

# Changes in Elastic Fibres in Yak Lungs at Different Developmental Stages

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## Research article

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# Abstract

## Background

Yaks have a strong adaptability to the plateau environment, which is closely associated with the effective oxygen utilization rate of their lung tissue. The elastic fibre is an important adaptive structure of alveolar tissue. However, there are few studies on the development of the structure of lung tissue and the changes in elastic fibres of yak after birth. The purpose of this study was to investigate the changes of elastic fibers in the lungs of yaks after birth and the relationship between these changes and adaptation to hypoxic environment.

## Results

In this experiment, a histological method was employed to observe the changes in the lung tissue structure of yaks at four ages: 1 day old, 30 days old, 180 days old and adult. There was no significant difference in the area of a single alveolus between the 1-day-old and 30-day-old groups ( $P > 0.05$ ). In yaks aged over 30 days, the single alveolar area gradually increased with age ( $P < 0.05$ ). The observation of elastic fibres showed that elastic fibres in alveolar tissue increased significantly from the ages of 30 days to 180 days ( $P < 0.05$ ) and stabilized after 180 days of age. Transcriptome analysis determined the highest levels of differentially expressed genes between 30 days of age and 180 days of age. KEGG analysis showed that the PI3K-Akt signalling pathway and MAPK pathway, which are involved in fibre formation, accounted for the largest proportion of differentially expressed genes between 30 days of age and 180 days of age. The expression levels of 36 genes related to fibre formation were analysed, and several genes related to elastic fibre formation and collagen fibre formation were determined to be highly expressed at the age of 30 days.

## Conclusions

The content of elastic fibres in the alveolar tissue of yaks increases significantly after birth, but this change occurs only from 30 days of age to 180 days of age to make better use of oxygen in the environment.

## Background

Yak is the only bovine animal that can multiply in the arctic-alpine pastoral area of the Qinghai-Tibet Plateau. This animal has strong adaptability to its ecological environment, and it is hard-working. Yak can live freely and multiply under harsh environmental conditions, such as hypoxia, cold and short herbage growing periods. Yak is important to plateau animal husbandry and is an essential means of livelihood and production for local people, being well-known as the "ship of the plateau" and "all-round livestock" [1]. With the constant development of yak resources, it can be stated that the sustainable development of the yak economy has become the highest priority of plateau animal husbandry.

Yak is a kind of animal with good adaptability in plateau environments, developing and reproducing in a special and harsh eco-geographical environment. After a long period of natural and artificial selection, yak has developed characteristics of morphology, physiology and heredity that are different from those of other animals [2]. Yak has already attracted wide attention from scholars, both in this country and abroad, due to its good adaptability to plateau environments. At present, there are many studies on the morphological structure of organs and tissues in adult yaks [3]. Anatomically, yak ribs are relatively long, and the intercostal spacing is large, which can increase the chest size and provide a useful space for the development of the heart and lungs. Also, the large diameter of the yak windpipe can increase the amount of air entering the body [4]. In microanatomy, a respiratory system histology study found that the yak trachea is rich in goblet cells, the alveolar diaphragm is thick, the pulmonary arterioles are thin, and the gas-blood barrier is relatively thin, which is conducive to the passage and diffusion of oxygen [5]. In the yak cardiovascular system, enhancing the conduction of excitement by increasing conduction fibres increases the length and density of capillaries in the heart, thereby increasing oxygen delivery [6]. Regarding skeletal muscle histology, yak muscle fibre diameter is relatively small, which increases the density of muscle fibre per unit area. In addition, the elastic fibre content in yak muscle fibre is relatively rich, which effectively improves its adaptability to hypoxia [7]. Physiologically, yak's red blood cell number and haemoglobin content are relatively high, and these values increase with increasing altitude, which helps to enhance yak's ability to carry oxygen in the blood [8]. However, there are few studies on the development of the structure of lung tissue and the changes in elastic fibres of yak after birth.

The elastic fibres have many branches and wide distribution, being interwoven into a net and arranged into a film in lung tissue. Elastic membranes are alternately combined to form elastic membrane units, that is, elastic arterial resilience units [9,10]. According to previously published research, ripe elastic fibres and elastic membranes are composed of homologous elastin macromolecules, which form scaffolds along microfibrils arranged in parallel [11,12]. Because of the elastic fibres, which are beneficial to gas exchange, the lungs have good elasticity [13]. Therefore, we investigated the changes in elastic fibres in yak lung tissues at different developmental stages from the perspective of histological observation. The mechanism governing formation was further elucidated by molecular biological detection to provide basic findings on the histological and molecular mechanisms underlying yak adaptation to hypoxia and to establish a foundation for future research in plateau medicine and other disciplines.

## Results

### **Observation of the basic structure of yak alveolar tissues at different developmental stages**

It was observed that the alveolar morphology of yak was similar at different ages, and most of the alveoli were irregular oblate and oval. One-day-old yaks had different alveolar sizes, which were smaller than those of 30-day-old, 180-day-old and adult yaks. Elastic fibres were determined to be evenly distributed in the alveolar septum, while elastic fibres at the top of the alveolar septum were obviously distributed. Some translucent structures could be observed in the alveoli of 180-day-old adults, and the number of elastic fibres increased significantly (Fig. 1A). According to quantitative analysis, the number of alveoli

per unit area was not significantly different between the 1-day-old and 30-day-old groups ( $P > 0.05$ ) but decreased significantly between the 30-day-old group and the adult group ( $P < 0.05$ ) (Fig. 1B). The average single alveolar area exhibited no significant difference between the 1-day-old group and the 30-day-old group ( $P > 0.05$ ), but it gradually increased significantly from the 30-day-old group to the adult group ( $P < 0.05$ ) (Fig. 1C). The percentage of elastic fibres in the lung parenchyma showed an increasing trend, but there was no significant difference between the 1-day-old group and the 30-day-old group ( $P > 0.05$ ), and it increased significantly from the 30-day-old group to the 180-day-old group ( $P < 0.05$ ), and there was no significant difference between the 180-day-old group and the adult group ( $P > 0.05$ ) (Fig. 1D).

## **Transcriptome detection**

The expression difference between two age samples in each group was analysed by difference analysis software DESeq2, and the test parameters were  $|\text{Foldchange}|$  (multiple difference)  $> 1.5$ ,  $\text{padj} < 0.05$ . The results showed that there were 17218 genes (69.71%) and 317 differential genes (1.28%) expressed at 1 day of age vs. 30 days of age, including 142 upregulated genes (0.57%) and 175 downregulated genes (0.71%). A total of 17404 (70.47%) genes were expressed at 30 days of age vs. 180 days of age, and 3190 (12.92%) genes were different, including 1775 (7.19%) upregulated genes and 1415 (5.73%) downregulated genes. A total of 17232 (69.77%) genes were expressed in 180-day-old yaks vs. adults, with 695 (2.81%) differentially expressed genes, of which 425 (1.72%) were upregulated and 270 (1.09%) were downregulated. Among these genes, those differentially expressed between 30-day-old yaks and 180-day-old yaks were the most abundant (Table 1 and Fig. 2).

## **GO analysis of differentially expressed genes**

Through GO enrichment analysis (Fig. 3), the differentially expressed genes of 1-day-old yaks vs. 30-day-old yaks, 30-day-old yaks vs. 180-day-old yaks, and 180-day-old yaks vs. adult yaks were analysed. The main biological processes (BP) involved in differential gene expression in each group include developmental processes and stimulus stress. Cell composition (CC) mainly concentrates action on several membrane and intimal systems. The molecular function (MF) mainly involves the functions of protein binding and ion binding.

## **KEGG analysis**

The differentially expressed genes screened in each group were analysed by KEGG to determine the main biochemical metabolic pathways and signal transduction pathways involved in differentially expressed genes. Analysis found that the PI3K-Akt signalling pathway was mapped in the differentially expressed genes between two adjacent ages (Fig. 4). Among the various pathways, the PI3K-Akt signalling pathway is the most important pathway involved in differentially expressed genes observed from 30 days of age to 180 days of age, and the second most important pathway is the MAPK signalling pathway (Fig. 4B).

## **Screening of Fibrogenic Genes**

By reviewing relevant studies conducted both in this country and abroad and combining them with GO annotation, we identified 36 genes involved in fibre production (Table 2). Among these genes, 22 were upregulated (61.11%), and 14 were downregulated (38.89%). Two genes were differentially expressed between 1-day-old and 30-day-old yaks, and 34 genes were differentially expressed between 30-day-old and 180-day-old yaks. There was no significant difference in the expression of these genes between 180-day-old and adult yaks.

### **Expression trend of fibrogenesis-related genes in different periods**

Five genes related to elastic fibre formation were selected, and all five genes promoted fibre formation. The expression levels of yak lung tissue in different stages was analysed, and it was found that the expression level was the highest at 30 days of age or 180 days of age (Fig. 5A). Seven genes related to fibroblasts were selected, and their functions may also promote fibrogenesis. The expression level was the highest in the 30-day-old and 180-day-old groups (Fig. 5B). Moreover, 24 genes related to collagen fibre formation were selected, among which 20 genes promoted fibre formation. The expression levels of 9 genes were the highest at 30 days of age (Fig. 5C), and 11 genes were the highest at 180 days of age (Fig. 5D). Then, 4 genes had inhibitory effects on fibrogenesis, and their expression levels all decreased continuously after 30 days of age (Fig. 5E).

## **Discussion**

### **Observation of yak alveolar tissue slices at different developmental stages**

The alveolus is the functional unit of the lungs, and gas exchange in the lungs largely depends on the size of the respiratory area of the lungs [14]. With the growth and development of the yak, individual volume and surface area increase, and the number of alveoli per unit area decrease (Fig. 1B). The total number of alveoli increases, which increases the area of gas exchange in the lung, accelerates the rate of gas exchange in lung tissue [15], and improves the utilization rate of oxygen, enabling yaks to adapt quickly to low-oxygen and high-altitude environments. The ability of yaks to adapt to low oxygen at high altitudes has representative significance [16].

The elastic fibres have a retractive force in alveolar tissue, and the adult yaks tend to ripen; therefore, it is more beneficial for yaks to exchange air between the outside atmosphere and the blood in the lungs by using their own retraction force. This property helps blood vessels bear the pressure of the heartbeat and blood flow to keep the blood flow constant [17]. The proportion of elastic fibres in yak alveoli was observed to increase significantly after 30 days of age ( $P < 0.05$ ) (Fig. 1D). Therefore, it can be seen that 30 days of age is the key period of yak alveolar development. The alveolar tissues of 180-day-old adults exhibit some ribbon alveolar septum structure of the semipermeable membrane, and the related literature presents similar reports [18]. The location of the elastic fibre that we observed was consistent with this translucent membrane structure, and it was inferred that it may be an elastic fibre.

### **Expression analysis of differentially expressed genes**

By distinguishing the biological information of transcriptomic data between two age groups, it was found that the comparison of the 30-day-old group with the 180-day-old group yielded the highest level of differentially expressed genes (Fig. 2B). Consequently, the stage from 30 days old to 180 days old was indicated to involve many gene expression changes, as this period is a significant stage of yak lung tissue development. This result was consistent with our previous morphological observations.

### **GO and KEGG annotations**

GO enrichment analysis showed that the biological process of differentially expressed genes in three age groups involved the development process, mainly in the membrane and nucleus. The main biological processes were biological regulation and metabolism, and the process involved the functions of protein binding and ion binding. The proportion of differentially expressed genes in the yak lung tissue was highest between the 30-day-old group and the 180-day-old group (Fig. 3B), which suggested that yak lung tissue underwent sustained development from 30 days of age to 180 days of age.

KEGG pathway analysis showed that the PI3K-Akt signalling pathway was an important cellular regulation pathway in three age groups and was related to the formation of fibres [19]. The signalling pathways involved in the formation of fibres were mostly observed between 30 days of age and 180 days of age [20]. Among these pathways, the PI3K-Akt signalling pathway accounted for the largest proportion (Fig. 4B) followed by MAPK, which was also closely related to cell growth and development [21].

### **Genes related to fibrogenesis**

Thirty-four of the 36 genes involved in fibre formation were differentially expressed from 30 days of age to 180 days of age. It was found that a large number of fibres were formed in this stage. Moreover, the expression levels of the genes related to promoting fibrogenesis increased significantly between 30 days of age and 180 days of age ( $P < 0.05$ ), while the expression levels of the genes related to inhibiting fibrogenesis decreased at this stage. The genes related to elastic fibrogenesis are FBN1, FBN2, EMILIN3, EMILIN2 and ELN; FBN1 and FBN2 belong to the fibrillin protein family [22]; and EMILIN3, EMILIN2 and ELN belong to the elastin family. The fibrillin family and the elastin family genes are closely related to the formation of elastic fibres [23]; therefore, we selected the ELN for analysis, and the expression level of ELN reached its maximum at 30 days of age (Fig. 5A); also, ELN was abundant in lung tissue. Elastin constitutes elastic fibre, and natural elastin is surrounded by a shell composed of microfibrils. Microfibrils are composed of some glycoproteins, and fibrillin is necessary to maintain the integrity of elastic fibres [24]. Elastic fibre is a stretched rubber-like fibre that can provide elasticity and tensile strength to tissues. Although collagen can provide strength and toughness to the extracellular matrix, it needs to be elastic for lung tissue, and the elasticity primarily depends on elastic fibres in the extracellular matrix.

There are 7 fibroblast-related genes, FGF1, FGF9, FGF18, FIBP, CNPY3, TLR3 and FN1, which can promote the formation of fibroblasts. Fibroblast growth factors have a wide range of biological activities that are closely related to cell proliferation and differentiation [25]. These factors can promote the mitosis of fibroblasts and the growth of mesodermal cells, stimulate the formation of blood vessels and play a role

in wound healing and limb regeneration [26]. Fibroblast-related genes can promote the growth of fibroblasts and subsequently cause them to develop into fibroblasts [27]. The expression levels of related genes reached a maximum at 30 or 180 days of age (Fig. 5B), which shows that fibre formation is upregulated at this stage.

There are 20 genes that promote the formation of collagen fibres. The collagen family is primarily associated with cell composition, and other related genes mainly participate in fibre formation by inducing related growth factors and various cytokines [28]. COL3A1 gene expression reached the highest value at 30 days of age (Fig. 5C). Type III collagen is a kind of high-molecular-weight protein. Filamentous collagen fibres are the bonding materials of connective tissue, which can keep the skin firm and elastic, participate in the migration, differentiation and proliferation of cells, and promote the generation of collagen fibres [29]. Glutathione peroxidase can improve the survival rate of cells and ensure the integrity of genetic DNA [30]. The GPX1 gene reached its maximum expression level at 180 days of age (Fig. 5D), confirming that GPX can promote collagen fibre formation [31]. Four genes, ADAMTS2, ACAN, TGF $\beta$ 2 and TGF $\beta$ 1, repress the formation of collagen fibres. These genes are mainly involved in inducing (Fig. 5E) and inhibiting the effects of growth factors and cytokines to inhibit fibre formation.

## Conclusions

In conclusion, this study proved that yak's good adaptability to plateau hypoxia environment was closely related to the elastic fibers in alveolar tissue. For example, during the development process, the differentially expressed genes were at the highest level from 30 days old to 180 days old, PI3K-Akt signaling pathway and MAPK pathway involved in fiber formation accounted for the largest proportion, and genes related to fiber formation were also highly expressed at 30 days old or 180 days old. To make better use of oxygen in the environment, the elastic fibers in the alveolar tissue of yaks increased significantly from 30 to 180 days of age, and stabilized after 180 days. The existence of elastic fibers makes the lungs have good elasticity, is conducive to gas exchange, improves the oxygen utilization rate of lung tissues, and enables yaks to better adapt to the environment.

## Methods

### Experimental animals

From the Haiyan area, Qinghai province of China (3200 m above sea level), there two 1-day-old and 180-day-old plateau yaks were studied, as well as three 30-day-old and adult plateau yaks. All yaks purchased from herders of Haiyan area. The respiratory systems of these yaks were healthy, regardless of sex. All plateau yaks were killed by exsanguinated via abdominal aorta in slaughter house after anesthesia via IV injection of pentobarbital sodium (200 mg/kg) according to the Animal Ethics Procedures and Guidelines of the People's Republic of China.

### Histological staining

## **Paraffin sections of conventional tissues**

Fresh tissues were collected, fixed with 4% paraformaldehyde for 24 hours, dehydrated with gradient alcohol, cleared with xylene, embedded in paraffin after wax immersion, sliced with a slicer to a thickness of 4  $\mu\text{m}$ , and placed on glass slides for later use.

## **HE staining**

For HE staining of tissue samples, reverse gradient alcohol rehydration was employed followed by staining with haematoxylin for 5 min, differentiating by diluted hydrochloric acid differentiation, rinsing fully in running water, treating tissues with 0.6% ammonia water until they turned blue, rinsing the tissues with running water, staining with eosin for 1-3 min, and sealing through gradient alcohol dehydration.

## **Elastic fibre staining**

Tissue samples were subjected to reverse gradient alcohol rehydration with Wiegert oxidant for oxidation for 5 min, washed with Wiegert bleach for 1~2 min, differentiated with acidic differentiation solution for 2~3 s, washed with running water for 10 min, re-dyed with VG staining solution for 30 s, and finally sealed through gradient alcohol dehydration.

## **Observation and measurement**

HE-stained sections and elastic fibre sections were observed with an Olympus BX51 microscope, and pictures were collected. Next, images were taken and measured with Image-Pro Plus 6.0, which was employed to measure the area of single alveoli and the number of alveoli per unit area in HE-stained sections; the areas of lung parenchyma and elastic fibres in different developmental stages were also measured. Excel was used to calculate the proportion of elastic fibres in alveolar tissue, and SPSS 19.0 was used to perform statistical analysis among multiple groups. The results are expressed as the mean  $\pm$  standard deviation ( $\bar{x} \pm \text{SD}$ ), with a P-value  $< 0.05$  indicating a significant difference.

## **Transcriptome data analysis**

### **Transcriptome sequencing**

Transcriptome sequencing was performed on the total RNA samples of yak lung tissue samples from 1-day-old, 30-day-old, 180-day-old, and adult yaks by Shanghai Liebing Biomedical Technology Co., Ltd. Sequencing was performed the NovaSeq sequencing platform by adopting double-end sequencing mode, carrying out quality control (QC) and pollution assessment on the sequencing data, and then analysing the expression of the genes after quality control.

### **Bioinformatic analysis**

GO (gene ontology) databases and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases were used to analyse transcriptomic data combined with verified transcriptomic data. For screening

differentially expressed genes, the parameters are 1.5 times the difference and FDR (false discovery rate)  $\leq 0.05$ . Screening and counting fibre generation-related genes of samples in different periods was performed. The change trend of fibrogenic gene expression in each period was measured and analysed.

## Abbreviations

HE: Hematoxylin Eosin; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes

## Declarations

### Ethics and consent to participate

This study was approved by the Institutional Animal Care and Use Committee of Qinghai University (Xining, China), and all methods were carried out in accordance with approved guidelines. No local regulations or laws were overlooked. I had obtained written informed consent to use the animals in this study from the owners of the animals.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

Conceptualization, Hongxian Yu and Qing Wei; methodology, Qing Wei; software, Lihan Wang; validation, Yang Yu, Jingyi Li and Xiangqiong Meng; formal analysis, Jingyi Li and Xiangqiong Meng; investigation, Jingyi Li; resources, Qing Wei; data curation, Xiangqiong Meng; writing—original draft preparation, Jingyi Li; writing—review and editing, Hongxian Yu and Qing Wei; project administration, Qing Wei; funding acquisition, Qing Wei. All authors have read and approved the manuscript.

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## Tables

Table 1 Analysis results of differentially expressed genes

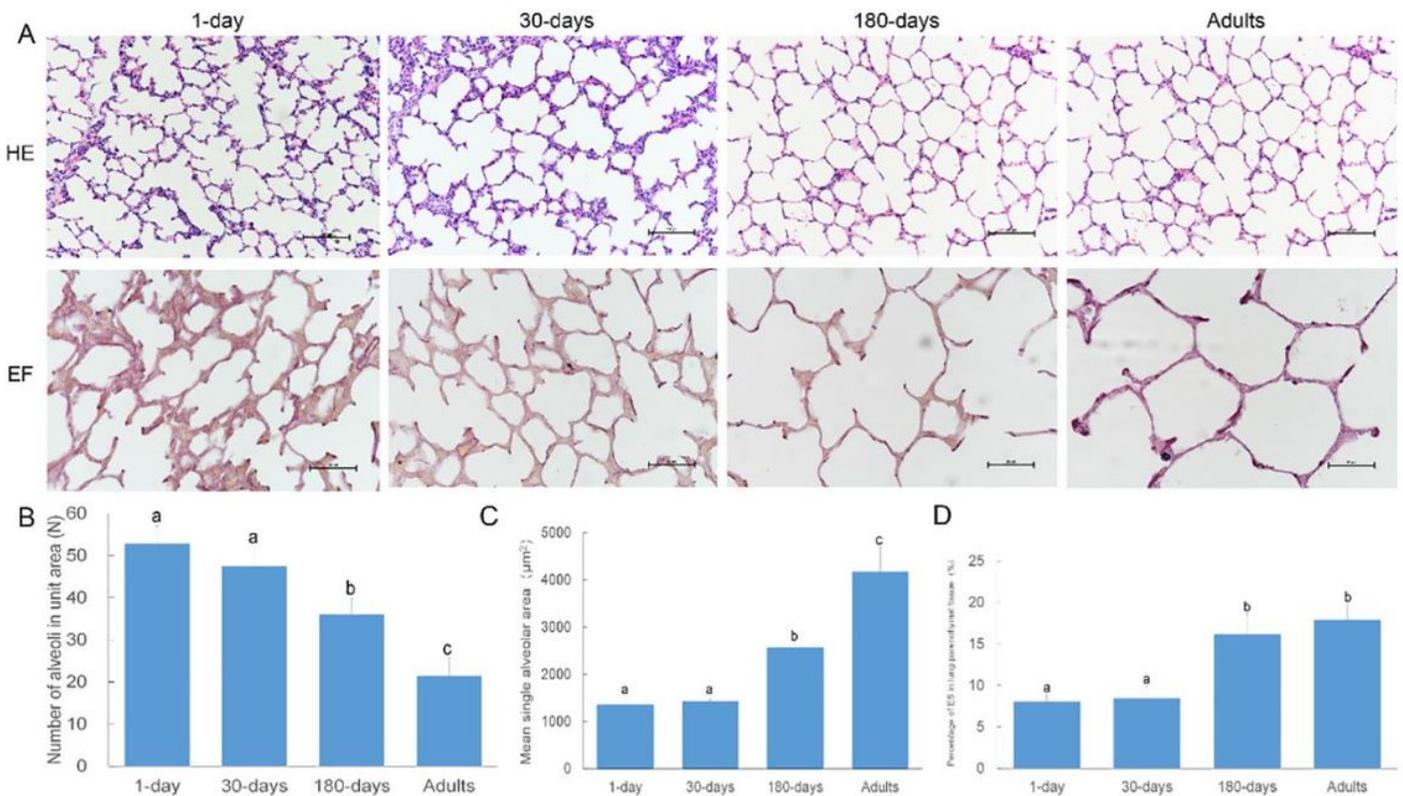
Age	Co-expression gene	Differential gene	Up-regulated gene	Down-regulated gene
1 day-old Vs 30-day-old	17218	317	142	175
30-day-old Vs 180-day-old	17404	3190	1775	1415
180-day-old Vs adult	17232	695	425	270

Table 2 Annotation of genes related to fiber formation

Gene name	KEGG ID	Description
COL3A1	102279325	Collagen, type III, $\alpha$ 1
COL11A1	102276187	Collagen, type XI, $\alpha$ 1
COL11A2	102285415	Collagen, type XI, $\alpha$ 2
COL1A2	102267202	Collagen, type I, $\alpha$ 2
ADAMTS2	102285627	ADAM metal peptidase and platelet reactive protein 1 type motif, 2
LOX	102276831	Lysyl oxidase
LOXL2	102282891	Lysyl oxidase like 2
VIL1	102272630	Villin 1
PHACTR2	102278950	phosphatase and actin regulators 2
TGF $\beta$ 1	102283357	Transforming growth factor, $\beta$ 1
ACAN	102278312	Aggrecan
TGF $\beta$ 2	102283491	Transforming growth factor, $\beta$ 2
TGFBI	102284294	Transformed growth factor, induced, 68kDa
CAMSAP3	102274353	Family of spectral-related proteins regulated by calmodulin, member 3
CDC42BPA	102284687	CDC42 binding protein kinase
BAIAP2	102286151	BAI1 associated protein 2
RASAL3	102269674	RAS protein activator 3
ITGB1	102273972	Integrin subunit, $\beta$ 1
DNM2	102279431	Motor protein 2
STIM1	102271938	Matrix interacting molecules 1
RNF44	102276069	Ring finger protein 44
NDRG1	102266961	Downstream regulation of N-MYC 1
FGF1	102287352	Fibroblast growth factor 1
FGF9	102273668	Fibroblast growth factor 9
FGF18	102287270	Fibroblast growth factor 18
FIBP	102276075	FGF1 intracellular binding proteins
CNPY3	102287727	Canopy FGF signal regulator 3
TLR3	102268437	Toll-like receptors 3

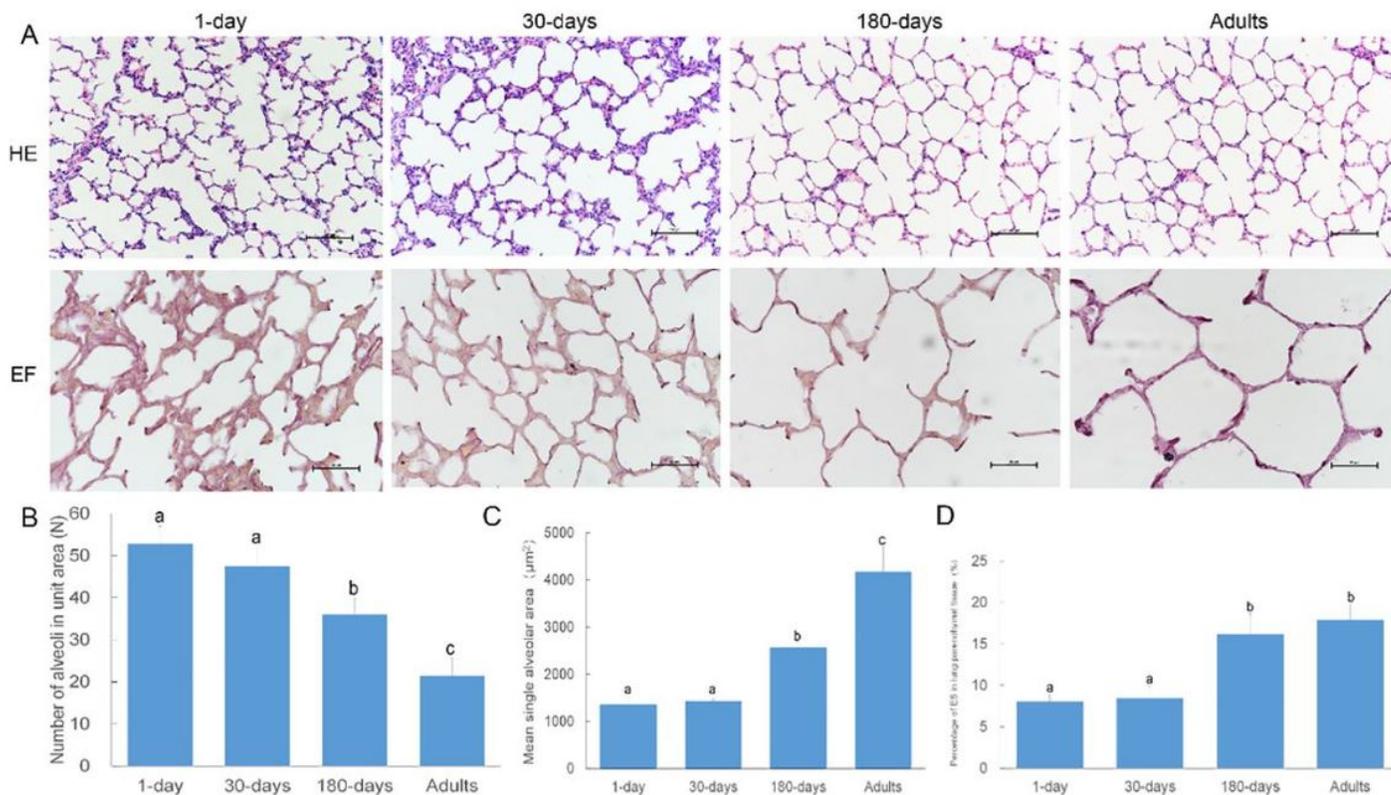
FN1	102280180	fibronectin 1
FAM65B	102273075	65 members of the sequence similarity family B
GPX1	102280278	Glutathione peroxidase 1
FBN1	102283369	fibrin 1
FBN2	102267459	fibrin 2
EMILIN3	102288344	Elastin microfiber junction 3
EMILIN2	102271699	Elastin microfiber junction 2
ELN	106700709	elastin

## Figures



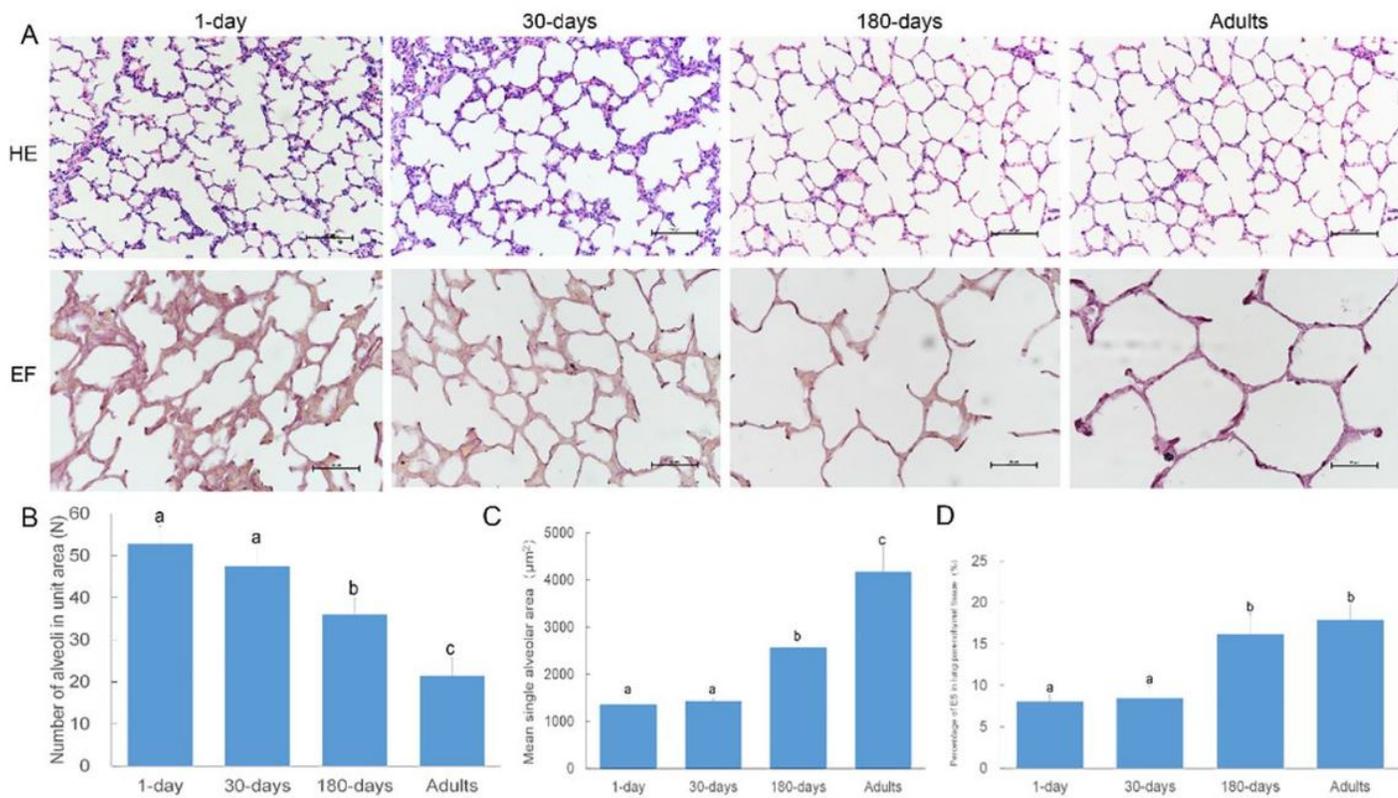
**Figure 1**

Basic structure and quantitative measurement of alveolar tissue in yak at different developmental stages. (A) HE staining and elastic fibre staining of alveolar tissue of plateau yak at four ages: 1 day old, 30 days old, 180 days old and adult; (B) the number of alveoli per unit area; (C) average single alveolar area; (D) percentage of elastic fibres in lung parenchyma



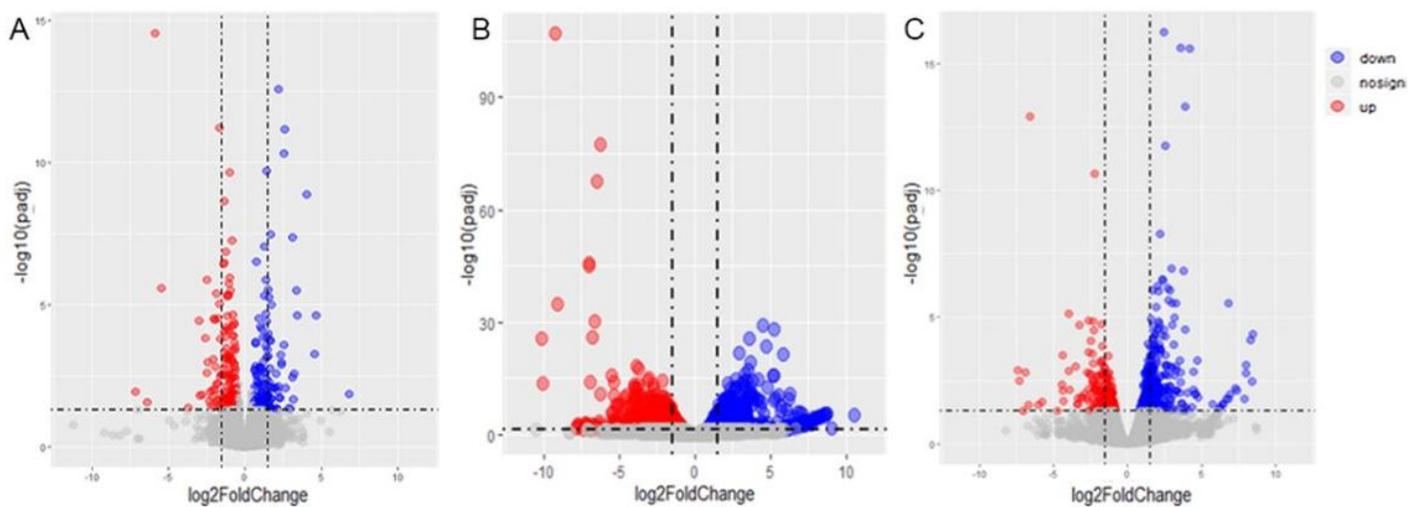
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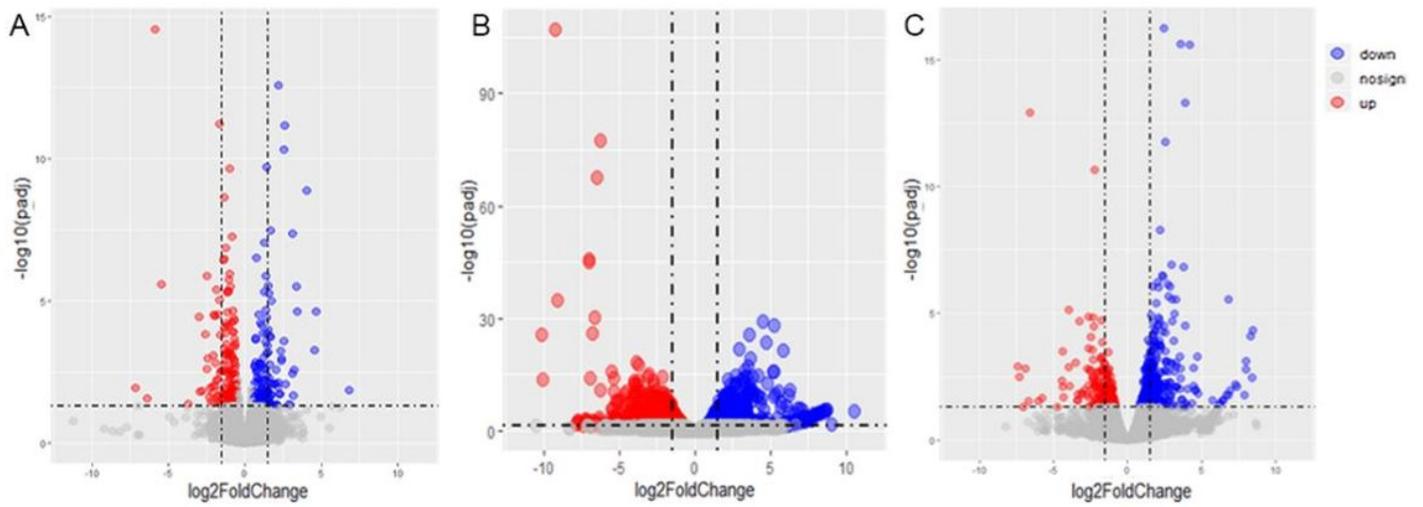
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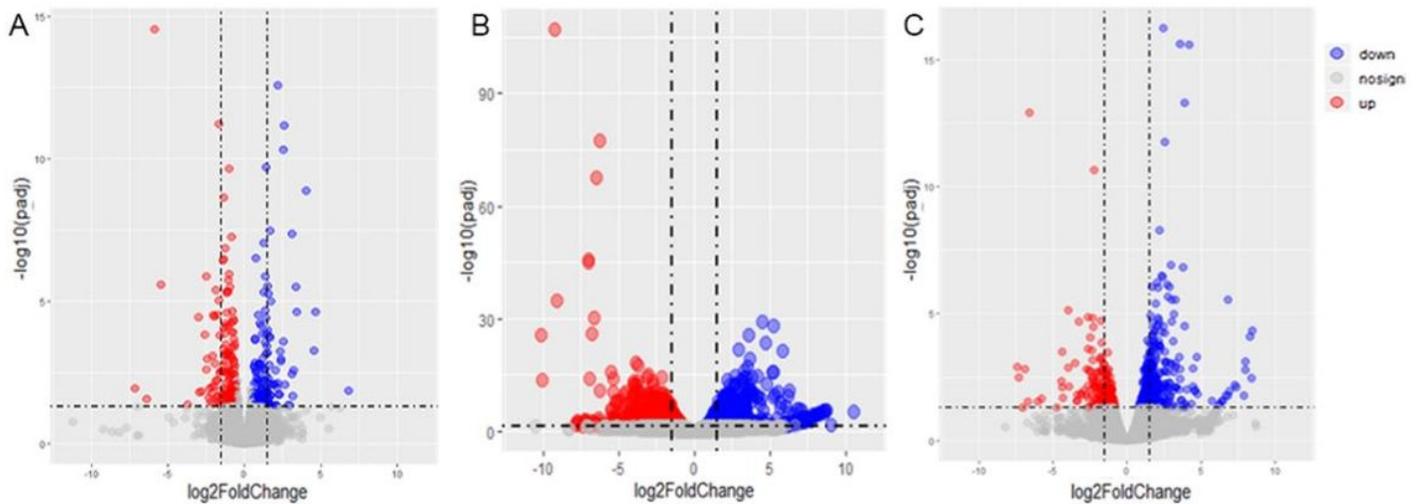
**Figure 2**

Differentially expressed genes in volcanic maps. (A) 1 day old vs. 30 days old; (B) 30 days old vs. 180 days old; (C) 180 days old vs. adult.



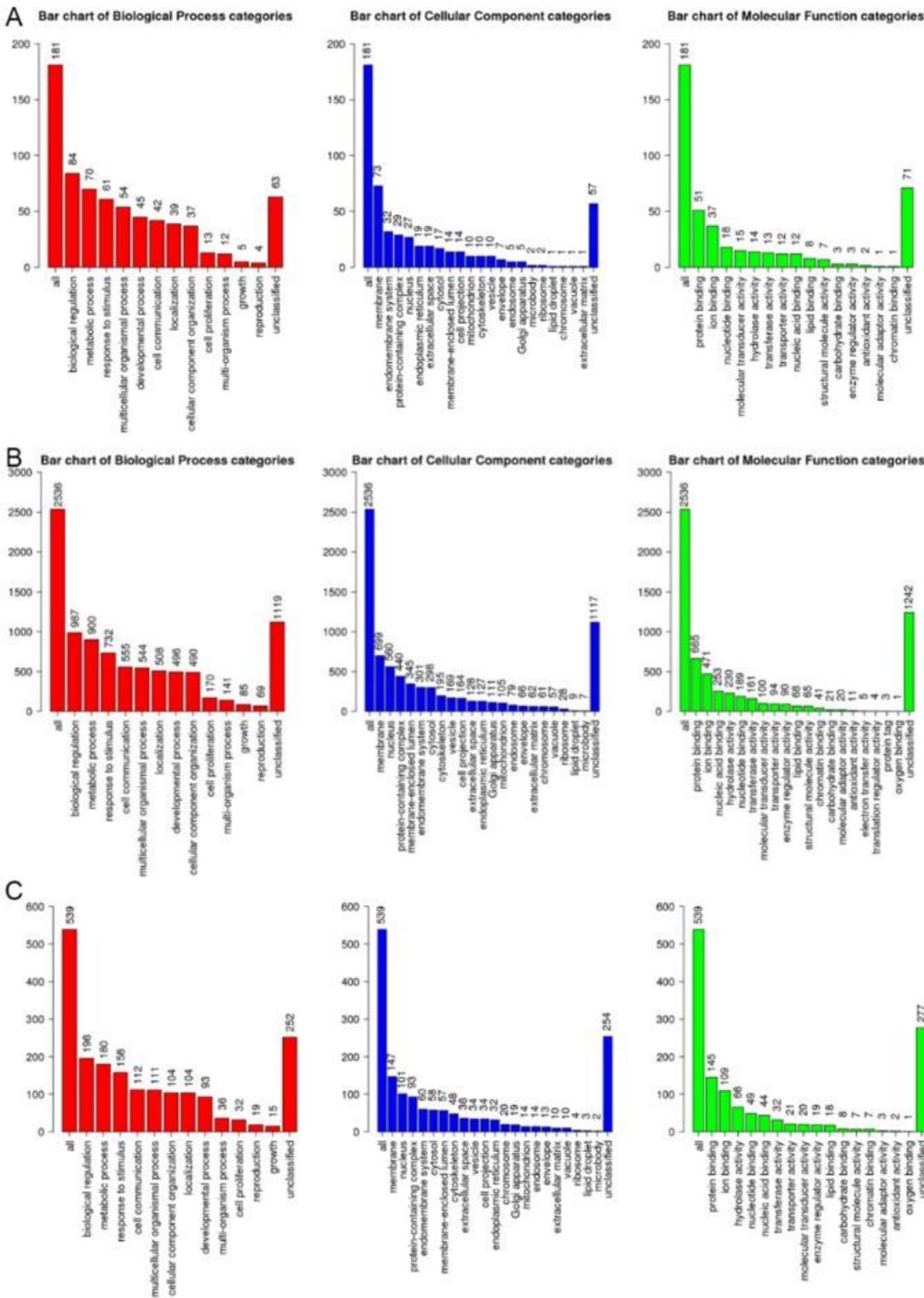
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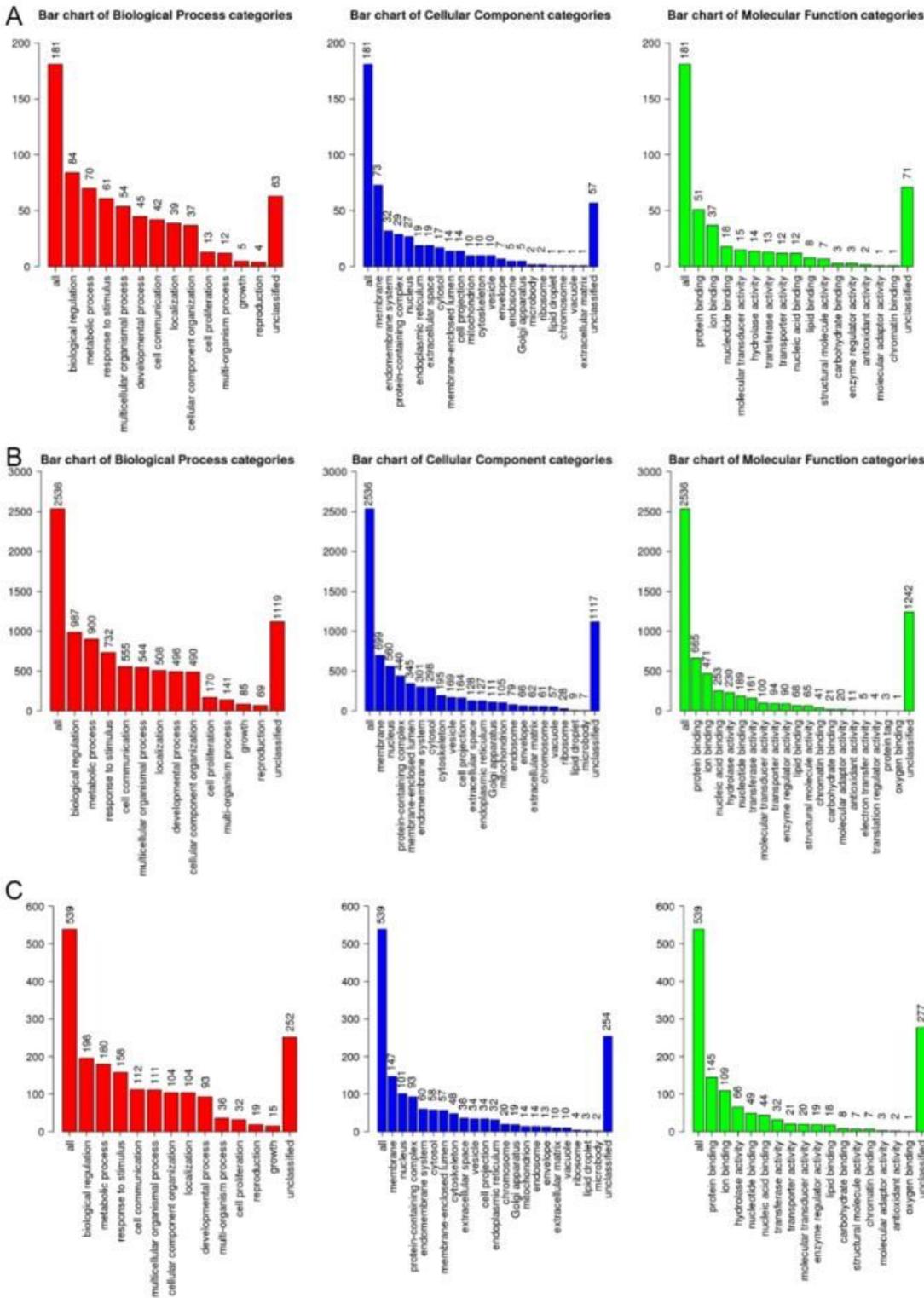
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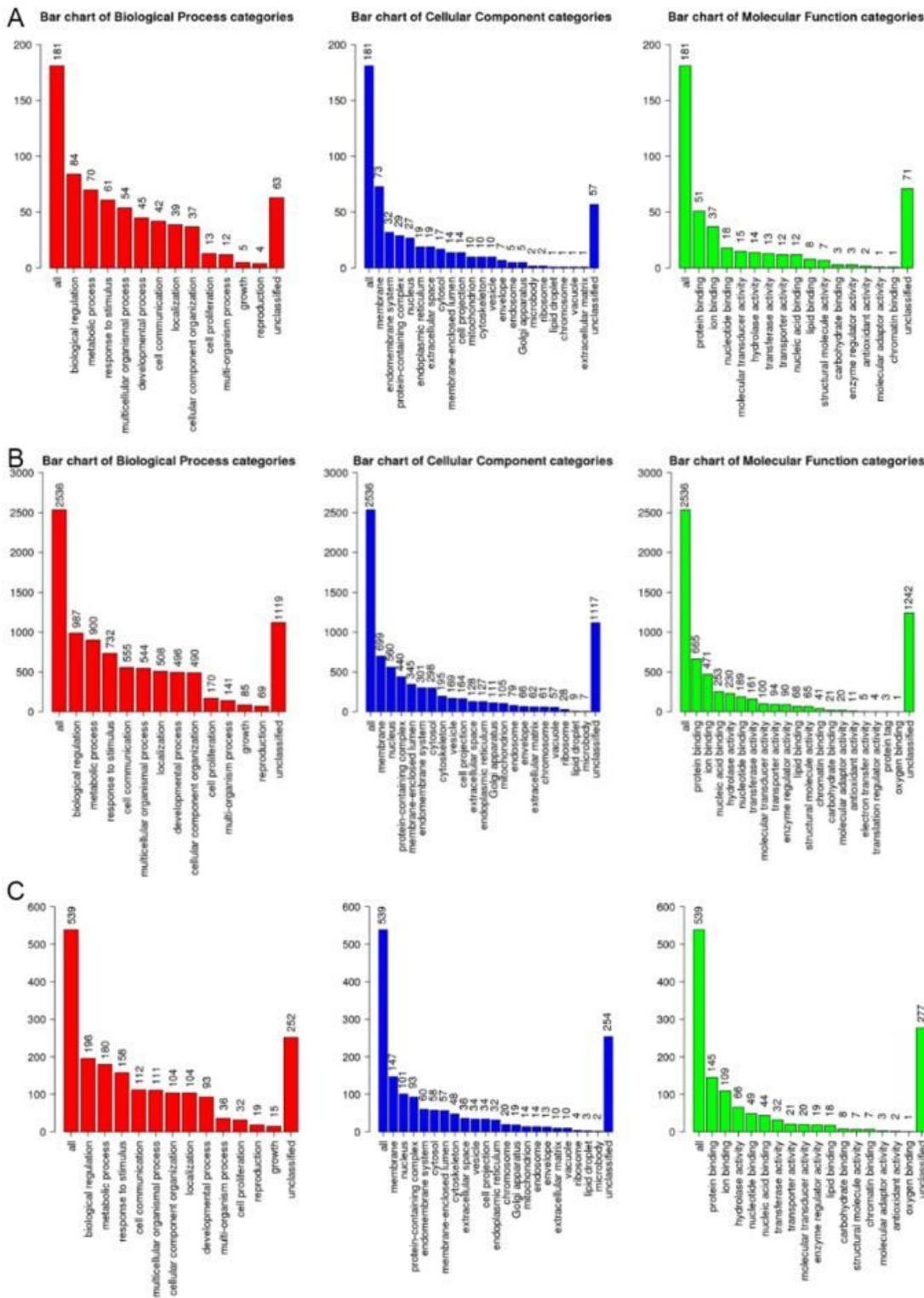
**Figure 3**

GO enrichment annotation of differentially expressed genes. (A) 1 day old vs. 30 days old; (B) 30 days old vs. 180 days old; (C) 180 days old vs. adult



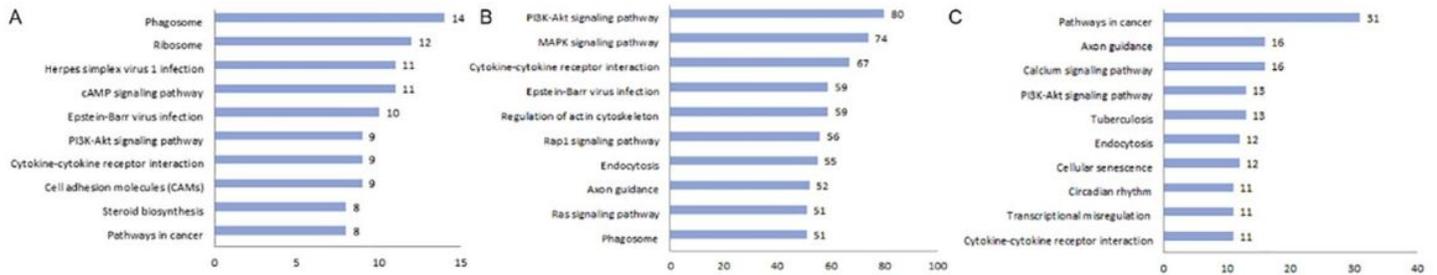
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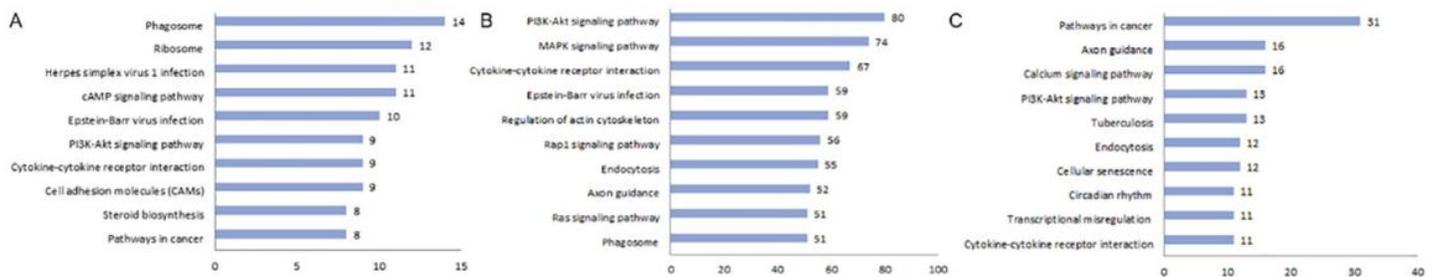
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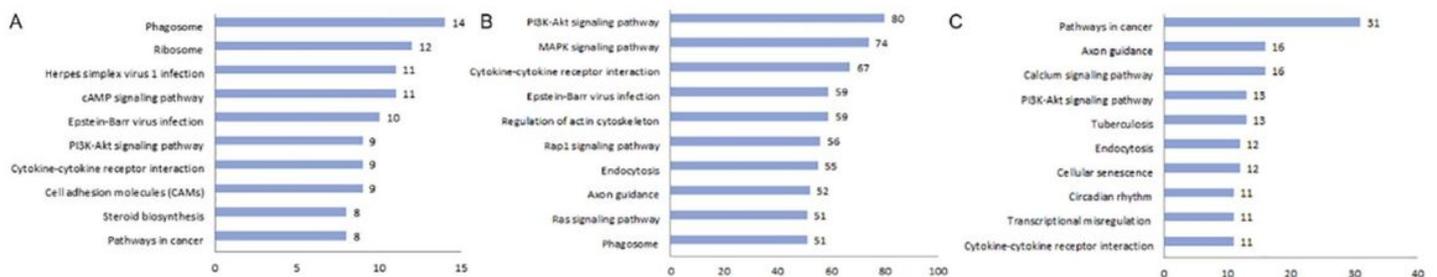
**Figure 4**

KEGG pathway analysis of differentially expressed genes. (A) 1 day old vs. 30 days old; (B) 30 days old vs. 180 days old; (C) 180 days old vs. adult; only the top 10 pathways are listed.



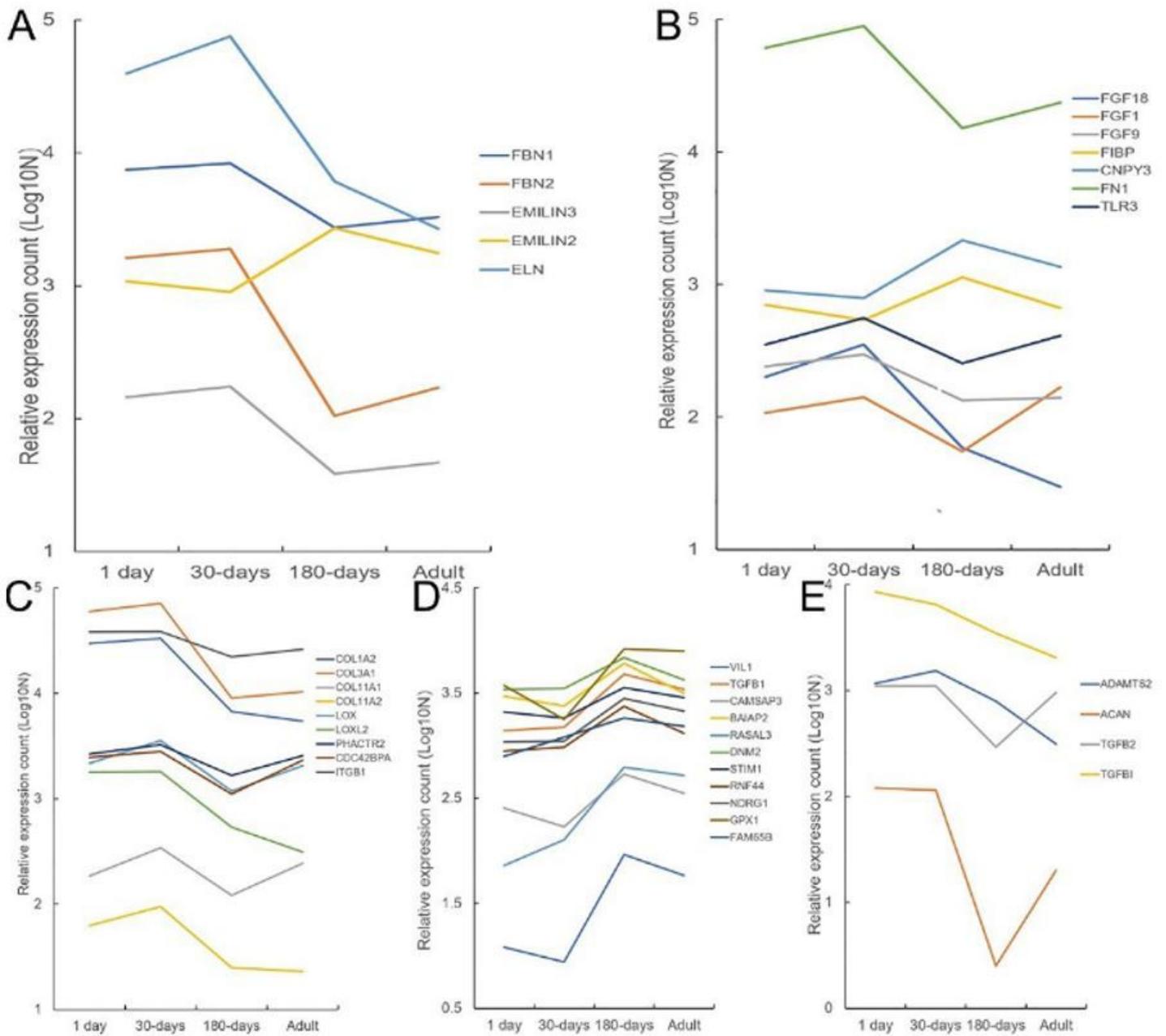
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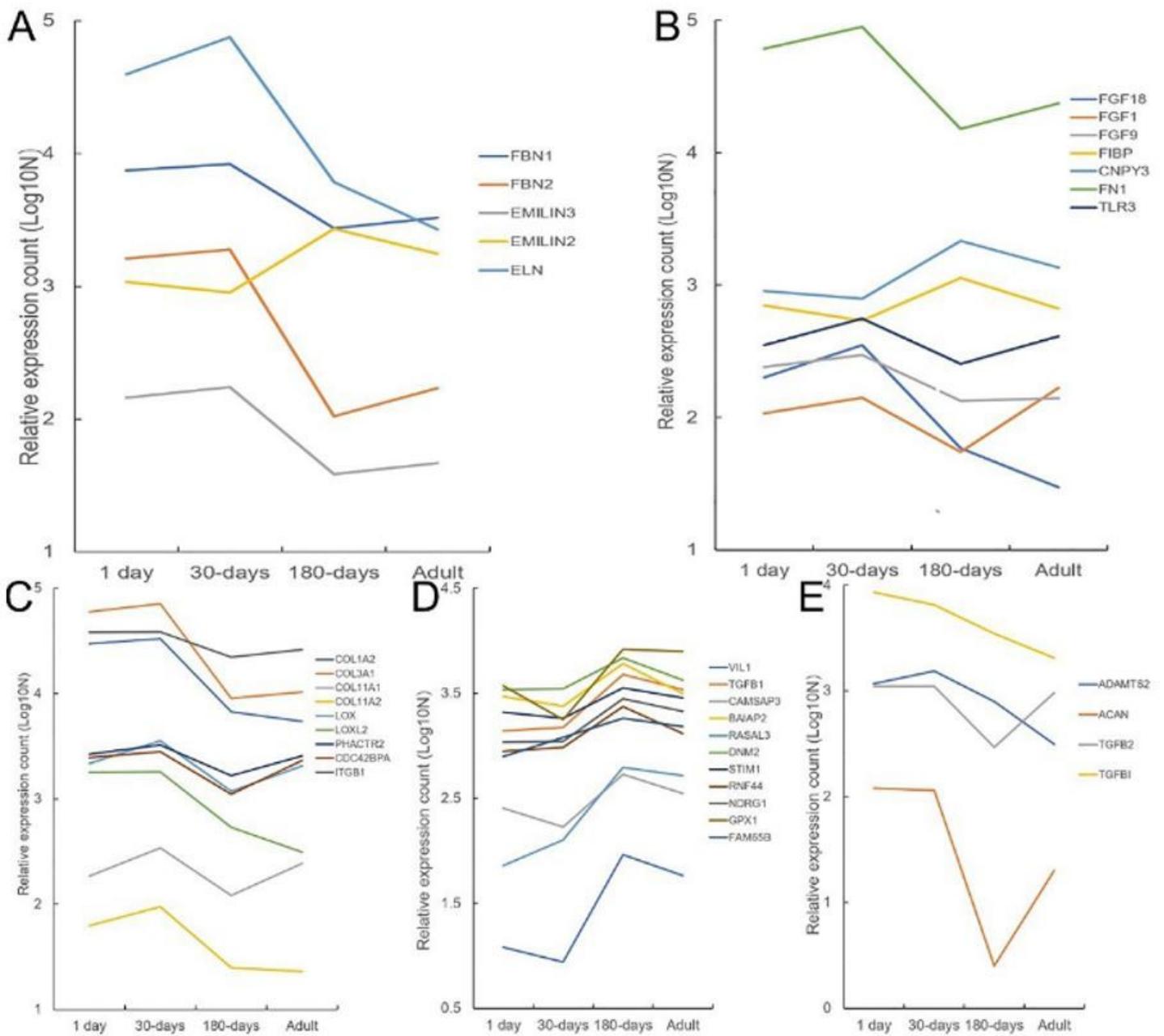
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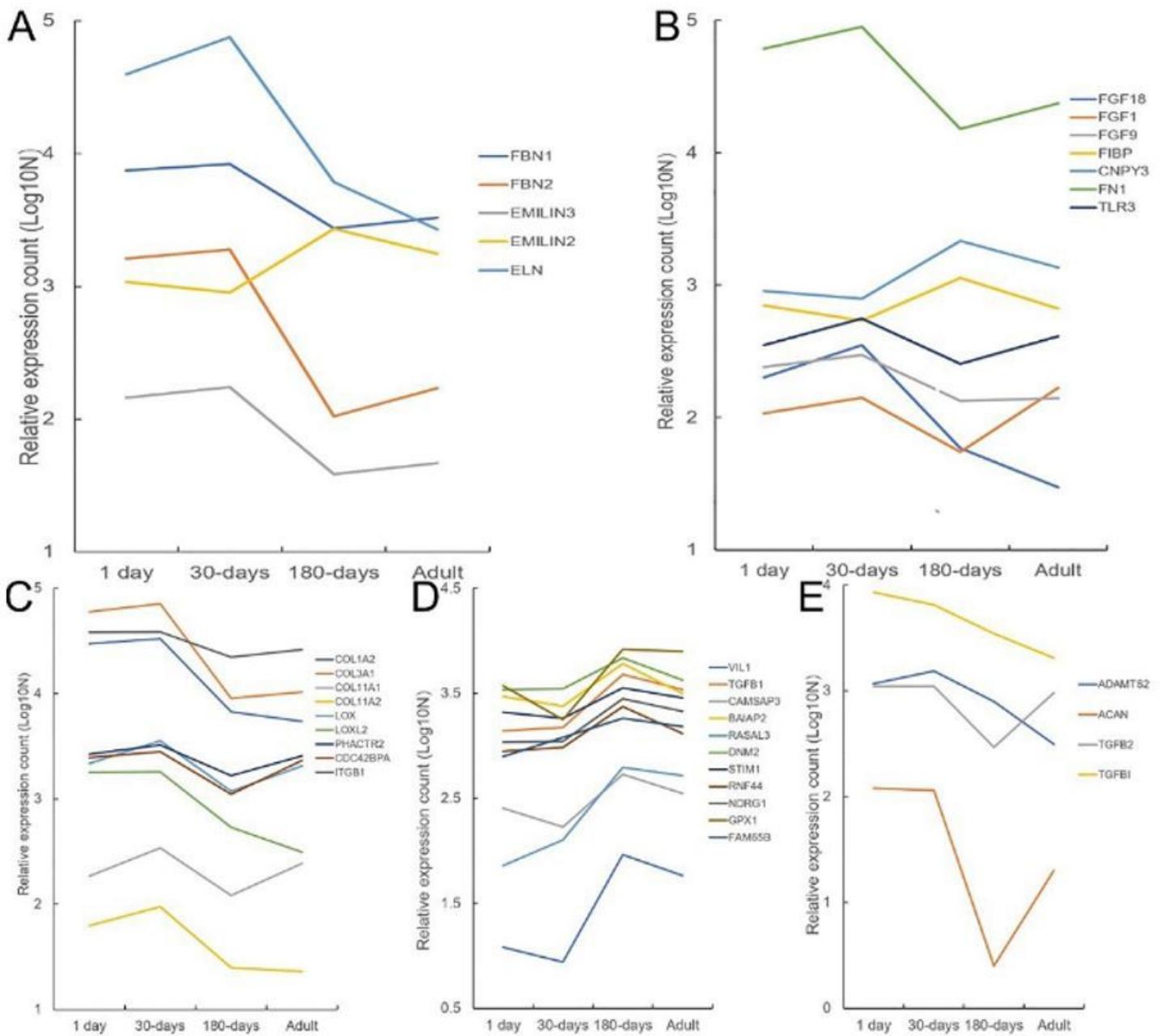
**Figure 5**

Expression levels of the genes related to fibre formation changed at different stages. (A) Elastic fibre formation-related genes; (B) fibroblast-related genes; (C) gene expression of collagen fibre formation-promoting genes reached a maximum at 30 days of age; (D) gene expression of collagen fibre formation-promoting genes reached a maximum at 180 days of age; (E) collagen fibre collagen fibre formation-inhibiting genes



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