



Lmbr1* Expression in Early Embryo Development Stages in White Leghorn and Chinese Silky

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ABSTRACT : *Lmbr1* is regarded as a key gene that controls the digital model formation in early developmental stages of the chicken. However, there are few reports of *lmbr1* expression levels and tendencies in 4-toe and 5-toe chicken species. Therefore, the objective of this study was to compare the *lmbr1* expression in White Leghorn (4-toe) and Chinese Silky (5-toe). Firstly, total RNA was extracted from 14 different embryonic development stages (HH3 to HH31) in White Leghorn and Chinese Silky. Secondly, dramatic gene expression changes of *lmbr1* were monitored by RT-PCR, which indicated a general up-down-up tendency with subtle differences between these two species. Moreover, Q-PCR reactions were performed to quantitate the expression level of *lmbr1* in the 14 selected developmental stages. These data demonstrated a first *lmbr1* expression peak of 18.68 and 15.32, a *lmbr1* expression trough of 6.61 and 1.80, and a second *lmbr1* expression peak of 22.33 and 12.48 in White Leghorn and Chinese Silky, respectively. Finally, embryonic *in situ* hybridization analysis identified that *lmbr1* expressed in the ectoderm in HH21, HH23 and HH24 developmental stages in both species. (**Key Words :** Polydactyly, *Lmbr1*, Embryo Development, White Leghorn, Chinese Silky)

INTRODUCTION

Polydactyly is a kind of common neonate limb abnormality, which has similar phenotypes in different vertebrates. In the chicken, the normal digital model is 4-toe, while the Chinese local chicken species typically have a 5-toe digital model.

It has been suggested that *lmbr1* is an important functional candidate gene which controls the polydactyly. The impact of *lmbr1* on chicken polydactyly was reported in several studies (Warren, 1944; Tickle, 1975; Tickle, 1981; Huang et al., 2004; Huang et al., 2006). Some researchers considered that the *lmbr1* gene located on the chicken 2nd chromosome was homologous with the *lmbr1* gene located on the mouse 5th chromosome and the C7orf2 gene located on the human chromosome 7q36 (Horikoshi et al., 2003). Other reports indicated that mutation in the above

chromosome regions resulted in human preaxial polydactyly, mouse *Hx*, *Hm* and *Ssq* limb abnormality, and chicken polydactyly. These experimental findings indicated that *lmbr1* played an important role in limb development, especially in chicken polydactyly development (Loomis et al., 1998; Clark et al., 2000; Pitel et al., 2000; Lettice et al., 2002).

However, few researchers have focused their attention on the *lmbr1* expression level in the 4-toe and 5-toe digital model of chicken species. In our research, total RNA in different embryonic development stages of the phenotypically normal 4-toe (White Leghorn) and 5-toe (Chinese Silky) chicken was extracted. The embryonic limb developmental pattern was observed (Jang et al., 2008), the *lmbr1* expression was monitored by RT-PCR (Wu et al., 2008), and further measured by Q-PCR. The embryonic *in situ* hybridizations were performed to determine the *lmbr1* expression region in three different development stages, so as to provide important information regarding the impact of *lmbr1* expression level on the chicken polydactyly trait.

MATERIALS AND METHODS

Experimental materials and procedures

All fertilized eggs of White leghorn (4-toe) and Chinese Silky (5-toe) were obtained from the China Agricultural

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University Experimental Chicken Houses. Primers consisted of oligoT18 (5'- TTTTTTTTTTTTTTTTTT -3'), chicken specific housekeeping GAPDH primers (GAPDH upper primer: 5'- GGAACACTATAAAGGCGAGAT -3', GAPDH lower primer: 5'- TCACAAGTTTCCCGTTCTCA -3, designed upon GenBank No. NM_204305), *Imbr1* cDNA specific primers (*Imbr1* cDNA upper primer: 5'- GATCAGAGCACGCATTCT -3', *Imbr1* cDNA lower primer: 5'- ACCAGCAACTGACCCATTA -3') and primers used for prepare the probes of *in situ* hybridization (Probe upper primer: 5'- TCCACAGCCAAGTGCGGGAGTACAC-3'. Probe upper primer: 5'- CAGCCAGTTAAACCTTCCGAA GTCT-3, designed upon GenBank No. AY316689). Besides, pGEM T Easy vector (Promega, WI, USA) was used to transcript the probes. Other reagents used in this research were DEPC water, Trizol (Invitrogen, CA, USA), SYBR Green PCR Kit (Appliedbiosystems, CA, USA), and DIG RNA Labeling Kit (Sp6/T7) (Roche, IN, USA).

All fertilized eggs were incubated at 37.6°C to 38°C and 55% to 60% humidity. Eggs were turned over every two hours. Light detections were performed twice on the 5 to 6th day and 18 to 19th day of each incubation cycle, so as to identify the egg embryos developing normally in the incubation stages.

50 to 100 mg tissue was collected from different development stages of the two chicken species, and was homogenized in liquid nitrogen. Each homogenate was transferred into a 1.5 ml tube, and quickly mixed with 1ml Trizol according to the manufacture's protocol. The total RNA was purified by DNase I (Takara, Dalian, China) and RNaseOUT (Invitrogen, CA, USA) and stored at -80°C until use.

500 µg purified RNA, Oligo T18 primers and DEPC water were mixed and incubated at 70°C for 5 min, and then cooled down in ice. 5 µl 5× first strand buffer, 2 µl 10 mM dNTP, 1 µl RNA inhibitor, 1 µl MMLV Reverse Transcriptase were mixed and DEPC water was supplemented to a final volume of 25 µl. The reaction system was incubated at 37°C for 60 min, then at 70°C for 15 min, and finally cooled down to 4°C to stop the reaction and the reverse transcription products were stored at 4°C. PCR reactions with GAPDH and *Imbr1* cDNA upper and lower primers were performed, with reaction conditions which consisted of 94°C, 5 min; 94°C, 30 sec, 54°C 30 sec, 72°C 20 sec, 30 cycles; 72°C 7 min and stored at 4°C.

We applied the 7900HT quantitative thermal cycler (Appliedbiosystems, CA, USA) to analyse the *Imbr1* expression level in different embryonic developmental stages, with reaction conditions which consisted of 55°C 2 min; 95°C 10 min; 95°C 30 sec, 54°C 30 sec, 72°C 30 sec, 40 cycles. The standard curves were performed twice, while all the samples were repeated four times. Average values

were calculated with the exclusion of irrational data. The dissociation curves were refreshed each time, so as to confirm the specificity of the reaction and the resultant products.

PCR products amplified from the *Imbr1* cDNA upper and lower probe primers were cloned into pGEM T easy vector. 3 µg correct transformant DNA confirmed by sequencing was linearized by *Sac*II digestion, purified and dissolved in DEPC water to a final concentration of 1 mg/ml. According to the manufacturer's protocol, 1 µg purified linear DNA, 2.0 µl 10× DIG labeled NTP mixture, 2.0 µl 10× transcription buffer, 1.0 µl RNase inhibitor and 2.0 µl Sp6/T7 RNA polymerase were mixed and incubated at 37°C for 1 h. 2.0 µl 0.2 M EDTA (pH 8.0) was added to stop the reaction and DEPC water was added after the purification process, so as to dissolve the pellet to a final concentration of 1 mg/ml. RNA probe aliquots were stored at -80°C, or added into 10 times volume of pre-hybridization solution to form the probe stock, and stored at -20°C.

Chicken embryos which developed within 5 days were collected, washed with ice prechilled PBS, and pooled to homogenize with a homogenizer or syringe (the less volume the better). 4 times volume of ice prechilled acetone were added into the homogenate, mixed and incubated in ice for 30 min. The mixture solution was then centrifuged at 4°C at 10,000 rpm for 10 min. The supernatant was discarded and the precipitate was washed once with the ice prechilled acetone. Again, the mixture solution was centrifuged at 4°C at 10,000 rpm for 10 min. The supernatant was discarded and the precipitates were spread on the filter paper and ground into powder. All powders were dried at room temperature and stored at 4°C.

3 mg chicken embryo powder was added to 1 ml TBST (pH 7.4). The mixture was incubated at 70°C for 30 min, and centrifuged at 3,000 rpm for 1 min. The supernatant was discarded carefully, TBST was added to rinse the precipitate which was then centrifuged at 3,000 rpm for 1 min. The above steps were repeated until the supernatant became clear. Then the supernatant was discarded again, the precipitate was resuspended with 1 ml pre-blocking buffer, an imperative amount of antibody was added and then incubated with RT for 2 to 3 h, with reversal of the tube at intervals. The solution was spun at 10,000 rpm for 3 to 5 min, and the supernatant was kept as the pre-absorbed antibody buffer. The antibody dilution was adjusted to 1:5,000 with the pre-blocking buffer, and the antibody buffer was stored at 4°C, which could be used 15 to 20 times.

Chicken embryos developed within 5 days were collected. The residuary yolks of the embryos were rinsed by PBS, and the vitelline membranes were dissected.

Embryos developed within 1 day were manipulated in a Petri dish, and submerged in fixation buffer (4% Paraformaldehyde); embryos developed within 2 to 5 days were treated with an insect-pin (0.1 mm diameter) to prick the cavities such as the cerebral vesicle, eye and heart etc. Fixation buffer was added and incubated with RT for 4 to 5 h or at 4°C overnight. The fixation buffer was discarded and 100% methanol was added. The fixed embryos were stored at -20°C till use.

Revised embryonic *in situ* hybridization (ISH) (Streit and Stern, 2001) was performed over a consecutive 3 days. On the first day, the fixed embryos were soaked for 20 to 30 min in the 75%, 50% and 25% methanol/PBT series. The embryos were rinsed twice in PBT for 10 minutes each and then soaked in 6% H₂O₂/PCT for 1 h to bleach the pigment in eyes. The embryos were rinsed 3 times for 10 min each. After the last rinse, the volume of PBT was measured and Protease K added to a final concentration of 10 µg/ml to digest in RT for 30 min. The Protease K was discarded and the embryos washed twice with PBT. Paraformaldehyde with 0.1% glutaraldehyde was added to the RT mixture so as to post-fix for 20 to 30 min. The post-fix solution was discarded and the embryos washed twice with PBT. Pre-hybridization buffer was added and the sample pre-hybridized at 37°C for 3 h. The pre-hybridization buffer was discarded, the hybridization solution added with RNA probes to a final concentration of 0.5 to 1.0 µg/ml and the mixture was hybridized at 65 to 70°C overnight.

On the second day, the hybridization solution with probes was removed and stored at -20°C for repetitive use. The embryos were washed 3 times with pre-hybridization solution preheated to 65 to 70°C and then rinsed 3 times at 70°C within 30 to 45 min, with TBST; prehybridization solution (1:1). The embryos were washed 3 times with TBST at RT for 30 min each. The TBST was discarded, enough pre-blocking solution added and pre-blocked at RT for 2 to 3 h. The pre-blocking solution was discarded, blocking solution added with antibody dilution of 1:5,000 and blocked at 4°C overnight.

On the third day, the blocking solution was removed and stored at 4°C for repetitive use. The embryos were washed 3 times with TBST within 30 to 60 min, and washed twice with NTMT solution for 10 min each. Coloration solution (1.5 ml) was added and developed away from light (5 min to several days). After color development, the embryos were washed twice with PBS for 10 min each to stop the coloration reaction and the results recorded.

RESULTS

Chicken embryo development could be divided into 46 stages according to Hamburg Hamilton Stages (Each stage

was presented as HH##), and all embryos at different stages provide recognizable developmental characteristics (Hamburger and Hamilton, 1951). In our research, we monitored the *lmbr1* expression level in 14 development stages from HH3 to HH31 in White Leghorn (4-toe) and Chinese Silky (5-toe).

Embryonic total RNA in 14 different stages between HH3 to HH31 from White Leghorn (4-toe) and Chinese Silky (5-toe) were extracted by Trizol, and detected by 0.7% gel electrophoresis after purification.

Reverse transcription and amplification of *lmbr1*

In order to detect the *lmbr1* expression at different development stages in these two chicken species, PCR amplifications were performed with the template obtained from embryonic cDNA. The online BLAST results indicated that the *lmbr1* cDNA shared a lower homology with other genes. Thus, a specific region was selected, in which the primers were designed to generate a 249 bp amplification product. The housekeeping GAPDH was used for the control (with a 218 bp amplification product).

The electrophoresis screen indicated that the *lmbr1* expression was clearly different in these two chicken species, which were confirmed by repetitive detection. Electrophoresis results showed that the *lmbr1* expression demonstrated a general up-down-up tendency in White Leghorn and Chinese Silky, but gave subtle differences in the two chicken species. In White Leghorn, the expression decreased at HH17, but increased sharply at HH18, and decreased rapidly again. In Chinese Silky, however, the same tendency existed, with a more decreasing extent than White Leghorn. Therefore, the fluorescent quantitative PCR reactions were performed to quantitate the exact expression.

Moreover, PCR products were sequenced and aligned, which indicated that the amplification products were *lmbr1* gene, there being no differences between the Chinese Silky and White Leghorn PCR products.

Quantitative PCR

Standard curve relative quantitation mode was applied in our research. Four individual samples in each developmental stage were selected, each sample was repeated 4 times and a standard curve was carried out twice. Average values were calculated in the analysis step with the exclusion of unreasonable data. A dissociation curve was performed each time to ensure the amplification specificity. Correlation between each reaction was guaranteed above 0.995.

In the mRNA quantitative analysis, the amount of cDNA were quantitated by housekeeping GAPDH (Q1), then the *lmbr1* were quantitated (Q2), and the sample results anticipated in our research should follow the formula $Q = Q2/Q1$. This ratio could be only used in the relative

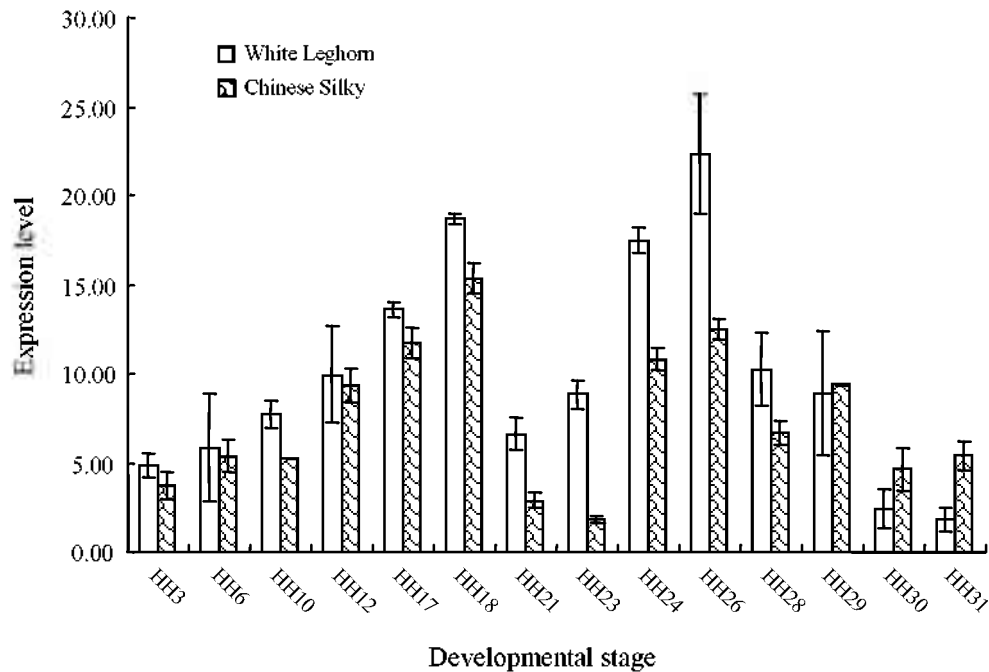


Figure 1. *Lmbr1* expression in different developmental stages in White Leghorn and Chinese Silky.

quantitative analysis, which should be applied to compare the expression level of the same gene in different samples. It does not represent the absolute quantity, and could not be applied in the contrast between different genes.

Figure 1 shows the results of Q-PCR. *Lmbr1* expression in chicken embryos was detected, and the expression level demonstrated an up-down-up tendency in different embryonic developmental stages. However, we need to determine the expression region of *lmbr1*, which is the key factor in limb development. Therefore, embryonic *in situ* hybridization experiments were needed.

Embryonic *in situ* hybridization

Due to technical limitations, the whole embryonic *in situ* hybridization could be only applied to the embryos developed within 5 days. So, we selected embryos at the HH21, HH23 and HH24 stage, whose limbs were developing and the *lmbr1* was expressing.

Probe length used for the RNA hybridization was 1.2 kb, which was designed from the *lmbr1* cDNA sequence (NCBI accession number AY316689). RT-PCR reactions were performed with the template of different developmental stage embryonic RNA pools. The amplification results were screened by gel electrophoresis. The 1.2 kb amplification fragments were collected and cloned into the pGEM T easy vector. Positive transformants were identified by enzyme digestion and sequencing after transforming the *E. coli*. The resultant recombination plasmids were linearized by *Sac*II, and the 1.2 kb RNA probes were produced by *in vitro* transcription under RNase-free conditions. Figure 2 shows

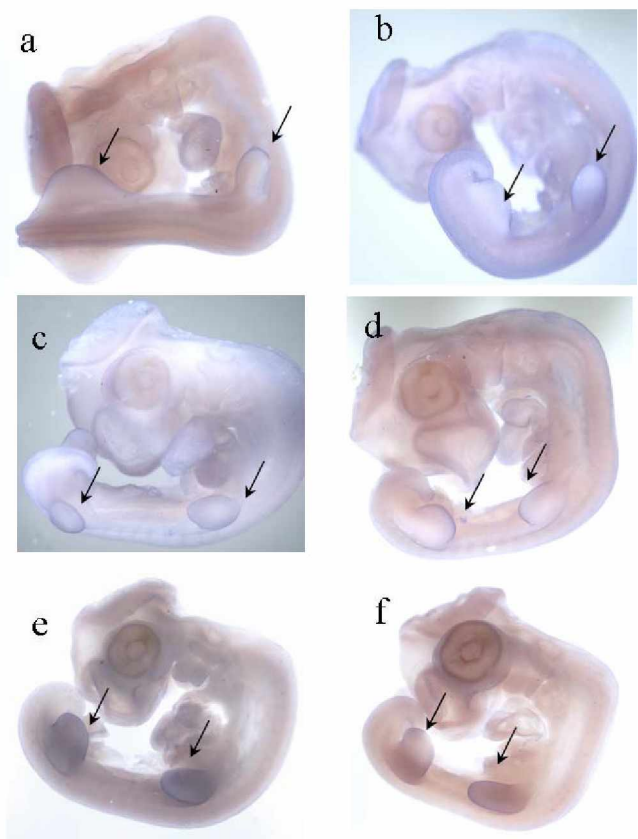


Figure 2. *Lmbr1* expression at HH21, HH23 and HH24 stages in White Leghorn and Chinese Silky. (a), (c), (e) show the *lmbr1* expression sites at HH21, HH23 and HH24 stages in White Leghorn. (b), (d), (f) show the *lmbr1* expression sites at HH21, HH23 and HH24 stages in Chinese Silky.

the hybridization results.

The *in situ* hybridization analysis indicated that the *lmbr1* expression levels were coincident with the Q-PCR results in HH21, HH23 and HH24 stages, with the expression sites located in ectoderm of the limb buds.

DISCUSSION

At the mRNA level, a general “up-down-up” *lmbr1* expression tendency was noted in both the White Leghorn and Chinese Silky species, as indicated in Figure 1.

In White Leghorn species, in developmental stages between HH3 to HH18, *lmbr1* expression increased smoothly from HH3 (4.87) to HH18 (18.68). *lmbr1* expression at HH18 corresponded to almost 4 times the expression in HH3. In developmental stages of HH18 to HH26, *lmbr1* expression presented a “V-shape” model, which indicated the expression level dramatically decreased from HH18 (18.68) to HH21 (6.61), almost 3 times falling of expression. Then the *lmbr1* expression re-increased at stages HH23 (8.84), HH24 (17.44) and reached the climax at HH26 (22.33), which corresponded to almost 4 times that of HH21. After developmental stage HH26, *lmbr1* expression decreased again. The expression at HH28 (10.21) and HH29 (8.87) decreased almost to about 1/2 to 1/3 of the expression of HH26, and continued to decrease stepwise at HH30 (2.40) until the expression nadir of HH31 (1.81).

A similar *lmbr1* expression profile was also noted in the Chinese Silky species. Firstly, although the *lmbr1* expression presented a smooth ascending tendency between developmental stages HH3 to HH18, the expression always maintained a lower level than that of the White Leghorn. When the *lmbr1* expression achieved the first peak at HH18 (18.68) in White Leghorn, the corresponding expression in Chinese Silky also reached the climax at 15.32, which equals nearly 80% of the White Leghorn. Secondly, although the same *lmbr1* expression tendency presented between developmental stages HH18 to HH26, their expression in Chinese Silky species decreased much more than that of the White Leghorn, to 2.88 and 1.80 in HH21 and HH23, and increased to 10.78 and 12.48 at HH23 and HH26, respectively. In these developmental stages, decline of *lmbr1* expression in White Leghorn and Chinese Silky did not appear synchronously, but presented different degree of descent. These expression differences were confirmed by the further embryonic *in situ* hybridization analysis (see Figure 2). Additionally, *lmbr1* expression gave a “W-shape” mode between developmental stages of HH26 to HH31 in Chinese Silky, which is quite different to that of the White Leghorn, and demonstrated a higher expression in HH29 (9.41), HH30 (4.64) and HH31 (5.39) than that of the White Leghorn.

When these expression tendencies were combined with the limb developmental schedules, we made some

surprising discoveries.

According to the Hamburger-Hamilton chicken embryonic developmental stage descriptions, limb bud should appear between developmental stages HH16 to HH18. In our research, both the White Leghorn and Chinese Silky species presented an increasing *lmbr1* expression tendency before and in HH16 to HH18, which showed that the limb bud development correlated closely to the *lmbr1* expression, and the high expression level between HH16 to HH18 triggered the development of the limb.

In HH21 to HH23 stages, the cartilage prosoma of phalanges should begin to appear, and the phalanges mode initiated to form. In our research, *lmbr1* expression in those stages quickly dropped, and demonstrated an up tendency in White Leghorn and a down tendency in Chinese Silky respectively. A generally lower *lmbr1* expression level between those stages indicated that the formation of the phalanges required a lower expression of *lmbr1*. In other words, *lmbr1* expression should be inhibited during the phalange formation stages. Moreover, the *lmbr1* expression nadir was observed at HH23 in Chinese Silky, which may undertake the bifurcation point of 4-toe and 5-toe digital formation in chicken.

Normally, the intact 4-digit dactyl plate should be recognized at stage HH26. In our research, the *lmbr1* expression level at this stage in White Leghorn was almost twice of that of the Chinese Silky. On one side, this reinforced that a higher *lmbr1* expression is required by the digital development, on the other side, the *lmbr1* expression climax at this stage in White Leghorn also eliminated the possibility of new digit formation.

However, a relatively higher *lmbr1* expression was observed at HH29 in Chinese Silky, which corresponded to the 5th digit formation. As far as we can conclude, the decreased *lmbr1* expression from HH26 to HH28 may start the 5th digit development in Chinese Silky, and may be the expectant digital model developmental results derived from the baseline *lmbr1* expression at HH23.

This phenomenon has also been reported in the mouse preaxial polydactyly mutant *Hx* (Clark et al., 2000). In 2004, Sarah cloned the chicken *lmbr1* gene coding sequences and conducted *lmbr1* expression analysis with the chicken limb deletion mutant *ozd*. In their research, *lmbr1* expression initiated in the limb polarization active regions in HH17 to HH18, reached the climax at HH19 to HH23, and dropped gradually till the expression could hardly be detected at HH24 to HH26 (Sarah et al., 2004). Their research results were totally contrary to ours on the *lmbr1* expression tendency in Chinese Silky, but with the same developmental stages and opposite phenotypes.

Moreover, there are few reports indicating the relationship between the polydactyly phenotype in Chinese Silky and the relative gene expression profile. As far as we know, it is the first time that the formation of the 5th toe in

Chinese Silky has been correlated with *lmbr1* expression changes. For instance, as shown in Figure 1, a higher *lmbr1* expression level at stage HH29 than at HH28 in Chinese Silky could be identified, which suggests that formation of the 5th toe in Chinese Silky requires a higher *lmbr1* expression level. Furthermore, the *lmbr1* expression level at stage HH29 in Chinese Silky first exceeds that in White Leghorn, which implies that a specific *lmbr1* expression level threshold is required (in our research it equals 9.41) for the 5th toe formation. Therefore, we could deduce that the *lmbr1* expression level at stage HH29 in Chinese Silky which exceeds 9.41 is essential for the formation of the 5th toe in Chinese Silky.

Polydactyly is a complex phenotype affected by more multiple factors than *lmbr1*. Previous studies indicated that the Sonic hedgehog (*Shh*) gene is required for patterning the limb (Hill et al., 2003), the misexpression of *Shh* is a common requirement for generating extra digits (Lettice et al., 2002), and regulators lying within the *Lmbr1* gene 1 Mb from *Shh* may be capable of causing congenital digital abnormalities (Lettice et al., 2008). Therefore, *Shh* should also be a key candidate gene for polydactyly but there is still no certain mechanism elucidating the relationship of *Shh* gene expression and polydactyly.

In addition, the preaxial polydactyly in chicken has been mapped to a homologous chromosomal region in the human and mouse, and the *Lmbr1* chicken chromosomal region is the critical candidate region for chicken preaxial polydactyly. However, only a few researchers have linked the *Lmbr1* effect to polydactyly (Clark et al., 2001; Mass et al., 2004; Huang et al., 2006). In those reports, a similar *Lmbr1* gene expression in the mouse preaxial polydactyly mutant *Hemimelic* with extra toes has been presented, but less developmental stages were selected, which could not define a satisfactory *Lmbr1* tendency (Clark et al., 2001).

The next step in our research is to perform RNAi in chicken embryos to investigate the function of *lmbr1*, and to combine the gene expression profile of *lmbr1* with other genes to determine in more detail the relationship between polydactyly phenotype changes and gene expression changes.

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