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Original Article

LAMA5: A new pathogenic gene for non-syndromic cleft lip with or without cleft palate

Zhenzhen Fu [a,b,1], Yan Qi [c,1], Ling-fa Xue [a,b], Yao-xiang Xu [a,b], Jin Yue [a,b], Jin-ze Zhao [a,b], Cong Li [a,b], Wenlin Xiao [a,b,*]

[a] Department of Oral and Maxillofacial Surgery, the Affiliated Hospital of Qingdao University, Qingdao, Shandong, China
[b] Department of Stomatology, Qingdao University, Qingdao, Shandong, China
[c] Department of Stomatology, The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital, Jinan, Shandong, China

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ABSTRACT

Background: This study aimed to investigate the effect of LAMA5 on palatal development in mice.
Methods: The palatine process of C57BL/6 J fetal mice on the embryonic day 13.5 (E13.5) was cultured *in vitro* via the rotating culture method. The LAMA5-shRNA adenovirus vector was constructed, then transfected into the palatal process of E13.5 for 48 h *in vitro*. A fluorescence microscope was used to visualize the fusion of palates. The expression of LAMA5 was also detected. The expression of ki67, cyclin D1, caspase 3, E-cadherin, vimentin and SHH signaling pathway-related signaling factors in the blank control group, the negative control group, and the LAMA5 interference group were detected after virus transfection.
Results: The bilateral palates in the LAMA5 interference group were not fused after virus transfection. PCR and WB showed that the mRNA and protein expressions of LAMA5 were decreased in the LAMA5 interference group. Furthermore, the mRNA and protein expressions of ki67, cyclin D1 and gli1 were decreased in the LAMA5 interference group, while the mRNA and protein expressions of caspase 3 were increased. However, the mRNA and protein expression of E-cadherin, vimentin, Shh and ptch1 did not significantly change in the LAMA5 interference group.
Conclusions: LAMA5 silencing causes cleft palate by inhibiting the proliferation of mouse palatal cells and promoting apoptosis, which may not be involved in EMT. LAMA5 silencing can also cause cleft palate by interfering with the SHH signaling pathway.

At a glance commentary

Scientific background on the subject

The non-syndromic cleft lip with or without cleft palate (NSCL/P) is a common maxillofacial malformation, which affect about 1/700 live births worldwide. NSCL/P poses a huge economic and psychosocial burden on the individual, family, and society. Therefore, the research on the pathogenesis of NSCL/P has been flourishing for a long time.

What this study adds to the field

Our study identified and confirmed that LAMA5 gene may be a new pathogenic gene of NSCL/P. The identification of new pathogenic genes would help elucidate the pathogenesis of NSCL/P and provide a scientific basis for the prenatal diagnosis, prevention, and treatment of NSCL/P.

Cleft lip and palate (CL/P) is a common maxillofacial malformation affecting about 1/700 live births worldwide [1]. CL/P is mainly caused by genetic and environmental factors, especially genetic factors [2]. Mammalian palate development involves palatal growth, elevation and fusion, which are achieved through a series of cellular biological events,

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* Corresponding author. Department of Stomatology, the Affiliated Hospital of Qingdao University, 16, Jiangsu Rd., Qingdao 266003, China.
E-mail address: xiaowenlin@qduhospital.cn (W. Xiao).
[1] These authors contributed equally to this work.

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such as cell proliferation, apoptosis, and epithelial-mesenchymal transformation (EMT). Therefore, interruption of these processes can result in a cleft palate (CP) [3]. In addition, palatal development is associated with multiple signaling pathways. Moreover, extensive crosstalk occurs between different signaling pathways. Shh signaling pathway plays a crucial role in craniofacial development, especially palatal development [3], indicating that both loss and gain of function of Shh signaling can lead to CP [4].

Laminin α5 (LAMA5), belonging to the Laminin (LM) family, is widely expressed and plays an important role in embryonic development [5]. LAMA5 −/− mice died at E13.5–16.5, indicating that LAMA5 mutation can severely affect embryos [6]. In addition, LAMA5 knockout causes multiple developmental defects in embryos, including extracerebral malformations, and abnormal development of lungs, kidneys, teeth, and hair follicles [7]. Laminin α5 can bind to receptors and mediate cell migration, proliferation, apoptosis and EMT [8,9]. Maltseva et al. found that Laminin α5 promotes EMT by binding to integrin 6 [10]. Wegner et al. found that LAMA5 deficiency can delay hair follicle development and hyperproliferation of basal keratinocytes, suggesting that LAMA5 inhibits keratinocyte proliferation and migration [11]. In addition, LAMA5 regulates various signaling pathways, such as Shh, WNT and PI3K/Akt. Furthermore, LAMA5 mutant can decrease the expression of Shh in dental epithelial cells of mice [12].

Our previous WES-based studies conducted in East China showed that LAMA5 is a novel candidate pathogenic gene of CL/P. LAMA5 is mainly expressed in the epithelial basement membranes (BM), and it is spatiotemporally specific in mice palate development [13]. In this study, inhibition of LAMA5 expression *in vitro* culture of palatal organs interfered with normal cell proliferation, apoptosis and Shh signaling pathway, resulting in CP, suggesting that LAMA5 is crucial for palatal development.

Materials and methods

Animals

C57BL/6 J mice were obtained from Ji'nan Pengyue Laboratory Animal Breeding CO., Ltd. The mice were housed in a temperature-controlled room (22–25 °C) under artificial lighting (lights on from 05:00 to 17:00) and 55 ± 5% relative humidity. The mice had access to food and water ad libitum. Two female mice were mated with one male mice at 8:00 p.m., and pregnancy was established based on the presence of vaginal plug the next morning (embryonic day (E) 0.5). Embryos on E13.5 were dissected and prepared for subsequent experiments. This study followed the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th edition, 2011) and was approved by the Ethics Committee for Animal Experiments of Qing Dao University (reference no. AHQU-MAL20200821).

shRNA preparation

Three siRNAs were obtained from Hanbio Biotechnology Co., Ltd (Shanghai, China) for targeting different regions of LAMA5 mRNA. shRNAs were designed and synthesized based on siRNA sequences. shRNA with the best inhibitory effect on LAMA5 was selected for subsequent experiments. A random nucleotide sequence with no effect on LAMA5 was selected as a negative control sequence. The siRNA sequences are shown in Table 1.

Table 1
siRNA sequence.

siRNA	siRNA sequence
negative control	TTCTCCGAACGTGTCACGTAA
LAMA5-siRNA1	CCCTGAACCAGGCCGTTAATA
LAMA5-siRNA2	CCAAGCTGTGATCGCTGTAAA
LAMA5-siRNA3	TGGCTGCATCAAGGGTATTAA

Palate organ culture and RNA interference

The pregnant mice were euthanized via cervical dislocation. Embryos were then harvested under sterile conditions for palatal anatomy [Fig. 1A]. Palatal explants on E13.5 were dissected using micro-scissors under a dissection microscope, as previously described [14]. The heads of embryos were cut off from the necks and placed upside down on a sterile drape [Fig. 1B]. The jaw and tongue were removed horizontally from the corner of the mouth, exposing the bilateral palatal shelf [Fig. 1C]. The head was resected above the palpebral fissure via horizontal incision. These procedures were conducted while avoiding damage to the surface of the palatal explants. The palate explants were randomly divided into the blank control, the negative control and LAMA5-shRNA groups and placed on culture dishes (diameter: 3 cm) containing sterile MF-Millipore membranes (Merck Millipore, Germany). DMEM/F12 (Hyclone, USA) supplemented with 10%FBS (Hyclone, USA) and 1% penicillin-streptomycin solution (Hyclone, USA), with or without recombinant adenovirus (Hanbio, China) was used as the culture medium. The palatal explants were cultured on a rotating culture instrument (25 rpm) in an incubator at 37 °C with an atmosphere of 5% CO_2 for 48 h [Fig. 1D]. The culture medium was changed every 24 h.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Trizol Reagent (Thermo Fisher Scientific, USA) was used to extract total RNA from the palatal explants. PrimeScript TM reagent Kit (TaKaRa, Japan) was then used to synthesize cDNA. qPCR was performed using the TB Green Premix Ex Taq ™ II (TaKaRa, Japan) and a Roche Light Cycler 480 System (Roche Diagnostics, UK) following the manufacturer's instructions. The primer sequences are shown in [Table 2].

Western blot analysis

RIPA buffer was used to extract total protein from the palatal explants. A BCA Protein Assay Kit (Solarbio, China) was then used to detect protein content in the supernatant. The protein was denatured at 100 °C for 5 min, then (~20 μg) electrophoresed on SDS-PAGE gel and transferred onto a PVDF membrane (Merck Millipore, USA). The membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.05% Tween-20 and incubated with primary antibodies at 4 °C overnight. The antibodies used were anti-Laminin α5 (Abcam, AB184330, 1:1000), anti-ki67 (Affinity, AF0931, 1:1000), anti-cyclin D1 (Affinity, AF0931, 1:1000), anti-cleaved caspase 3 (Cell Signaling Technology, 9664, 1:1000), anti-gli1 (Affinity, AF0931, 1:1000), anti-β-actin (Elabscience, E-AB-20058, 1:1000). Then the membranes were incubated with a secondary antibody at room temperature for 1.5 h. An ECL kit (Cell Signaling Technology, USA) was used to detect the protein bands following the manufacturer's protocols. β-actin was used as the load control. The grey level was quantified using ImageJ v1.8.0 software (National Institutes of Health).

Histology and immunohistochemistry staining

The palate explants were embedded in OCT (Solarbio, China) compound using conventional methods, then sectioned (thickness; 5 μm) onto Superfrost Plus slides (Tissue-Tek, Japan). The frozen sections were used for virus transfection analysis under a fluorescence microscope.

The fetal head on E13.5 and the palate explants cultured for 48 h *in vitro* were fixed in 4% paraformaldehyde (Beyotime, China) for 48 h, then dehydrated using gradient ethanol, and embedded in paraffin in the coronal orientation for sectioning by routine procedures. Paraffin

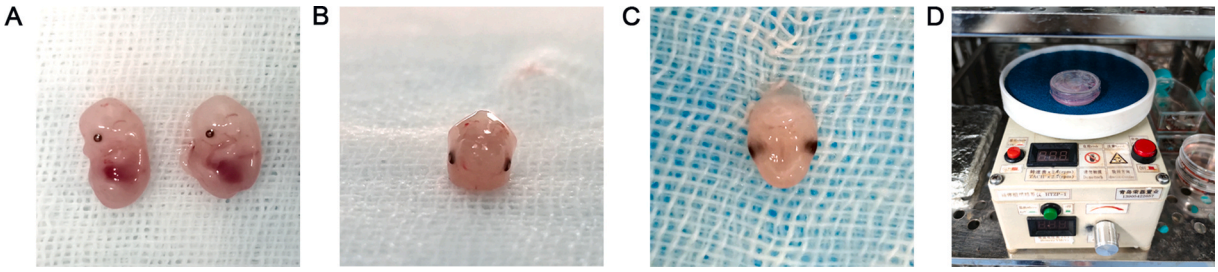

Fig. 1. The anatomy and culture of mice palates. (A) The mice embryos collected from pregnant mice under sterile conditions (B) The heads of mice embryos were cut off from the necks and placed upside down on a sterile drape. (C) The jaw and tongue were removed horizontally from the corner of the mouth, exposing the bilateral palatal shelf. (D) The palatal explants were cultured on a rotating culture instrument (25 rpm) in an incubator at 37 °C with an atmosphere of 5% CO2.

Table 2
The primer sequences.

Gene	sequence
LAMA5	F: GGACCTCTACTGCAAGCTGGT R: ATAGGCCACATGGAACACCTG
ki67	F: CACAGAGAACAAAGGTGTGAA R: GGAGACTGCAGAGCTATTTTTG
cyclin D1	F: CGTATCTTACTTCAAGTGCGTG R: ATGGTCTCCTTCATCTTAGAGG
caspase 3	F: GAAACTCTTCATTCAGGCC R: GCGAGTGAGAATGTGCATAAAT
E-cadherein	F: CTCAGAAGACAGAAACGAGACT R: AACCAGGTTCTTTGGAAATTCG
Vimentin	F: CAGCCTCTATTCCTCATCCC R: GGTGTTGAACTCAGTGTTG
Shh	F: ACGATTTAAGGAACTCACCC R: TTGTCTTTGCACCTCTGAG
ptch1	F: GACTTCAGGATGCATTTGACAG R: GTAGAAAGCGCTCGGATTAATG
gli1	F: GGTGTGTAATTACGTTCAGTCG R: GGATAGGAGCCTGATTTGTGAT
Gapdh	F: ATCACCATCTTCCAGGAGCGAG R: GACCCTTTTGGCTCCACCCTTC

sections (3 μm) were stained with hematoxylin-eosin (HE) and immunohistochemical (IHC) staining for histological analysis. Standard HE staining was performed on paraffin sections as described previously [15], while IHC staining was performed following the manufacturer's instructions (DAKO, Denmark). The sections were deparaffinized and hydrated stepwise, followed by antigen retrieval and blocking with goat serum (GSGB-BIO, China). Non-specific peroxidase was blocked in a 3% hydrogen peroxide solution (Beyotime, China). The sections were then incubated with primary antibodies (anti-Laminin α5, Elabscience, E-AB-31903, 1:400; anti-ki67, Affinity, AF0931, 1:900; anti-cyclin D1, Affinity, AF0931, 1:400; anti-cleaved caspase 3, Cell Signaling Technology, 9664, 1:2000; anti-gli1, Affinity, AF0931, 1:600) at 37 °C for 1 h. The sections were also incubated with the secondary antibody (Beyotime, China) at 37 °C for 30 min. The sections were subsequently visualized using 3,3′-diaminobenzidine (GSGB-BIO, China) and hematoxylin (Beyotime, China).

Statistical analyses

All the experiments were performed in triplicates. The data were expressed as mean ± standard error. All statistical analyses were performed using GraphPad Prism7.0 software. One-way ANOVA and t-test were used for comparison between groups. $p < 0.05$ was considered statistically significant.

Results

Effects of LAMA5 gene silencing on palate development

The effects of the three chemosynthetic shRNAs (1, 2 and 3) after 48 h of palatal organ culture with shRNA adenovirus were confirmed to verify whether the LAMA5 gene was silenced by the designed shRNA. shRNA 1 did not decrease LAMA5 mRNA expression, while shRNA 2 and 3 decreased LAMA5 mRNA expression, especially shRNA3 [Fig. 2A]. Western blotting results were consistent with PCR results [Fig. 2B and C]. Therefore, shRNA 3 was selected to study the function of LAMA5.

The follow-up experiments were divided into three groups (blank control group, negative control group, and LAMA5-shRNA group) to investigate the function of LAMA5 during palate development. The palates in the negative control and LAMA5-shRNA groups emitted homogenous green fluorescence after culturing, indicating that the RNA interference (RNAi) vector containing EGFP and shRNA was successfully introduced into the palates. The bilateral palatal shelf was completely fused in the negative control group, while those in the LAMA5-shRNA group were not contact with each other [Fig. 2D]. The efficiency of shRNA for LAMA5 was verified via PCR, Western blotting and IHC. Compared with the blank control group, the mRNA expression levels of LAMA5 did not significantly change in the negative control group. In contrast, the mRNA expression levels of LAMA5 in the LAMA5-shRNA group decreased by 74.48% [Fig. 2E]. Similarly, Western blotting results were consistent with PCR results [Fig. 2F and G]. IHC staining showed that Laminin α5 was mainly expressed in the epithelial BM [Fig. 2H]. Furthermore, LAMA5 expression was higher in the blank control and negative control groups than in the LAMA5-shRNA group. Also, Laminin α5 positive cell rate of epithelial BM was not significantly different between the blank control and negative control groups, while it was significantly decreased in the LAMA5-shRNA group [Fig. 2I]. PCR and Western blotting found similar results.

The mouse palates were elevated to a horizontal position and close to each other, then fused to form a complete secondary palate at E15.5 days. The samples underwent HE staining after 48 h of culture to assess the effect of LAMA5 on the development of mice palate. The palate in the two control groups had normal development, while palates in the LAMA5-shRNA group were underdeveloped. The bilateral palatal shelf contacted and fused to form a complete secondary palatal in the two control groups, while the bilateral palatal shelf in the LAMA5-shRNA group did not fuse [Fig. 2J].

Effect of LAMA5 on cell proliferation, apoptosis and EMT during palate development

Apoptosis and proliferation are crucial for proper palate development. In this study, the expression levels of cell proliferation and cell apoptosis markers were detected to examine whether CP was caused by impaired cell proliferation and apoptosis. Cyclin D1 and ki67 regulate cell proliferation during mammalian palate development, while caspase

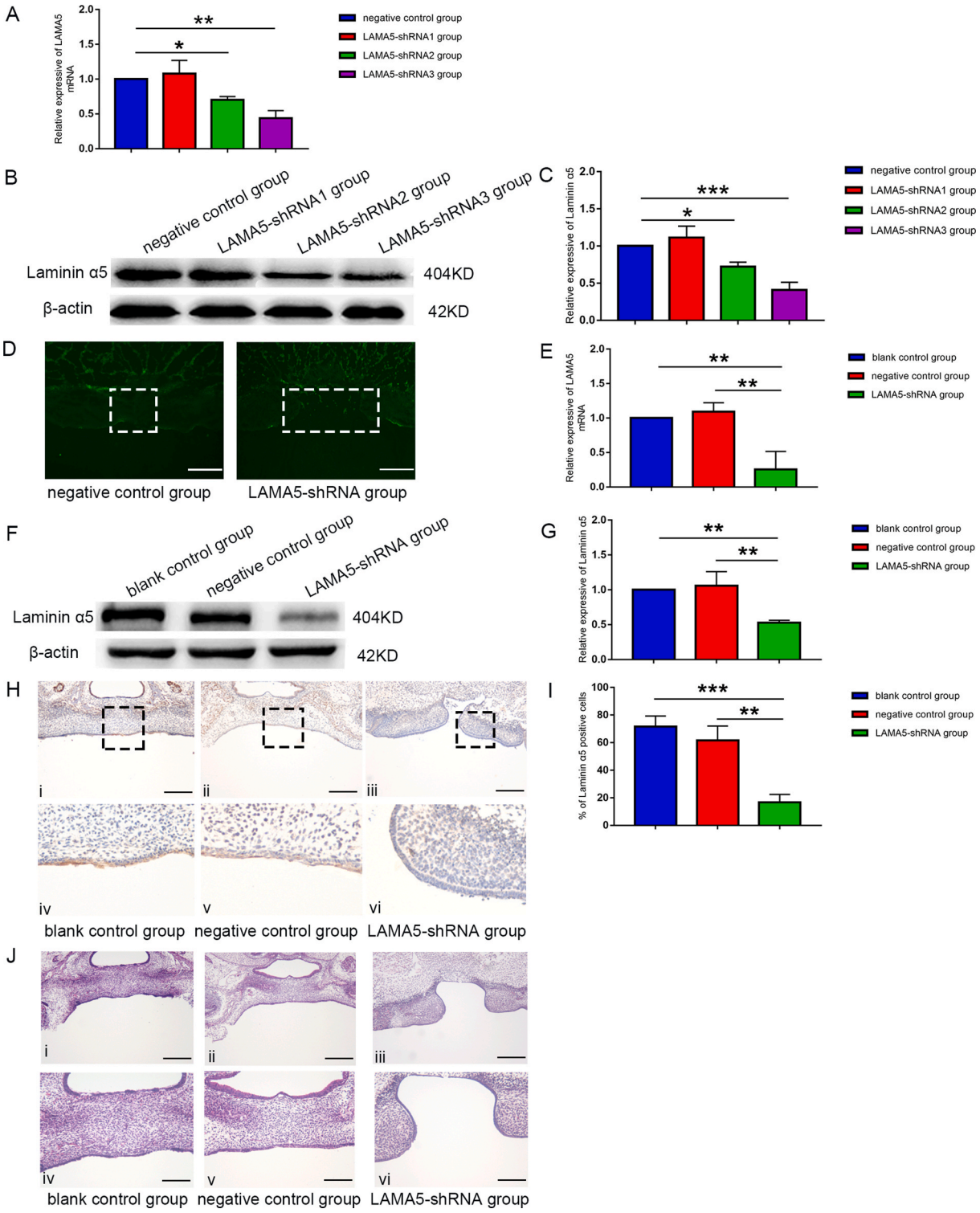

Fig. 2. Inhibition of LAMA5 expression with RNAi technology affects palatal development. (A) The mRNA level of LAMA5 was determined by PCR after 48 h of palatal organ culture with shRNA adenovirus. (B–C) The protein level of laminin α5 was quantified by Western blot after 48 h of palatal organ culture with shRNA adenovirus. (D) Virus transfection and palatal fusion level were shown after 48 h of palatal organ culture with shRNA adenovirus. (E) The inhibition efficiency of shRNA adenovirus on LAMA5 mRNA was detected by PCR. (F–G) The protein level of laminin α5 after 48 h of palatal organ culture with shRNA adenovirus was quantified by Western blot assays. (H–I) Immunohistochemical staining was performed to detect the expression level and location of laminin α5 in mice palates after 48 h of palatal organ culture with shRNA adenovirus. (iv-vi) are magnification of the square boxes in (i-iii), respectively. (J) Palatal fusion level was detected by HE staining after 48 h of palatal organ culture with shRNA adenovirus. (iv-vi) are the magnification of (i-iii), respectively. Bars: J (iv-vi),100 μm; other, 50 μm $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

3 is essential for canonical apoptosis. PCR results showed that the expression of cyclin D1, ki67, and caspase3 was not significantly different between the negative control and the blank control groups [Fig. 3A and F] and [Fig. 4A]. However, the expressions of ki67 and cyclin D1 were significantly decreased in LAMA5-shRNA group (by 56.88% and 83.63%, respectively, compared with the control group [Fig. 3A and F], while the expression of caspase 3 was significantly increased (by 157.47% compared with the control group) [Fig. 4A]. Western blotting also showed similar results [Fig. 3B, C, G and H , [Fig. 4B and C]. IHC staining showed that ki67 and cyclin D1 are mainly expressed in the nucleus while cleaved caspase 3 is mainly expressed in the cytoplasm of palatal cells [Fig. 3D and I], and [Fig. 4D]. The positive cell rate of ki67 and cyclin D1 was significantly reduced, consistent with PCR and Western blotting results. Furthermore, IHC score of cleaved caspase 3 was significantly increased after shRNA treatment [Fig. 3E and J], and [Fig. 4E].

EMT is involved in mammalian palate development. E-cadherin is commonly used as a specific marker for palate epithelial cells. Vimentin is an intermediate filament protein found in mesenchymal cells. In this study, the expression levels of these markers were detected to assess the effect of LAMA5 on EMT during palate development. PCR results showed that the expressions of E-cadherin and vimentin in the three groups did not significantly change [Fig. 4F and G].

LAMA5 inhibition disrupts shh signaling pathway in developing palate

The Shh signaling pathway plays a crucial role in organ development. For example, Shh positively regulates ptch1 and gli1 in palate development [16,17]. In this study, the expression of Shh, gli1 and ptch1 was evaluated to investigate the effect of LAMA5 on Shh signaling pathway in mice during palate development. Although Shh and ptch1 expression did not change in each group, gli1 expression decreased in the LAMA5-shRNA group (by 67.73% compared with the blank control group) [Fig. 5A–C]. Western blotting and PCR results showed similar results [Fig. 5D and E]. IHC staining showed that gli1 is mainly expressed in both the nucleus and cytoplasm of palatal cells [Fig. 5F]. The positive cell rate of gli1 was significantly reduced after LAMA5 inhibition, consistent with PCR and Western blotting results [Fig. 5G].

Discussion

CL/P has a complex etiology, mainly due to the interaction of genetic and environmental factors. Genetic factors are the major cause of CL/P. In this study, results showed that LAMA5 participates in palate development in mice. Therefore, the identification of new pathogenic genes and their pathogenesis can provide a scientific basis for the prenatal diagnosis, prevention, and treatment of CL/P.

Humans and mice have similar palate development process. Besides, the gene homology between mice and humans is extremely high. Therefore, the mice CP model can be used to study the etiology and embryology of palate development in humans [3,18]. Palate organ culture *in vitro* can simulate the whole palate development process *in vivo* [19]. *In vitro* culture of palate organs can eliminate the influence of many other factors *in vivo* and the defects of single palatal epithelial cell or palatal mesenchymal cell culture. Therefore, *in vitro* culture of palate organs can be used to study the growth and development of palatal organs as a whole. RNAi technology can specifically reduce or silence the expression of specific genes. As a result, this technology has been widely used to explore the pathogenesis of diseases and gene therapy. In this study, we chose the palate organ culture *in vitro* combined with RNAi technology to construct a mice CP model *in vitro*.

First, we detected the interference effect of three shRNAs on LAMA5, and found that shRNA3 had the best inhibitory effect on LAMA5. Therefore, shRNA3 was chosen to study the function of LAMA5 on palate development. The experiments were divided into three groups (blank control group, negative control group, and LAMA5-shRNA group) to study the function of LAMA5 on palate development. The palate organ was cultured with shRNA adenovirus carrying EGFP for 48 h, and homogenous green fluorescence was detected in the palates in the negative control and LAMA5-shRNA groups, suggesting that the RNAi vector containing EGFP and shRNA was successfully introduced into the palates. The bilateral palate shelf in the negative control group fused to form normal secondary palate, while bilateral palate shelf in the LAMA5-shRNA group, still had a large gap. These results indicate that adenovirus vector did not affect palate development of mice in the negative control group, while LAMA5-shRNA interfered with normal palate development. Furthermore, PCR, Western Blotting and IHC showed that LAMA5 expression was not significantly different between the negative control and the blank control groups. In contrast, LAMA5 expression was significantly decreased in the LAMA5-shRNA group, indicating that adenovirus did not affect LAMA5 expression, while LAMA5-shRNA interfered with LAMA5 expression. HE staining showed that the palates in the blank control and the negative control group had normal development, and the bilateral palate shelf contacted and fused with each other to form a complete secondary palate. However, the palates in the LAMA5-shRNA group were underdeveloped, and the bilateral palate shelf did not fuse, causing CP. These results indicate that LAMA5 silencing induces CP in mice *in vitro*.

Mammalian palate development involves growth, elevation, and fusion of the palate shelves. Furthermore, palate development is associated with a series of cell behaviors, such as cell proliferation, apoptosis and EMT, and is strictly controlled in time and space [3]. Abnormal expression of ZEB family interferes with normal palate development. For example, ZEB1 and ZEB2 inhibition up-regulates proliferation markers ki67 and cyclin D1, down-regulates apoptosis marker caspase 3, and persistent MEE [20]. Furthermore, Foxf2 inhibition in the palate cultured *in vitro* significantly inhibits the proliferation of palatal mesenchymal cells, leading to the underdevelopment of palate shelves [21]. A study found that the number of ki67 positive cells is decreased in TCDD-induced CP mouse model, while the number of TUNEL positive cells is increased, indicating that TCDD causes CP by promoting cell apoptosis and inhibiting cell proliferation [22]. Abnormal Gpr177 expression interferes with normal palate development. Also, cell proliferation and apoptosis are significantly reduced and increased in Gpr177 mutant palatal shelves, respectively [15]. In this study, Gpr177 and TCDD interfered with normal palate development by promoting apoptosis and inhibiting the proliferation of palatal cells, resulting in CP since the bilateral palate shelves were too small to contact and fuse. Previous studies have also indicated that abnormal expression of AMBRA1, Sox9, TCF7L2 and PDGFRb can also lead to palatal dysplasia by interfering with EMT process [23–25].

Laminin α5, as a component of ECM, participates in cell adhesion, migration, proliferation, apoptosis and EMT by activating or inhibiting signaling pathways. It also participates in organ and tumor development [26,27]. The proliferation of middle dental epithelial cells was inhibited in LAMA5−/− mice, resulting in abnormal dental development. Culturing primary dental epithelial cells with LAMA5-containing LM trimer (LM-511/LM-521) can significantly promote the proliferation of epithelial cells [12]. Moreover, LAMA5 inhibition can decrease the total number of lung cells in lung epithelial cells of mice. IHC staining showed that LAMA5 inhibition decreased the number of ki67 positive cells and increased the number of cleaved caspase 3 positive cells, suggesting that LAMA5 inhibition interferes with lung development by decreasing cell proliferation and increasing apoptosis [28]. To explore whether LAMA5 is also involved in regulating cell proliferation and apoptosis during palatal development, we used RNAi technology to inhibit LAMA5 expression in palate *in vitro*, and detected the expression of ki67, cyclin D1, caspase 3, and cleaved caspase 3. Consistent with the above studies, our results showed that LAMA5 inhibition down-regulates ki67 and cyclin D1 in the palates of mice while up-regulating caspase 3 and cleaved caspase 3. These results indicate that LAMA5 silencing inhibits the proliferation and promotes the apoptosis of palatal cells. Therefore,

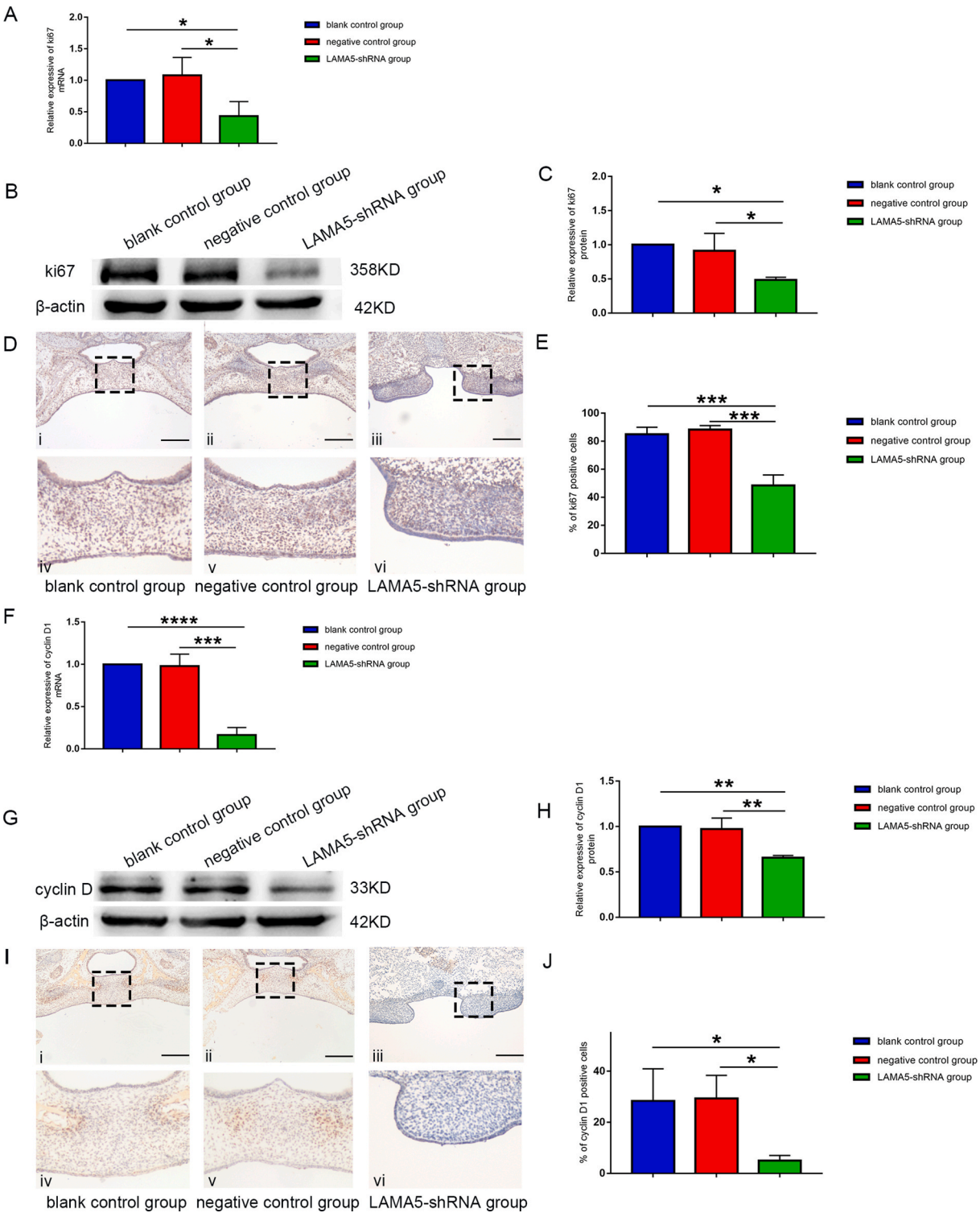

Fig. 3. Inhibition of LAMA5 expression suppressed the expression of proliferation markers. (A) The mRNA levels of ki67 were quantified by PCR after 48 h of palatal organ culture with shRNA adenovirus. (B–C) The levels of ki67 protein were detected by Western blot after 48 h of palatal organ culture with shRNA adenovirus. (D–E) Immunohistochemical staining was performed to detect the expression level and location of ki67 protein after 48 h of palatal organ culture with shRNA adenovirus. (iv-vi) are magnification of the square boxes in (i-iii), respectively. (F) The mRNA levels of cyclin D1 were detected by PCR after 48 h of palatal organ culture with shRNA adenovirus. (G–H) The protein expression level of cyclin D1 protein was quantified by Western blot after 48 h of palatal organ culture with shRNA adenovirus. (I–J) Immunohistochemical staining results showing the expression level and location of cyclin D1 protein after 48 h of palatal organ culture with shRNA adenovirus. (iv-vi) are magnification of the square boxes in (i-iii), respectively. Bars: 50 μm $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$. $^{****}p < 0.0001$.

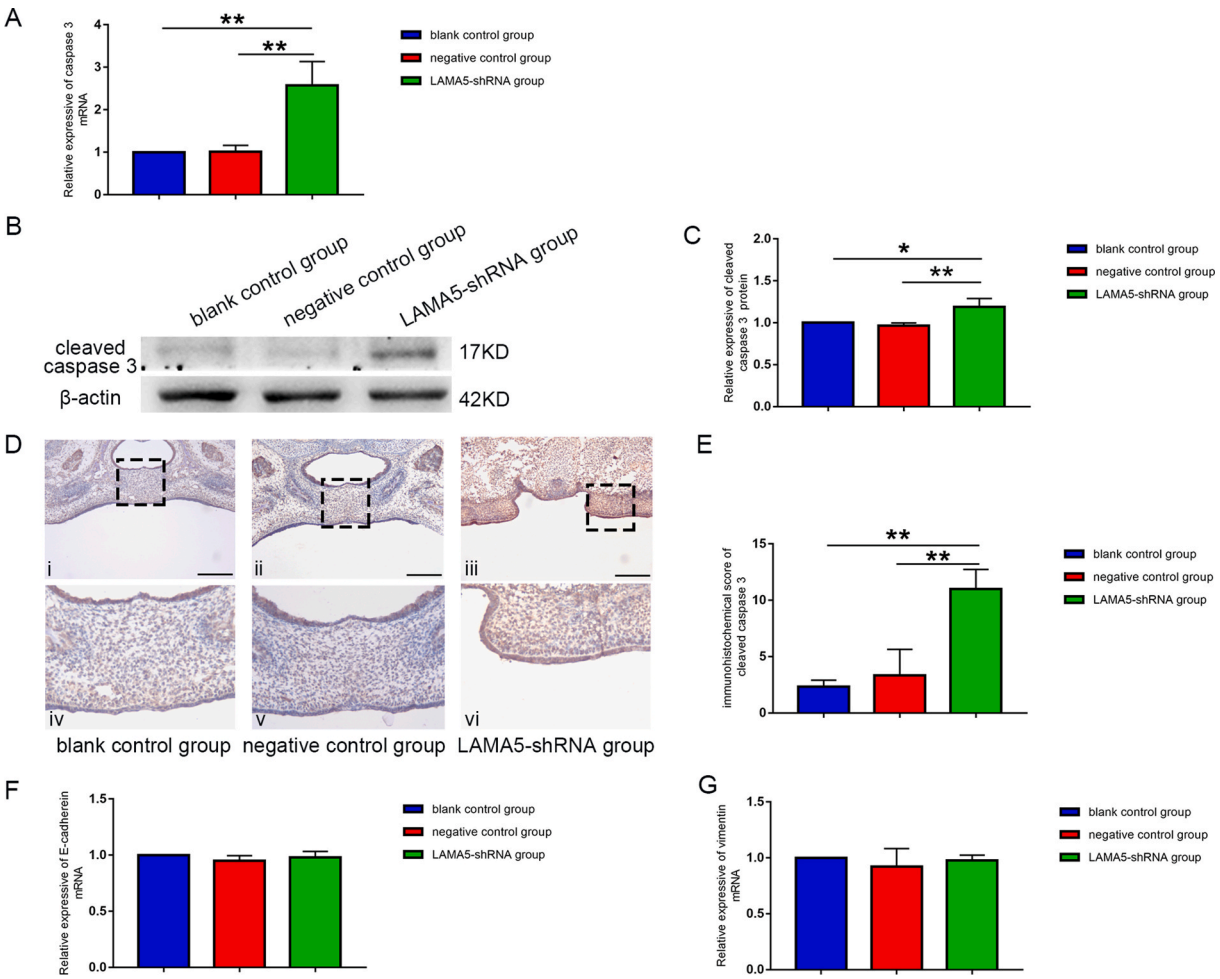

Fig. 4. Inhibition of LAMA5 expression promoted the expression of apoptosis regulators and had no effect on epithelial mesenchymal transformation. (A) The mRNA level of caspase 3 was quantified by PCR after 48 h of palatal organ culture with shRNA adenovirus. (B–C) The protein expression level of cleaved caspase 3 protein was detected by Western blot after 48 h of palatal organ culture with shRNA adenovirus. (D–E) Immunohistochemical staining results showing the expression level and location of cleaved caspase 3 protein after 48 h of palatal organ culture with shRNA adenovirus. (iv-vi) are magnification of the square boxes in (i-iii), respectively. (F–G) The mRNA levels of E-cadherin and vimentin were quantified by PCR after 48 h of palatal organ culture with shRNA adenovirus. Bars: 50 μm $^{*}p < 0.05$. $^{**}p < 0.01$.

we speculated that LAMA5 can regulate cell proliferation and apoptosis during palate development. A lung cancer-related study found that LAMA5 can activate EMT, possibly due to the poor prognosis of lung cancer [29]. A colorectal cancer-related study has also shown that LAMA5 knockdown can inhibit the migration of HT29 cells on LM substrates and alter expression of EMT-related genes, such as CDH1, SOX4, and VEGFA [10]. Although these studies suggest that LAMA5 regulates EMT in cancer, the role of LAMA5 in EMT during palate development is unclear. Therefore, after suppressing LAMA5 expression in mice palate *in vitro*, we tested the expression changes of epithelial marker E-cadherin and mesenchymal marker vimentin. However, there were no changes in the expression of E-cadherin and vimentin, indicating that LAMA5 does not regulate EMT during palate development.

Shh signaling pathway is crucial in craniofacial development, especially palate development [3]. Shh positively regulates the expression of ptch1 and gli1 during palate development [17]. In this study, All-Trans Retinoic Acid (ATRA) can significantly increase cell apoptosis in mouse palate and decreased the expression of Shh, ptch1 and gli1. However, Shh signaling agonist SAG can normalize Shh signaling and reduce the incidence of ATRA-induced CP [30]. A study also found that LAMA5 mutation can interfere with Shh signaling pathway, resulting in unbalanced expression of Shh and gli1. Shh expression is reduced in the dental epithelium of LAMA5 mutant mice, reducing the proliferation of dental epithelium and mesenchymal cells, thus tooth germs became smaller. These results show that LAMA5 maintains normal tooth development by regulating Shh signaling pathway and cell proliferation [12]. A study showed that LAMA5−/− mice have BM defects, abnormal hair follicle morphology, and hair embryo elongation failure. In situ hybridization has also shown that Shh and gli1 expressions are reduced in LAMA5−/− mice, suggesting that LAMA5 can regulate Shh signaling pathway [31]. However, the relationship between LAMA5 and Shh signaling pathway during palate development is unknown.

In this study, LAMA5 inhibition did not significantly change Shh and ptch1 expression but significantly down-regulated gli1. Various signaling pathways regulate palate development. Moreover, several crosstalk occurs among the pathways, such as BMP-Shh [32], Wnt-Shh [33,34], FGF-Shh [16]. Studies have revealed a new non-canonical mechanism of gli1 regulation, MEKK2 and MEKK3 reduce gli1 stability and suppress gli1 transcriptional activity through phosphorylation on multiple Ser/Thr sites of gli1. It was also found that MEKK2/3 mediates the crosstalk between Shh and FGF pathways, and FGF2–MEKK2/3–gli1 signaling axis is independent of canonical Shh signaling in the regulation of gli1 activity [35]. Therefore, we speculate that crosstalk in LAMA5-induced CP can directly regulate Shh signaling pathway by acting on gli1 instead of Shh and ptch1.

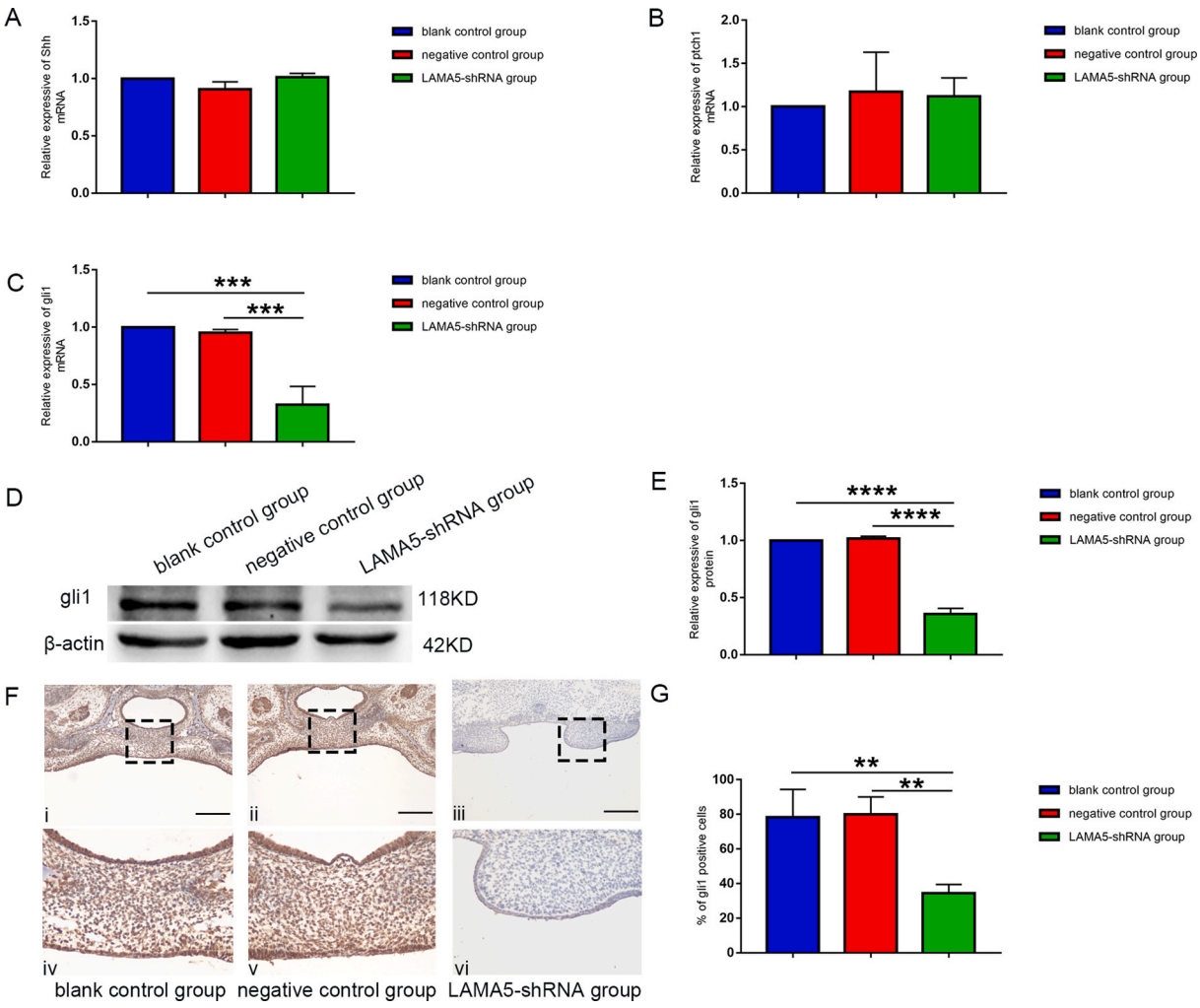

Fig. 5. LAMA5 inhibition suppressed the Shh signaling pathway. (A–C) The mRNA levels of Shh, ptch1 and gli1 after 48 h of palatal organ culture with shRNA adenovirus were quantified by PCR. (D–E) The expression level of gli1 protein after 48 h of palatal organ culture with shRNA adenovirus was measured by Western blot. (F–G) Immunohistochemical staining results showing the expression level and location of gli1 protein after 48 h of palatal organ culture with shRNA adenovirus. (iv-vi) are magnification of the square boxes in (i-iii), respectively. Bars: 50 μm $^{**}p < 0.01$. $^{***}p < 0.001$. $^{****}p < 0.0001$.

Conclusions

LAMA5 is crucial in palate development. In vitro culture of mice palate organ culture, LAMA5 silencing causes CP by inhibiting cell proliferation and promoting cell apoptosis. In addition, LAMA5 regulates Shh signaling pathway during palate development, and there may be a crosstalk of signaling pathways. However, further studies should investigate relevant crosstalk between signaling pathways, and the relationship between signaling pathways with proliferation and apoptosis.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bj.2023.100627.

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