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REVIEW ARTICLE

Identification and Validation of FGF10, SP-B and FOXM1 Genes for Regulating Histogenesis of the Fetal New Zealand White Rabbit (*Oryctolagus cuniculus*) Lungs

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Abstract

The current work was designed to observe gene expression of Fibroblast Growth Factor 10 (*FGF10*), Surfactant protein B (*SPB*), and Forkhead Box M1 (*Foxm1*) that regulated histogenesis of rabbit's fetus's lungs from the 20th- 29th days prenatally. During the course of the experiment, the relative expression of these three genes in rabbit's fetus's lungs was detected in the presence of internal reference for normalization, the housekeeping gene *GAPDH*, by using quantitative reverse transcriptase PCR (qRT- PCR). The histological study of the fetal lungs revealed that from the 20th-22th gestational days; the lung of rabbit fetuses coexisted in pseudoglandular stage and then enter the canalicular stage from the 25th- 26th gestational days. On the 27th day of gestation, the fetal lung developed to the succeeding stage termed the saccular stage. The final stage, the alveolar stage, began to be differentiated at the end of the gestation period. The transcription level of these three genes in rabbit's fetus's lungs was detected In conclusion, the manifestation of FoxM1 mRNA in early rabbit fetus's lungs was higher than that in the lungs of late-term rabbits. Also, lung maturation and the representation of surfactant proteins were in reverse relation with FoxM1 expression in the rabbit's fetuses prenatally.

Key words: FGF10, SPB, FOXM1, qRT-PCR, Fetal rabbit lungs, and Histogenesis.

Introduction

Rabbit are the most frequently used animals in a variety of biomedical research fields. The latter fields include: neuroscience, oncology, cardiovascular, dermatology, reproduction and embryonic development [1-3].

Embryonic, fetal, and postnatal developmental phases of lungs are the foremost phases. The embryonic one includes the appearance of lung bud. Whereas lung differentiation and maturation occurr at the fetal phase and involves the pseudoglandular, canalicular and saccular developmental stages correspondingly. In the end, the alveolar stage begens before birth and then extends postnatally [4, 5].

In rabbit fetuses, the tall columnar epithelium is the suitable lining of proximal airway tubes of pseudoglandular lungs. The height of these cells is reduced continuously near their subdivision. They become cuboidal with rounded nuclei in the terminal airways. The epithelium of the terminal bud remains lined by undifferentiated cuboidal form until the branching of pulmonary bronchi termination [6].

In related studies, the most characteristic event at the canalicular stage of lung development is the arising of the acinus. The formation of several short generations of clustered respiratory acinar canals is the result of branching of the terminal bronchiole. If this developmental stage is missed, failure of gas exchange will occur so, immaturely innate child fails to live [7].

Type II alveolar cells synthesiz only a specific marker called surfactant protein c (Sftpc)[8]. However, surfactant protein a (Sftpa) and surfactant protein b (Sftpb) are produced by Clara cells. The maintenance of alveoli and host defense depends upon these surfactants [9]. Secretory Clara cells in peripheral airways and basal cells in cartilaginous airways serve as epithelial progenitors that self-renew and give various types of airway epithelial cells, including ciliated, goblet and Clara cells [10-12].

During lung development, the identified FGF10 expression induces basal cell differentiation in the cartilaginous airways. Fgf10 expression in mesenchyme is stimulated by Wingless-related integration (Wnt) but is suppressed by sonic hedgehog (Shh) and epithelial Fgf10 signals triggered beta-catenin (β-catenin) signaling [13].

FoxM1 is not only closely connected with critical structural maturation of lungs, but also with differentiation of Clara cells, production and homeostasis of surfactant proteins (SP-A, SP-B, SP-C, and SP-D). Expression of latter proteins is declined by loss of Foxm1 that is required for adaptation to air breathing [14, 15].

The genes affecting the developmental stages of lungs have been reviewed broadly in recent years, generating new understandings of the origin of the different cell lineages. The latter exists in lungs, as well as, the molecular pathways that regulated these lineages [16]. Therefore, we were spurred to elucidate the developmental changes in lungs of New Zealand rabbits during specific fetal ages with a special focus on the detection of alteration in gene expression of FGF-10, FoxM1, and SP-B in different prenatal ages by using quantitative reverse transcriptase PCR (qRT- PCR).

Materials and Methods

Ethical statement

The Institutional Animal Care and Use Committee, Zagazig University revised and approved the research protocol (approval number ZU-IACUC/2/F/87/2022). All surgical approaches were done under anesthesia to minimize the animal pain.

Sample's collection

The present work was carried out on 42 fetuses of both sexes at the last third of pregnancy from apparently healthy white New- Zealand pregnant does, two-three years old. The latter does were collected from the unit of experimental animals of the Faculty of Veterinary Medicine, Zagazig University and rabbit farm in Benha city. The pregnant does were anesthetized by injection of 35 mg/kg ketamine Hcl (KETALAR, 100 mg/ml, Pfizer, NY) and 5 mg/kg of xylazineas (Xylaject, 20 mg/ml, ADWIA, Egypt) intramuscular. The fetuses at 20^{th}, 22^{th}, 25^{th}, 26^{th}, 27^{th} and 29^{th} days of prenatal life were collected after laparotomy. Then, the fetal rib cage was dissected under a dissecting microscope to take the lung carefully. After that, the collected specimens were subjected to qRT-PCR and histological examination.

Histological examination of lung tissue

The obtained fresh specimens of rabbit fetal lung were fixed in 10% neutral buffered formalin for at least 24 h and Bouine`s fluid for 8 -24 h then transferred to 70% ethyl alcohol with 2^{nd} fixative. The fixed specimens were processed in a normal histological technique then sectioned at 3-5 micrometers thickness by using a microtome (Rotary Manual Microtome M380, made in Germany). The prepared sections were stained with Harris's haematoxylin and eosin and examined microscopically [17].

Quantitative reverse transcriptase PCR

The qRT-PCR was done at the molecular biology laboratory center, Faculty of Veterinary Medicine, Benha University, Egypt. Fetal rabbit lungs were

collected at 20th, 22th, 25th, 26th, 27th, and 29th days of gestation. The specimens were frozen at -80°C until RNA extraction. The extraction of total RNA from the tissue was performed by using 1 mL of TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) per 100 mg of tissue sample. The absorbance in a SPECTRO star Nano absorbance plate reader (BMG Lab Tec GmbH, Ortenberg, Germany) at optical density of 260 and 280 nm was measured indicating concentration and purity of RNA. Five micrograms of RNA were converted into cDNA using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The latter was stored at -20°C until further use. q RT-PCR was performed by means of a SYBR Green qPCR master mix (TOPreal qPCR 2X PreMIX) following the manufacturer's protocol and gene specific primers. The designed sequences of primers in the gene bank were listed in Table 1. qRT-PCR was carried out in a thermal cycler (Eppendorf, Germany) with the following cycle profile: holding stage at 95°C for 10 min, and 40 cycles of 94°C for 30 sec of denaturation, annealing at 64°Cfor 30 sec, elongation at 72°C for 30 sec, and final extension at 72°C for 5min. Each PCR cycle was followed by a melting curve analysis [18].

Statistical analysis

Data analysis was carried out using the SPSS software program (version 16.0; Chicago, USA). Statistics were expressed as mean values ± standard error (SE). The unpaired Student's t-test was employed to assess statistically significant differences among the samples in the qRT-PCR in different prenatal ages with a significance level set at $P \leq 0.05$; n = 3 per each studied ages**.**

Table (1): The specific designed primers sequences used for qRT-PCR

Gene	primers sequences	accession no.
	F: 5`**-CAGGCACCACCAAAAAGAGC **-3`	XM_008262183.2
FGF10	**R: 5`**-AAAAAGGTCTCCTGGTCCCC **-3`**	
	F: 5`**-TAAGCAGCAGAAACGACCCA **-3`	XM_002712808.3
FOXM1	**R: 5`**- GCCCAGTGGGAGTTCAGTTT **-3`**	
	F: 5`**-TTGCACTGGTGGATGCAAGA **-3`	NM_001082343.1
SP-B	**R: 5`**-CAGGACAGAAGTGGCTCTGG **-3`**	
	F: 5`**- GTCAAGGCTGAGAACGGGAA **-3`	NM_001082253.1
GAPDH	**R: 5`**- CCAGCATCACCCCACTTGAT **-3`**	

Results

q RT-PCR study and Histological observation

The relative expression changes in transcription levels of *FGF10, SPB* and *FOXM1* genes were measured by conducting RT-PCR in the foetal rabbit lung from 20th till 29th days prenatally.

Relative change in expression of FGF10 gene in rabbit lung from 20th to 26th days prenatally

The data obtained from the qRT-PCR revealed a gradual decrease in the manifestation level of the *Fgf10* gene in fetal lung from 20th to 26th days of pregnancy (Table 2 and Figure 1). The obtained result ensured the histological findings at this period. At 20th day of prenatal life, the H& E stained sections of white New-Zealand rabbit fetal lung revealed the branching of bronchial buds of simple tubules invested in a highly vascular loose mesenchymal connective tissue, conferring a "pseudoglandular" appearance (Figure 2A). The more proximal branches (primitive bronchi) lined with undifferentiated pseudostratified epithelium with prominent highly vesicular cytoplasm, clear circumscribed layer of myoblast and undifferentiated mesenchymal cells. However, the more distal branches (primitive bronchiole) lined by simple low cuboidal to high cuboidal with prominent highly vesicular cytoplasm and peripherally circumscribed with a layer of myoblast and undifferentiated mesenchymal cells (Figure 2B).

At 22th day of prenatal life , the H& E stained sections of white New-Zealand rabbit fetal lungs revealed the end of pseudoglandular stage and the begin of the early canalicular stage that manifested by branching and widening of lung tubules with appearance of some elongated and widened branches in the form of canaliculi forming primitive airspaces that associated with the beginning of capillarization and remain abundant mesenchymal tissues (Figure 3A). The most proximal branches (primitive bronchi) lined by ciliated pseudostratified columnar epithelium with highly vesicular cytoplasm and enveloped partially by myoblast layers and condensed mesenchymal tissue (cartilage formation) (Figure 3B). The most distal branches (primitive bronchiole) exhibit differentiation of lining epithelium to form ciliated simple low cuboidal to high cuboidal epithelium with interspersed non-ciliated clara cells of tall, dome-shaped epithelial cells with large, rounded nuclei and vacuolated cytoplasm. The primitive myoblast cells of the future smooth muscle layer were spirally arranged around the most distal branches. The primitive airspaces lined by simple cuboidal non vacuolated epithelium (Figure 3C).

From 25th - 26th days of gestation, of the white New-Zealand rabbit fetal lungs exhibited the late canalicular stage of obvious growth of vasculature, undulating and complex airspaces with reduction of mesenchymal tissues as a characteristic features (Figure 4A). Moreover, distal branches were recognizable segmented among primitive airspaces as primitive terminal bronchioles, respiratory bronchioles, and alveolar duct that lined by simple cuboidal epithelium (Figure 4B)

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Table (2): Relative change in expression of *FGF10*, SPB and FOXM1 genes in fetal rabbit lung from 20th to 29th day prenatally

Genes	Fold change		
	20D	**22D**	**26D**
FGF10	$1.013^{a} \pm 0.110$	$0.553^{b} \pm 0.047$	$0.310^{c} \pm 0.016$
	25D	**27D**	**29D**
SPB	$1.060^{b} \pm 0.040$	$4.680^{a} \pm 0.280$	$0.211^{c} \pm 0.034$
FOXM1	$1.030^{a} \pm 0.180$	$0.540^{b} \pm 0.047$	$0.147^{c} \pm 0.006$

The different mean values within the same column carrying altered superscript letters were significantly different.

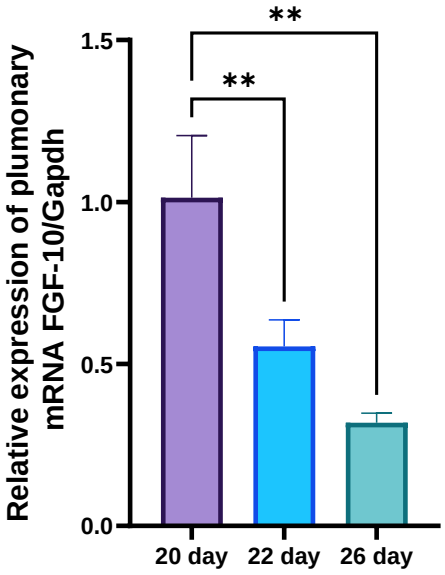


Figure 1: Histogram presentation of qRT- PCR study of the expression of *Fgf10* gene in fetal rabbit lung from 20th to 26thday prenatally. The superscript symbol ** illustrating the significance difference between ages at $P \leq .05$.

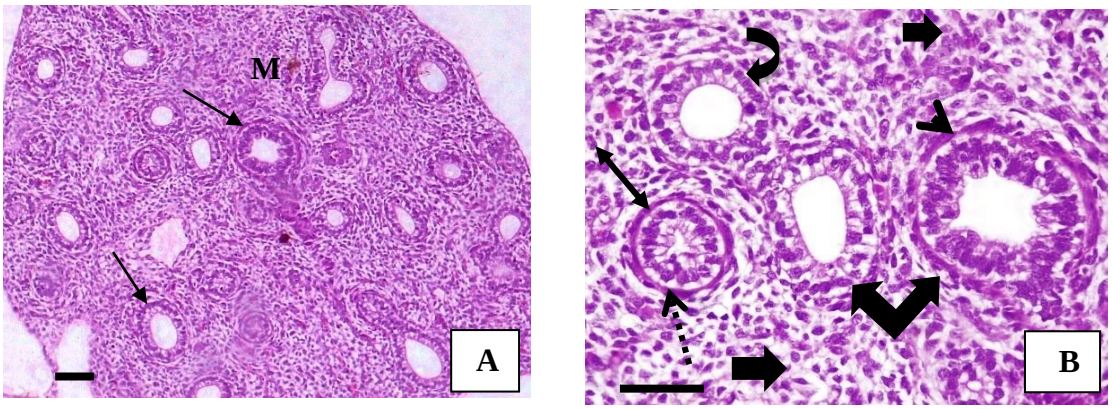
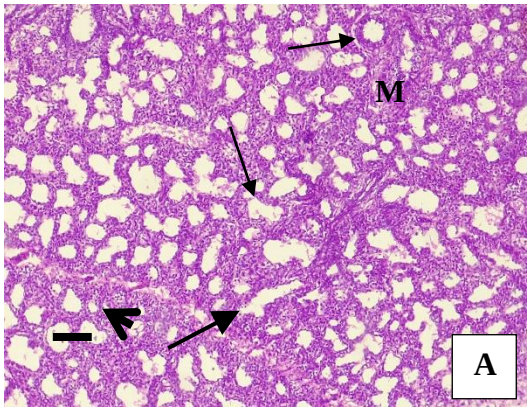


Figure (2): Photomicrographs of 20th days old white New-Zealand rabbit fetal lung (A) showing lung parenchyma at pseudoglandular stage with primitive bronchial buds of simple tubules (thin arrows) invested in a highly vascular mesenchymal connective tissue (M). (B) a higher magnification of (A) showing the more proximal branches (primitive bronchi) of undifferentiated pseudostratified lining epithelium (biforked arrow), circumscribed layer of fibroblast cells which were the myoblast and chondroblast cells (arrowhead) and the more distal branches (primitive bronchiole)of simple low cuboidal (curved arrow) to high cuboidal(double thick arrows) with prominent highly vesicular cytoplasm and peripherally circumscribed with a layer of myoblast (dashed arrow). Notice the undifferentiated highly vascular mesenchymal connective tissue cells (thick arrows). Stain: H & E; scale bar A= 200 µm; B= 50 µm.



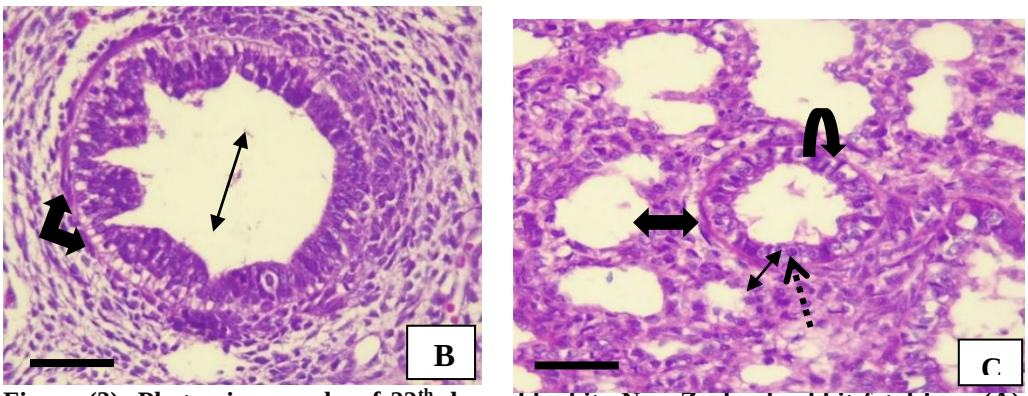


Figure (3): Photomicrographs of 22th days old white New-Zealand rabbit fetal lung (A) showing the end of pseudoglandular stage and the beginning of early canalicular stage with branching of primitive lung airways (thin arrows) with appearance of canaliculi forming primitive airspaces (thick arrow) that associated with the beginning of capillarization (arrowhead). Notice abundant highly vascular mesenchymal C.T. (M). (B,C) a higher magnification of A showing the most proximal branches with the beginning of ciliated pseudostratified columnar epithelium with highly vesicular cytoplasm (double thin arrow), enveloped partially by primitive myoblast and chondroblast layers (biforked arrow). The most distal branches lined by ciliated simple low cuboidal to high cuboidal (curved arrow) with the beginning of non-ciliated clara cells of large, rounded nuclei (dashed arrow) and highly vesicular cytoplasm (double thin arrows). The primitive myoblast cells layer is enveloped the most distal branches(closed arrow). Stain: H & E; scale barA= 50 µm; B&C= 200 µm.

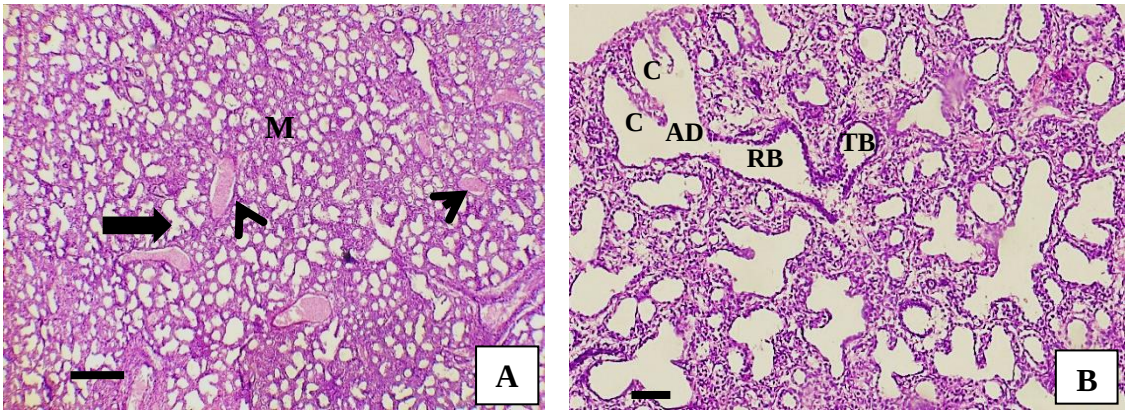


Figure (4): Photomicrographs of 25th -26th days old white New-Zealand rabbit fetal lung (A) showing the late canalicular stage of a highly vasculature (arrowhead), undulating airspaces (thick arrows) with reduction of mesenchymal C.T. (M). (B) A higher magnification of (A) showing the primitive terminal bronchiole (TB) divided into primitive respiratory bronchioles (RB) with clear alveolar ducts (AD). Each of which performed more bifurcation forming secondary tubules called canaliculi(C). Stain: H & E; scale bar: A= 100 µm; B= 50 µm.

Relative change in SPB gene expression in rabbit lung from 25thto 29thdays prenatally

The data obtained from q RT-PCR revealed an increase in the transcription level of the *SPB* gene in lung of rabbit feti at 25th days of gestation period then reached its peak at 27th days of prenatal life. After that, the expression declined gradually till reach the 29th days of pregnancy (Table 2 and Figure 5).

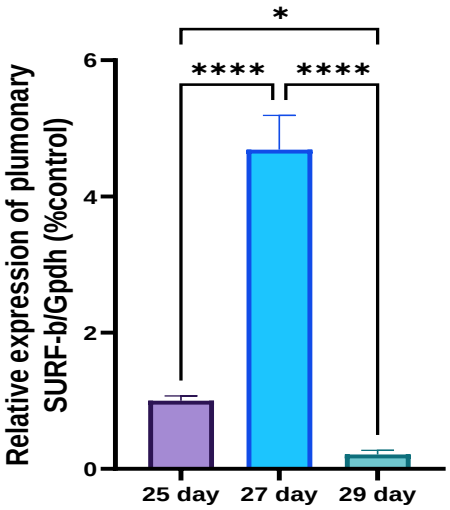


Figure 5: Histogram presentation of qRT-PCR study of the expression of SURF-b *(SPB)* gene in fetal rabbit lung from 25thto 29th day prenatally. The superscript symbol* illustrating the significance difference between ages at *P* ≤ .05.

Relative change in FOXM1 gene expression in rabbit lung feti from 25th to 29th days prenatally

A gradual decrease in the expression level of the *FOXM1* gene in fetal lung from 25th to 29th days of gestation (Table 2 and Figure 6) was detected.

These results ensure the obtained histological results at this period. At 27th day of prenatal life, the fetal rabbit lung was considered in saccular stage of lung development. Numerous future alveolar ducts were the result of division of the respiratory bronchioles that terminated by characteristic clusters of expanded and complex airspaces (Figure 7A). There was substantial condensation and thinning of the mesenchymal interstitium forming the thick wall of these complex air spaces, called primary septa. Limited little septal ridges of epithelial-surfaced mesenchyme contain a capillary network, termed secondary crest, were the in growth from the primary septa into air spaces, subdividing them into more complex structures termed air saccules. These events concomitant with progressive differentiation of airspace lining epithelium to flattened pneumocyte (Figure 7B).

At 29th day of gestation, the primitive lung presented the developmental alveolar stage. Alveolar sacs observed at the termination of an alveolar duct. The interstitial mesenchyme markedly decreased to form thin interalveolar septa. The latter partitioned the air saccules into smaller units called primitive alveoli (Figure 8A). Thin interalveolar septa formed of single layers of interalveolar capillaries with directly coat of epithelium lining alveoli. Mature alveoli displayed a polyhedral shape with their back-to-back configuration and were lined by pneumocyte type I of a flat squamous shaped cell and the pneumocyte type II of a cuboidal cells with rounded nuclei and obvious amount of cytoplasm. The alveolar lining epithelium was interrupted by capillaries bulge (Figure 8B).

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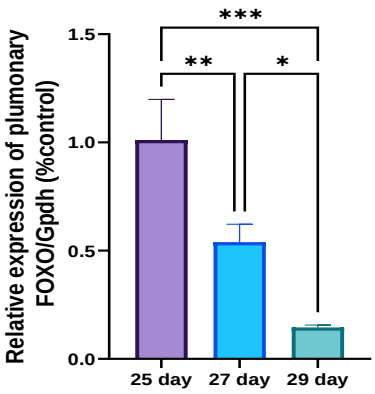

Figure 6: Histogram presentation of qRT-PCR analysis of the expression of FOXO (*FOXM1*) gene in rabbit lung foeti from 25th to 29th days prenatally. The superscript symbol * illustrating the significance difference between ages at $P \leq .05$.**

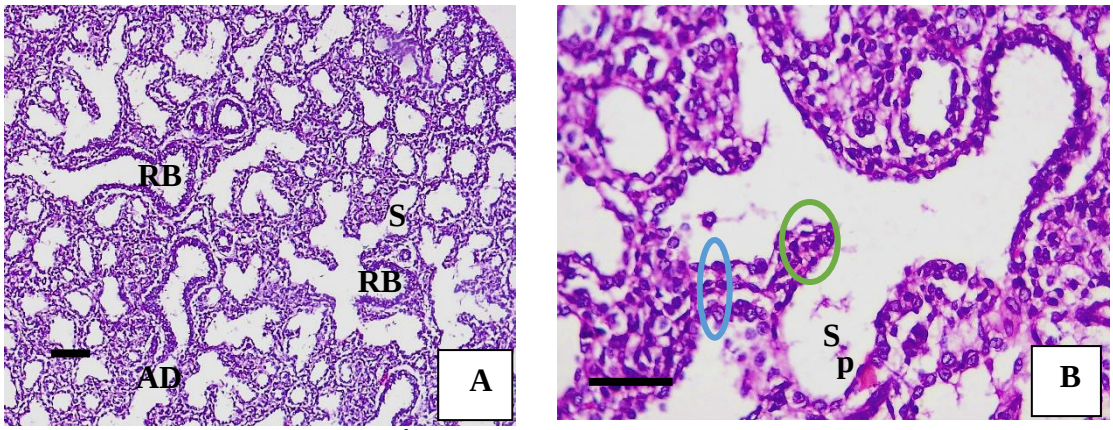

Figure (7): Photomicrographs of 27th days old white New-Zealand rabbit fetal lung (A) showing the primitive respiratory bronchioles (RB), alveolar ducts (AD), and characteristic complex clusters of air saccules (S). (B) A higher magnification of (A) showing the primary septae (blue circle) of saccules (S) and few low secondary septal ridges (green circle) protruding from the primary one. Notice progressive thinning of the interstitial mesenchyme (thick arrow) with the beginning of differentiation of to become flattened and considered as first observation of pneumocyte type I lining saccular epithelial cells (P). Stain: H & E; scale bar A= 50 µm; B= 200 µm..

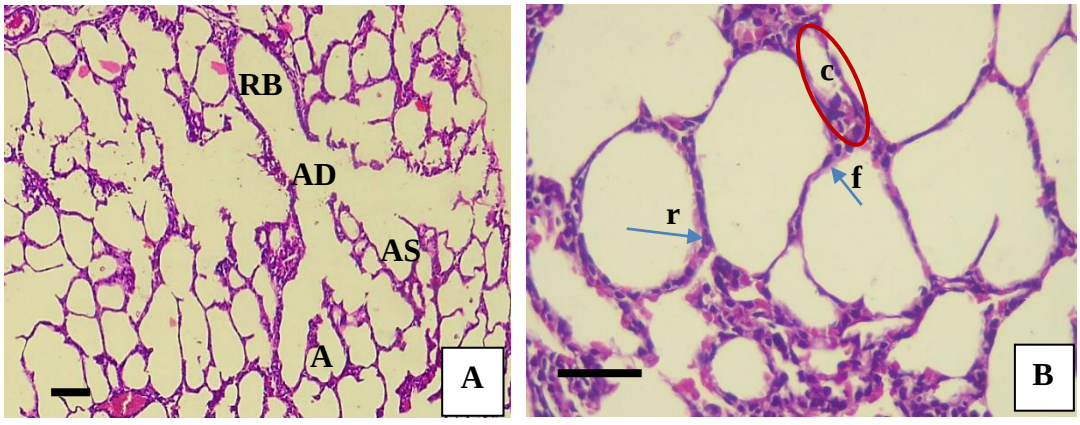

Figure (8A): Photomicrographs of 29th day`s old white New-Zealand rabbit fetal lung showing the respiratory bronchioles (RB), future alveolar ducts (AD) which ended by characteristic clusters of enlarged airspaces termed alveolar sacs (AS) surrounded by primitive alveoli. (B) a higher magnification of A showing theinteralveolar capillary (red circle), pneumocyte type I cell with flat nucleus (f) and pneumocyte type II cell with rounded nucleus (r). Notice direct contact between the interalveolar capillary (c) and the alveolar epithelial cells (red circle). Stain: H & E; scale bar:A= 50 µm; B= 200 µm.

Discussion

Coordination of epithelial branching and differentiation of the lung airways during development in rabbit fetuses need to be occurred to make a purposeful lung. Various signals that guide this development were produced from mesenchyme; however, several studies had identified other factors that act as autocrine or juxtacrine signals within the lung epithelium not, from the mesenchyme [19]**.**

Epithelial proliferation and proximal-distal patterning were achieved due to the expression of *Bmp4* in pseudoglandular stage epithelial tips [20]. Also, expression of *Wnt7b* in lung epithelium regulated canonical Wnt signaling, *Bmp4*, and *Fgfr2* expression [21-22]**.** Here, we focused on FGF10, the factor that was produced in mesenchyme in between the developing airways and guides proximal-distal specification, differentiation, branching, and outgrowth through the FGFR2b receptor located on the epithelium.

Fgf10 was critical for the appropriate differentiation of the pneumocyte type II cells and for the formation of the suitable number and ratio of pneumocyte type II versus pneumocyte type I cells. So, the differentiation of pneumocyte type II cells and expression of pneumocyte type II markers were reduced due to deletion in the *Fgf10* expression [23]**.**

Our results showed a gradual decline in the expression level of the *Fgf10* gene in fetal lungs from the 20^{th} to 26^{th} days of pregnancy. The level reached its peak on the 20^{th} day of prenatal life. After that, the expression declined gradually till reached the 26^{th} day of pregnancy. So, these results clarified the findings of the Chao and coauthors [23]**.**

In addition, these findings came in parallel with the gene signals in mouse. This expression was due to branching morphogenesis with the increase of epithelial cell proliferation and entry of mesenchymal cells into the parabronchial smooth muscle cell lineage [24-26]**.**

Recently, Rock and coauthors reported that the lung growth and branching morphogenesis or epithelial proliferation of the developing pulmonary epithelium in a mouse model had not been changed by deletion FoxM1 [14]. Nevertheless, inhibition of maturation of the lung was occurred resulting in serious respiratory failure after birth. In this study, we found that the expression of FoxM1 mRNA in fetal lung had a decreasing trend with gestational age from the 25^{th} day to 29^{th} day of pregnancy with the highest level at the 25^{th} day prenatally. This result was confirmed by Wang *et al*., [27] in mouse.

Within these consequences, it was potential to accept that the expression of *FoxM1* was great in the premature lung, declined quickly till birth, retained its level during definite postnatal periods, and then reduced in the developed lung later. This suggested that extra growth and development of the lung may be coordinated by *FoxM1* during these postnatal periods. Here the expression of *FoxM1* mRNA was higher in the lung of rabbit fetuses compared with full-term rabbit fetuses. Moreover, negative relations were observed between *FoxM1* and SP-B. The *FoxM1* gene seemed to be an essential genetic factor for lung maturation and expression of surfactant proteins (SPs).

Rabbits were recognized to have alveolar developmental stages and processes of surfactant proteins synthesis that were much related to human. The latter had the same chromosomal homology as rabbits [7].

Our data recorded an increase in the expression level of the *SPB* gene in the lung of rabbit feti on the 25^{th} day of gestation then reached its peak on the 27^{th} day of prenatal life. After that, the

expression declined gradually till reached the 29th day of pregnancy. These findings came in parallel with the results of Hahn and co-authors that this expression due to the adsorption and distribution of surfactant onto the surface of the alveolar epithelium to reduce the surface tension of these alveoli and prevent their collapse during the first breath just after birth [28].

Our findings revealed that the wall of the alveoli came in direct contact with the capillary wall around it. On each side, where the capillary wall faced the alveoli, a flat squamous cell had interfered between the capillary and the air spaces called AECI cell. Also, the second cell type, the AECII cell was observed to line the alveolar air space indicating the area of gas exchange after birth. These findings agreed with previous studies that the alveolar wall was the site of the air–blood barrier. It formed from a thin layer of surfactant, an AECI with its basal lamina, and a capillary endothelial cell with its basal lamina. Often, these two basal laminae fused together [29].

Conclusion

A rabbit model was the most applicable example, associated with other animal models; in the condition of studying human lung development. The *FoxM1* gene is an essential genetic factor for lung maturation principally in the aspect of SPs expression.

Conflict of interest: No conflict of interest.

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الملخص العربي

التحديد والتحقق من جينات FGF10, SP-B , FOXM1 لتنظيم تكون أنسجة أجنة الأرنب الابيض النيوزيلاندي

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تم تصميم الدراسة الحالية لمراقبة التعبير الجيني لFGF10, SP-B and FOXM1 التي تنظم تكوين نسيج رئة أجنة الأرنب من اليوم العشرين٧ لي اليوم التاسع والعشرين قبل الولادة. وكشفت الدراسة الهستولوجية لنسيج رئة الأجنة انه من اليوم العشرين إلي اليوم الثاني والعشرين من أيام الحمل ,كانت رئة الأرنب الجنينية في المرحلة الغدية الكاذبة ثم دخلت المرحلة القنوية من اليوم الخامس والعشرين إلي اليوم السادس والعشرين من أيام الحمل .وفي اليوم السابع والعشرين من الحمل، تطورت رئة الأرنب الجنينية إلي المرحلة التكيسية وبدأت المرحلة السنخية في التمييزفي نهاية فترة الحمل .تم استخدام جهازالبوليميراز المتسلسل للكشف عن التعبير النسبي ل FGF10, SP-B and FOXM1 والذي يعكس التغيرات في مستوي نسخ هذه الجينات الثلاثة في رئة الأرنب الجنينية .حيث قمنا أولا بعزل الحمض النووي الريبوزي الكلي من أنسجة الرئة. وتمت مراجعة الحمض النووي المعزول ونسخه إلي الحمض النووي المكمل الذي كان يستخدم كنموذج لتفاعل البوليميراز المتسلسل . وتم إستخدام ترميز الجين المرجعي كمرجع داخلي طوال التجربة. وفي الختام كان تعبيرFOXM1والبروتين الخاص به في رئة الجنين المبكر أاعلي من ذلك في رئة الأرانب كاملة النموأيضا كان نضج الرئة والتعبير عن SP-B مرتبط عكسيا مع التغيرات في تعبير FOXM1 في أجنة الأرانب قبل الولادة.