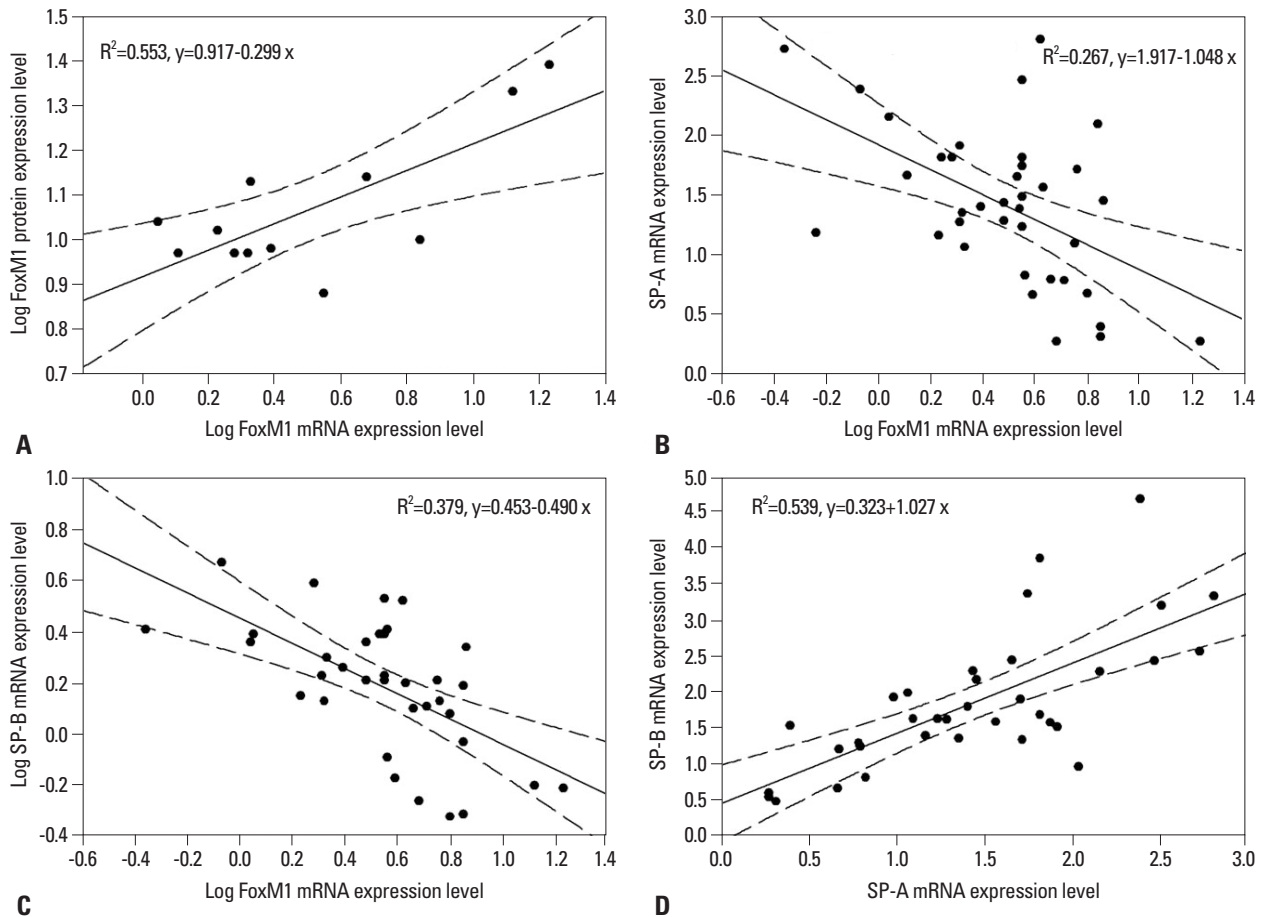


Fig. 4. Univariate linear regression analysis of the expression levels of mRNA and protein of Surfactant protein (SP)-A/B and Forkhead box M1 (FoxM1) genes. The diagonal solid lines are the regression lines and the dashed lines are approximate 95% confidence intervals. Significant positive correlations between FoxM1 expression levels of mRNA and protein (A), and mRNA expression levels of SP-A and SP-B (D) were found. Moreover, significant negative correlations between mRNA expression levels of FoxM1 and SP-A/B were found (B and C).

models in which FoxM1 was conditionally deleted in the developing pulmonary epithelium did not alter lung growth, branching morphogenesis, or epithelial proliferation in a mouse model. However, maturation of the lung was inhibited and severe respiratory failure developed after birth. Maturation defects were associated with decreased expression of T1- α and aquaporin 5, which was consistent with a delayed differentiation of type I alveolar cells. They also found that the expression of SP-A, -B, -C, and -D was decreased by deletion of FoxM1. Furthermore, the FoxM1 transcription factor was found to bind directly with the promoter of the SP-A and -B genes to enhance the mRNA expression of these genes.¹⁸ With these findings, they concluded that FoxM1 is closely associated not only with structural maturation of the lung, but also with the synthesis of SPs, which is an essential feature of neonatal respiratory distress syndrome.

In the present study, we revealed that the expression of FoxM1 mRNA had a significant increasing tendency during the prenatal period. This is consistent with a recent mouse

study by Wang, et al.,³⁰ even though the postnatal change in FoxM1 expression differed from the present study. They found that the expression of FoxM1 was reactivated just after birth and rapidly decreased at day 10 of the postnatal period, while no significant change was found in the FoxM1 expression levels just before and after birth in the present study. Moreover, adult rabbits had very low FoxM1 expression levels in lung tissues compared with the term and postnatal groups in the present study. With these results, it is possible to assume that the expression of FoxM1 is high in the premature lung, decreases rapidly until birth, maintains its level during certain postnatal periods, and then decreases in the developed lung. This implicates that additional growth and development of the lung may be regulated by FoxM1 during certain postnatal periods.

Several studies support the possible roles of FoxM1 in the recovery process from postnatal injury of the lungs in addition to its involvement in lung development. In a study with FoxM1 deleted mice, FoxM1 was shown to be impor-

tant in the recovery process from endothelial injury of pulmonary vasculature. They concluded that the abnormal function of FoxM1 may be one of the important causes of pulmonary edema by permanent vascular injury.⁴¹ Moreover, the expression of FoxM1 was significantly increased in type II alveolar cells in a pneumonia mouse model, and FoxM1 was found to be important in the transition to type I alveolar cells, maintenance of intra-alveolar barriers, and recovery from lung injuries.⁴² Therefore, it may be worthy to study the possible roles of FoxM1 in the pathogenesis of postnatal lung diseases, such as neonatal chronic lung disease and various infective lung diseases.

The steady decreasing tendency of FoxM1 protein expression without postnatal increase did not correspond with the expression levels of FoxM1 and SP-A/B mRNA. Moreover, FoxM1 mRNA expression correlated inversely with SP-A/B expression during the prenatal period, which was positively correlated with SP-A/B expression in the postnatal period. This would indicate that there are some other factors that differently regulate the action or expression of FoxM1 and SPs between the prenatal and postnatal periods. It will be necessary to investigate such factors in future studies.

Investigating the roles of FoxM1 in experimental models or patients with RDS and neonatal chronic lung disease would be helpful for understanding the pathogenesis of these diseases. However, further studies are necessary to identify the exact location of FoxM1 expression and additional factors that regulate the expression of SPs other than FoxM1.

In conclusion, the present study revealed that the expression of FoxM1 mRNA and protein was highest and the expression of SP-A/B mRNA was lowest in the lungs of preterm rabbits, compared with term rabbits. Moreover, significant correlations were observed between these genes. The FoxM1 gene seems to be an important genetic factor for lung maturation in the aspect of SP-A/B expression.

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