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THE INVOLVEMENT OF HORMONE-SENSITIVE LIPASE IN ALL-TRANS RETINOIC ACID INDUCED CLEFT PALATE

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ABSTRACT

Abnormally high concentrations of all-trans retinoic acid (atRA) induce cleft palate, which is accompanied by abnormal migration and proliferation of mouse embryonic palatal mesenchyme (MEPM) cells. Hormone-sensitive lipase (HSL) is involved in many embryonic development processes. The current study was designed to elucidate the mechanism of HSL in cleft palate induced by atRA. To establish a cleft palate model in Kunming mice, pregnant mice were administered atRA (70 mg/kg) by gavage at embryonic Day 10.5 (E10.5). Embryonic palates were obtained through the dissection of pregnant mice at E15.5. Hematoxylin and eosin (H&E) staining was used to evaluate growth changes in the palatal shelves. The levels of HSL in MEPM cells were detected by immunohistochemistry, quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and western blotting. RNAi was applied to construct vectors expressing HSL small interference RNAs (siRNAs). The vectors were transfected into MEPM cells. Cell proliferation and migration were evaluated by the cell counting kit-8 (CCK-8) assay and wound healing assay, respectively. The palatal shelves in the atRA group had separated at E15.5 without fusing. In MEPM cells, the expression of HSL was reversed after atRA treatment, which caused cleft palate *in vivo*. In the atRA group, the proliferation of HSL siRNA-transfected cells was remarkably promoted, and the migration rate significantly increased in the HSL siRNA-transfected MEPM cells. These results suggested that HSL may be involved in cleft palate induced by atRA and that atRA enhances HSL levels to inhibit embryonic palate growth.

Key words: *Cleft palate; atRA; HSL; MEPM*

INTRODUCTION

Cleft lip and palate is among the most common congenital diseases in humans, resulting from environmental and/or genetic factors of facial development in utero (Alois et al., 2020). Facial morphogenesis occurs at the early stages of embryonic development. In this process, the paired maxillary and mandibular and the medial and lateral nasal prominences constitute the first partly separate facial primordia (Worley et al., 2018). Palatogenesis starts at week 4 in human embryonic development and is not completed until approximately week 12 (Mossey et al., 2009). The palate consists of three elements: the primary palate, which derives from the frontonasal process, and the lateral protrusion of the maxillary processes will form a pair of secondary palates; the development of the secondary palate is a multistep process that consists of palatal shelf growth, elevation of palatal shelves, fusion between the paired palatal shelves, and the disappearance of the medial epithelial seam; and the closed palate separates the oropharynx from the nasopharynx, the palatal shelves grow into the midline, and palatal shelves are fully formed (Abramyan et al., 2015; Nohara et al., 2022). Mice have widely been used for palatal development studies. Palate formation and related molecular mechanisms in mice are similar to those in humans (Suzuki et al., 2018). In mice, secondary palate development begins at embryonic Day 11.5 (E11.5), and at E14.5 and 15.5, the two palatal shelves meet and fuse each at the middle of the oral cavity. Palate formation drastically changes between E14.5 and E15.5, from the cell proliferation phase to differentiation, not only morphology but also the gene expression is altered according to cellular events (Bush et al., 2012). Any imbalance in embryonic palatal mesenchyme cell proliferation and apoptosis can result in cleft palate formation (Gao et al., 2016).

Lipids, due to their multiple biological roles in cell biology, physiology and pathology, are crucial for energy storage and are also involved in cell signaling as well as gene expression during embryonic development. Several human diseases have been found to be associated with abnormal lipid levels (Knittel et al., 2022). Hormone-sensitive lipase (HSL) is a key enzyme in the regulation of lipid metabolism. According to its physiological properties, HSL is an intracellular neutral lipase that is highly expressed in adipose tissue and can catalyze the hydrolysis of triacylglycerol and diacylglycerol (Sztrolovics et al., 1997). According to some studies, the catalytic function of HSL is essential for fertility in male mice (S. P. Wang et al., 2014), and 2,4-dienoyl-CoA reductase (DECR1) directly activates HSL to promote lipolysis in cervical cancer cells to support cervical cancer cell growth (H. Zhou et al., 2022). A previous study also showed the relationship between the incidence of cleft palate in fetuses and the change in endogenous small molecular metabolites. Additional research on the expression of related genes in lipid metabolism will likely determine the pathogenetic mechanisms of craniofacial malformation (J. Zhou et al., 2011).

Retinoic acid (RA) is a synthetic retinoid used for the treatment of psoriasis. All-trans retinoic acid (atRA) is an oxidant of RA and supports normal pattern formation during embryogenesis (Al Tanoury et al., 2013). In both experimental animals and humans, abnormally high concentrations of atRA induce fetal malformations, including cleft palate (Ackermans et al., 2011). Previous *in vivo* studies have demonstrated that atRA induces growth retardation of the palatal shelves (W. Wang et al., 2017). In the present study, we created cleft palate models in Kunming mice using abnormally high concentrations of atRA to test whether the expression of HSL differs from that of the control group. In addition, we investigated whether HSL affects the migration and proliferation of MEPM cells in the atRA group.

MATERIALS & METHODS

Animals

The study protocol involving animals was approved by the Laboratory Animal Ethical Committee of the Medical College of Shantou University (Shantou; China). Sixty Kunming mice (20 males and 40 females; age 8–10 weeks) were purchased from the Shantou University Laboratory Animal Technology Co., Ltd. (Shantou; China). Females were mated with fertile males with similar weights and ages overnight. The presence of a vaginal plug on the next morning was considered indicative of embryonic gestation Day 0.5 (E0.5). We obtained a total of 22 pregnant females, and they were randomly divided into two oral gavage groups at E10.5: atRA-treated groups received 70 mg/kg atRA (Sigma; USA) dissolved in corn oil, and an equivalent volume of corn oil alone was given to the control group. At E15.5, pregnant mice were sacrificed by cervical dislocation. The fetuses were removed from the uterus, and 108 embryos were examined.

Tissue fixation and section preparation

Seventy embryonic heads were dehydrated and greased using an ascending ethanol series and xylene after spending the night in 4% paraformaldehyde (PFA). Then, the embryonic heads were embedded in paraffin and processed into 5 μm -thick serial coronal sections of the fetal heads.

Histological observation

Hematoxylin and eosin (H&E) staining was performed on coronal sections of the embryonic heads to evaluate changes in palatal shelves during palatogenesis according to standard procedures. The results were compared between the control and atRA-treated groups.

Immunohistochemistry (IHC)

The coronal sections were exposed to microwave irradiation for 30 min by applying citric acid buffer (pH 6.0) at 95 °C for antigen activation. Normal donkey serum (10% v/v in PBS, Vector) was used to block nonspecific staining for 1 h. Following optimization, the HSL primary antibody (1:50; 17333-1-AP; Proteintech; China) was used at 4 °C overnight. The sections were washed using phosphate-buffered saline (PBS; Gibco; USA) and incubated with secondary antibodies for two hours at room temperature. Staining was visualized following incubation with 3,3'-diaminobenzidine (DAB; OriGene Technologies, Inc.; USA). The emergence of brown granules was regarded to indicate positive staining. Sections were counterstained with hematoxylin for two seconds. Images were obtained by a Zeiss Axioskop 40 (Leica Microsystems; Germany).

Cell culture and treatment

Pregnant mice were euthanized at E15.5, and the fetuses were collected. Only the palate mucosa of the fetal mice was selectively isolated under a microscope and digested with dispase II (Godo Shusei Co.; Japan) and trypsin EDTA (Biological Industries, Israel) at 37 °C for 30 min. After centrifugation, mouse embryonic palatal mesenchymal (MEPM) cells were isolated using a cell strainer with a pore size of 70 µm (BD Falcon; USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; USA) containing 10% fetal bovine serum (FBS; Gibco; USA) and 1% penicillin/streptomycin (Sigma; Germany) in an atmosphere with 5% CO₂ at 37 °C. The medium was replaced every 2-3 days and then subcultured at the exponential growth stage (80% confluence).

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated with TRIzol from MEPM cells or transfected cells (Sigma; Germany). cDNA was synthesized using the PrimeScript RT reagent kit (TaKaRa; Japan), and the qRT-PCR mix was prepared with the SYBR Premix Ex Taq kit (Takara; Japan). Then, qRT-PCR was performed using the SLAN-96S qPCR system (Hongshi; China).

Table. List of primers used in this study

Primer Name	Primer Sequence (5'-3') F=Forward, R=Reverse
HSL gene	F=5'-TGAGATGGTAACTGTGAGCC-3'
	R=5'-ACTGAGATTGAGGTGCTGTC-3'
GAPDH	F=5'-AGAACATCATCCCTGCCTCTACTG-3'
	R=5'-AAATGAGCTTGACAAAGTGGTCGT-3'

Western blot (WB) analysis

All lysates were prepared from MEPM cells or transfected cells using $2 \times$ SDS lysis buffer supplemented with protease inhibitors (M250, Amresco, Ohio, United States) and phosphate inhibitors (WB0117, Weiao Biotech, China). Protein content was determined using a standard BCA protein assay kit (Dingguo, Beijing, China); 50 μ g of protein was fractionated by 12% SDS-PAGE and electroblotted to nitrocellulose membranes. After blocking with 5% nonfat milk, the membranes were incubated with anti-HSL (1:1000; 17333-1-AP; Proteintech; USA) and anti-GAPDH (1:5000; 10494-1-AP; Proteintech; USA) primary antibodies at 4 °C and secondary antibodies (1:10000; SA00001-2; Proteintech; USA) for 1 h at room temperature. Blots were created using the enhanced chemiluminescence reagent (Beyotime; China), and ImageJ software was used to assess band intensities (NIH, Bethesda; USA).

Transfection with small interfering RNAs (siRNA)

atRA-treated group cells were cultured in 6-well plates (1×10^4 cells/well) in DMEM containing 10% FBS. According to the manufacturer's instructions, the MEPM cells were transfected with a final concentration of 70 pmol HSL siRNA (Huzhou HippoBio Co., Ltd.; China) using Lipofectamine RNAiMAX Reagent (Invitrogen; USA) for 48 h. A negative siRNA (Huzhou HippoBio Co., Ltd.; China) was used as the control group. These cells were collected to confirm whether the genes were silenced by the designed siRNA. The effectiveness of the target gene knockdown was determined using qRT-PCR and WB.

Cell counting kit-8 (CCK8) assay

atRA-treated group cells (5×10^3 cells/well) were cultured in a 96-well plate (Corning; USA) in DMEM containing 10% FBS at 37 °C. Following 24, 48, and 72 h, incubation with 10 μ l CCK8 solution (Beyotime Biotechnology; China) at 37 °C for an additional 2 hours was added to each well. The absorbance at 450 nm was measured using a microplate reader.

Wound healing assays

atRA-treated group cells (5×10^5 cells/well) were collected and seeded into 12-well plates. After transfection for 48 h, cells were starved for 12 h. The DMEM was replaced with medium containing 2.5% FBS. A line-shaped scratch was made by passing a pipette tip after the cells merged into the monolayer cells. The width between the borders of the scratches was photographed in each group at 0, 12 and 24 h using an inverted microscope connected to a digital camera (Olympus Corporation; Japan).

Statistical analysis

Data for each parameter were obtained in triplicate. All data were evaluated and analyzed for significance by SPSS version 22.0. Comparisons between groups were performed using one-way analysis of variance (ANOVA) or Student's t test. $P < 0.05$ was deemed a statistically significant difference.

RESULTS

Histology of embryonic palate shelves

Embryonic palate shelf tissue was collected from pregnant mice. The palate shelf tissue and histological sections of the control group showed that the palatal shelf fused and formed a complete palate. The midline epithelial seam (MES) had disappeared in the mid and anterior region at E15.5. The atRA-treated group showed that the palatal shelf was separated without fusion, and the palatal processes on both sides were short. A wide cleft phenotype was present in the atRA group (Fig.1).

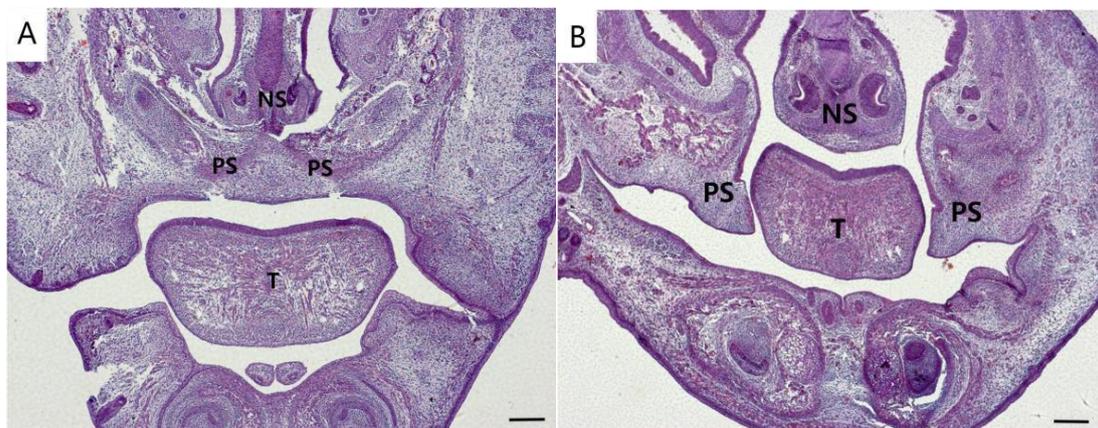


Figure 1. Hematoxylin and eosin (H&E) staining of palate shelf tissues at E15.5 between control vs atRA-treated mice

(A) The palatal shelf had been fused and eventually formed a complete palate. The midline epithelial seam (MES) disappeared in the midanterior region; (B) Unfused, separated palatal shelf from an embryo of an atRA-treated mouse, the palatal processes on both sides were short. A wide cleft phenotype was eventually presented. (A, B) H&E staining images (scale bar, 400 μm). PS, palatal shelf; T, tongue; NS, nasal septum; H&E, hematoxylin and eosin.

HSL significantly increases the MEPM of cleft palate

At E15.5, the palate shelves had fused in the control group (Fig.2A). In contrast, the palatal shelves appeared abnormally small and failed to undergo elevation and fusion, finally developing into cleft palate in the atRA-treated group at E15.5 (Fig.2B). There were more HSL-positive cells in the atRA group than in the control group (Fig.2C, Fig.2D). The expression of HSL protein was upregulated in the MEPM cells in the atRA treatment group compared with the control group (Fig.2E; $**P<0.01$). The qRT-PCR results indicated that the mRNA level of HSL was upregulated at E15.5 in the atRA group compared with the control group (Fig.3A; $**P<0.01$). The western blot results showed that the protein levels of HSL were significantly increased in the atRA-exposed MEPM cells compared with the control MEPM cells at E15.5 (Fig.3B, 3C; $**P<0.01$).

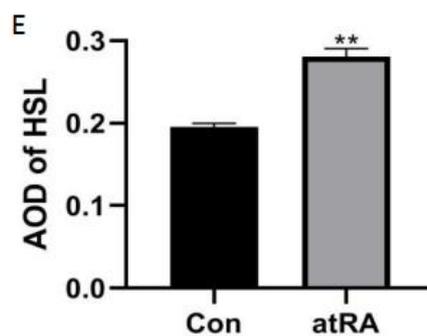
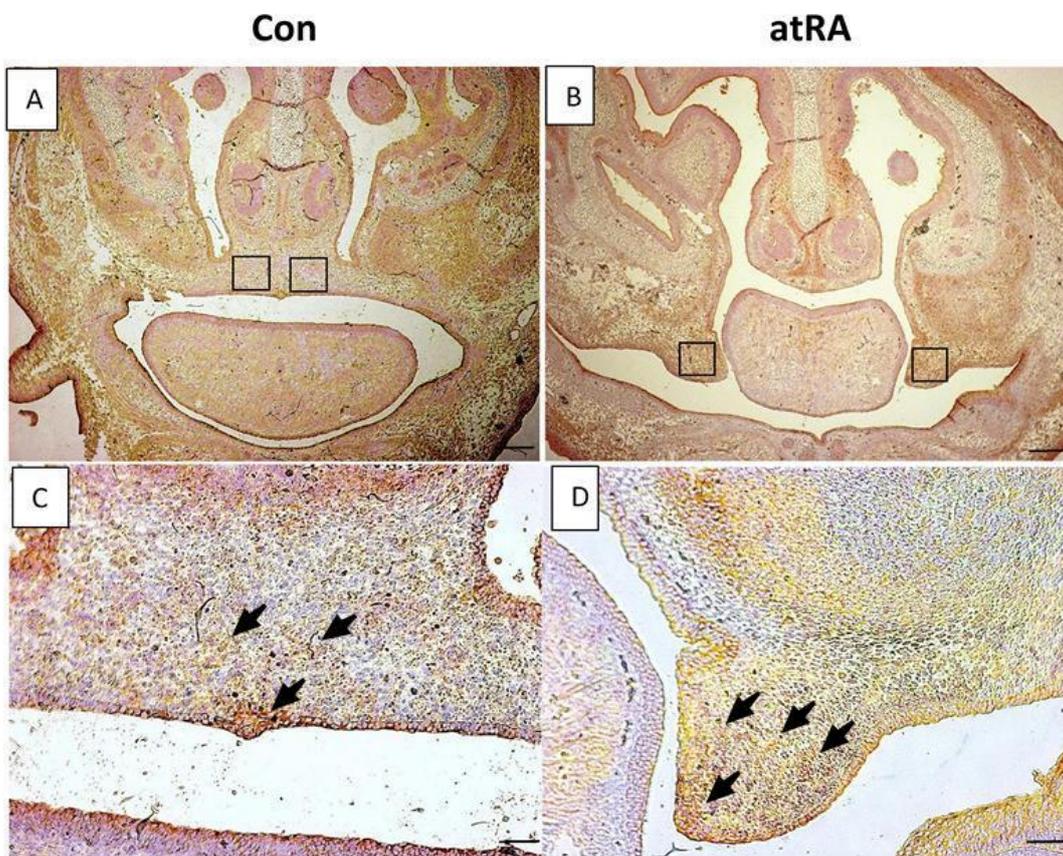


Figure 2. Immunohistochemistry assay demonstrating HSL expression in the EPM cells

HSL was significantly increased at E15.5 in the MEPM cells in the atRA group when compared with the Con group. (A, B) Immunohistochemistry results (scale bar, 400 μm). (C, D) Immunohistochemistry results (scale bar, 100 μm). (C, arrowhead) In the control group, HSL-positive cells were found in the palatal fusion area, (D, arrowhead) In the atRA group, the surface of the unilateral palatal process epithelium showed obvious yellowish-brown, and scattered brown yellow areas were observed in the palatal process mesenchyme. (E) HSL AOD quantification. $**P<0.01$. HSL, hormone-sensitive lipase; AOD, average optical density; MEPM, mouse embryonic palatal mesenchyme; E, embryonic day; atRA, all-trans retinoic acid; Con, control.

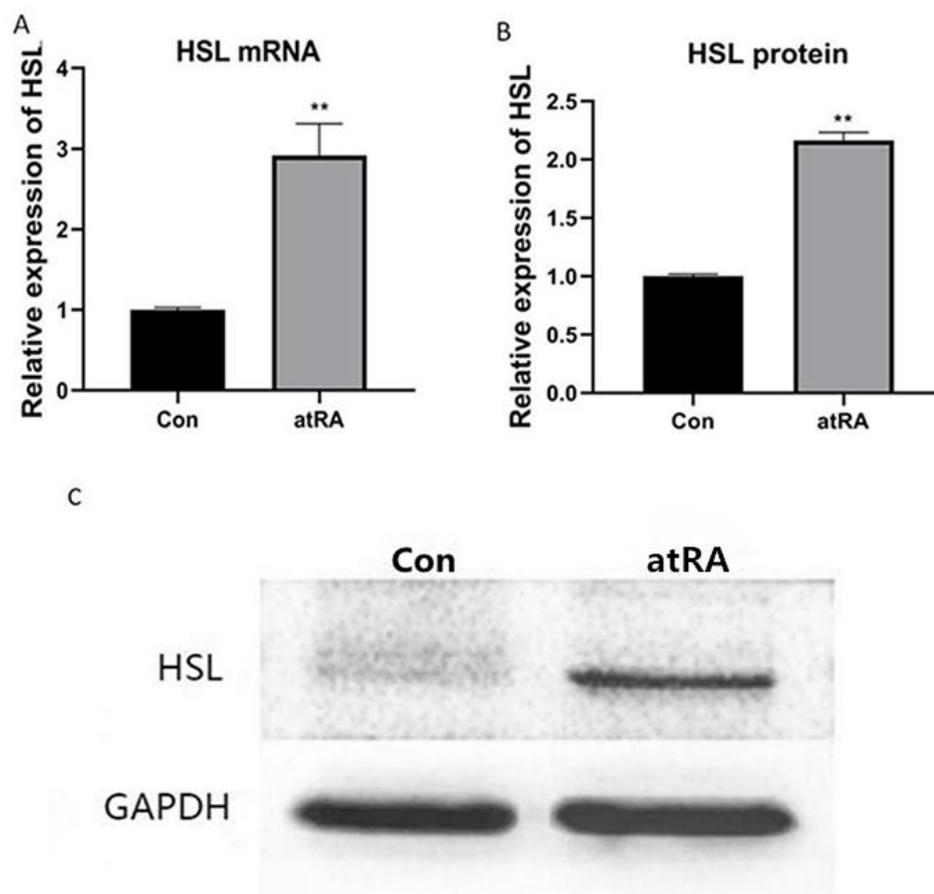


Figure 3. The expression of HSL was upregulated in the atRA group

(A) When compared with the Con group, mRNA expression of HSL was increased in the atRA group at E15.5. (B, C) Protein expression of HSL was higher in the atRA group compared with the Con group. $**P<0.01$. HSL, hormone-sensitive lipase; MEPM, mouse embryonic palatal mesenchyme; atRA, all-trans retinoic acid; E, embryonic day; Con, control.

Silencing of the HSL gene by RNAi

To verify whether the HSL gene was silenced by the designed siRNA in the atRA group, we determined the effects of the two chemosynthetic siRNAs (#2 and #3) in MEPM cells after transient transfection. The two siRNAs against HSL reduced mRNA and protein expression, and cells transfected with siRNA#3 showed the most significantly reduced mRNA and protein levels of HSL (Fig.4). Therefore, siRNAs #2 and #3 were selected to study the function of HSL.

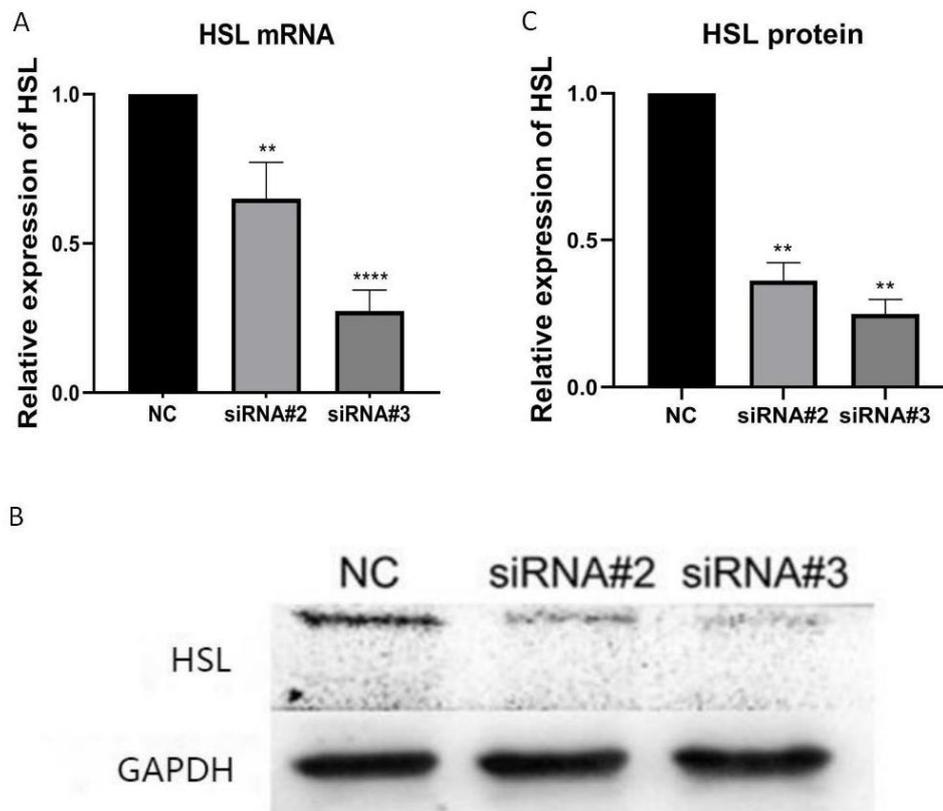


Figure 4. RNAi knockdown of HSL in MEPM cells

(A) qRT-PCR was used to detect the effect of HSL RNAi on the mRNA expression of HSL. (B) The protein level of HSL in MEPM cells was detected by western blot. (C) Quantitative densitometry evaluation of HSL was shown. ** $P < 0.01$, **** $P < 0.0001$ versus the negative control groups. HSL, hormone-sensitive lipase; MEPM, mouse embryonic palatal mesenchyme; atRA, all-trans retinoic acid; NC, negative control.

Promotion of cell proliferation by HSL siRNA

In the atRA group, the CCK-8 assay was used to assess the effect of HSL siRNA on the proliferation of MEPM cells cultured in 96-well plates. After 48 h of transfection, the HSL siRNA-transfected cells showed an increase in proliferation compared to the negative siRNA-transfected cells (Fig.5).

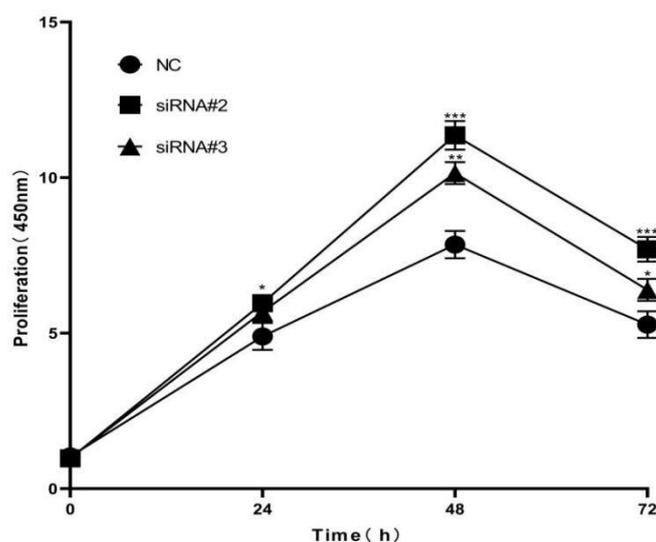


Figure 5. The downregulation of HSL expression promoted the proliferation ability of MEPM cells

MEPM cells transfected with negative control and HSL siRNAs were used for the CCK8 assay. Cell viability in HSL siRNA-transfected cells was significantly increased compared with corresponding controls. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the control groups. HSL, hormone-sensitive lipase; MEPM, mouse embryonic palatal mesenchyme; atRA, all-trans retinoic acid; NC, negative control.

Promotion of cell migration by HSL siRNA

In the atRA group, we studied the influence of HSL knockdown on MEPM cell migration capacity by the wound scratch assay. After 48 h of transfection, the migration of cells transfected with HSL siRNA was significantly increased compared with that of the negative siRNA-transfected cells (Fig.6).

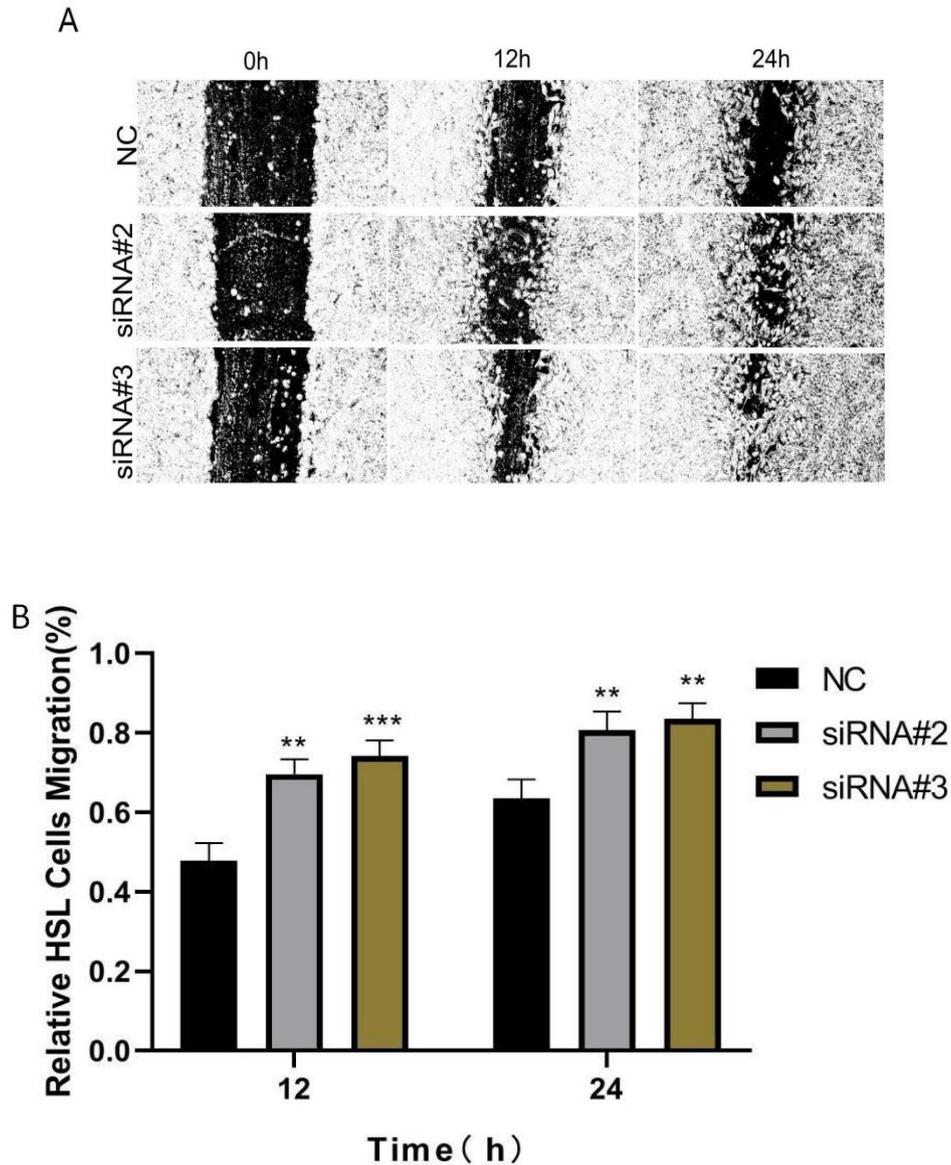


Figure 6. Knockdown of HSL promoted MEPM cells migration as determined by the scratch wound assay

Representative photomicrographs of MEPM cells migration were taken, and the relative distances of migration were measured by inverted fluorescence microscope. (A) The typical photomicrographs of MEPM cells' migration. (B) The results showed that the relative mobility of cells in siRNA group was significantly higher than that in the negative control group. Data were expressed as mean \pm SD from three separate experiments. ** $P < 0.01$, *** $P < 0.001$ versus the negative control. Original magnification 100 \times . HSL, hormone-sensitive lipase; MEPM, mouse embryonic palatal mesenchyme; atRA, all-trans retinoic acid; NC, negative control.

DISCUSSION

Cleft lip and palate is the most prevalent orofacial birth defect, occurring in approximately 1:700 live human births worldwide each year (Salari et al., 2022). Therefore, it is of significant clinical importance to explore the pathogenesis of cleft lip and palate. Since morphological events in mice closely resemble those seen in humans, mouse genetic models with palatal defects have been valuable for analyzing the genes that regulate palatogenesis and for determining the genetic causes of human cleft palate (Oliver et al., 2021).

atRA plays an essential role in regulating morphogenesis, cell differentiation and proliferation during embryonic development (Clagett-Dame et al., 2002). Many animal models and cytotoxicity tests have found that atRA is widely used as a teratogen to determine the pathogenesis of cleft palate (Han et al., 2006; H. Zhang et al., 2017). Cleft palate occurred in mice and humans when exposed to both deficiency and overdoses of atRA (Ackermans et al., 2011; Wilcox et al., 2007). Previous studies have confirmed that embryos exposed to atRA (70 mg/kg) at E10.0 show a higher incidence of cleft palate, the palatal shelves of which become growth retarded and fail to contact one another (Abbott et al., 1990; Shu et al., 2019). In this study, we observed that the palatal shelves were abnormally small and dysplastic, and the results indicated that palatal shelf growth was permanently impeded in atRA-exposed embryos on gestation Day 15.5.

HSL is a key enzyme in the regulation of lipid metabolism and is highly expressed in adipose tissue (Pajed et al., 2021). Previous studies confirmed that a defect in lipolysis was detected in the cleft palate of rats (Stefanovich et al., 1971), and modulation of lipid metabolic defects rescued cleft palate in gene mutant mice (Iwata et al., 2014). These results indicated that lipid metabolism plays a significant role in normal physiological function and development, particularly in the maxillofacial region and palate. During embryonic development, mesenchymal cell proliferation is the main process in shelf outgrowth, and cleft palate occurs when this process is disrupted (H. Zhang et al., 2017). However, whether HSL is present in MEPM cells and the mechanism of HSL in cleft palate remain unclear. The present study showed that HSL was strongly expressed at E15.5 in cleft palate MEPM cells induced by atRA, while it was downregulated in the normal embryonic palate. These observations indicated that HSL is involved in the development of MEPM cells. The increase in HSL may result in alterations in lipolysis activities and other lipid metabolic pathways, causing reduced mesenchymal cell proliferation.

MEPM cell migration and proliferation play significant roles in the formation of facial structures (He et al., 2008). Many studies have investigated whether atRA inhibits MEPM cell proliferation (Liu et al., 2016; Liu et al., 2021; Yoshioka et al., 2021; Y. Zhang et al., 2017). However, the molecular mechanism by which HSL affects cell

migration and proliferation needs to be fully clarified. In the present study, the CCK-8 assay results showed that the knockdown of HSL by siRNA promoted MEPM cell proliferation in the atRA group. Furthermore, HSL siRNA significantly promoted the migration of MEPM cells in the atRA group. We provide evidence for the first time that HSL contributes to altering MEPM cell migration and proliferation. Our data suggest that the promotion of cell proliferation and migration induced by HSL siRNA may be related to the development of the palatal shelf in mice.

In summary, this study confirmed that HSL is involved in cleft palate induced by atRA, and the expression of HSL was upregulated in the atRA group. The downregulation of HSL using RNAi promotes the proliferation and migration of MEPM cells in the atRA group. Therefore, key lipolytic enzymes may affect the development of the palatal shelves, as atRA enhanced HSL levels to inhibit embryonic palate growth. Our study had several limitations. First, we investigated the function of HSL in cleft palate at E15.5; thus, it is not known whether our results are applicable to other stages of palatogenesis. Furthermore, we examined a simple gene in a very complex signaling pathway. Further studies should be applied to verify the relationship between HSL and lipid metabolism-related pathways in MEPM cell migration and proliferation and to determine whether the increase in cellular HSL levels may contribute to alterations of lipid metabolic activity in the palatal mesenchyme. Our findings provide new insights into the underlying mechanism of cleft palate formation and may help identify new candidate markers for prenatal screening for cleft palate and new targets for its diagnosis and treatment.

COMPETING INTEREST

There are no conflicts of interest.

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