Development and gene expression of C57BL/6 mouse embryo palate shelves in rotary organ culture

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Abstract. The aim of the present study was to improve methods for the suspension culture of mouse palatal shelves by comparing the expression of platelet-derived growth factor receptor (PDGFR)-α in palatal shelves *in vivo*, to that *in vitro*. The palatal shelves of C57BL/6 mouse embryos were obtained on gestation days (GDs) 13.5, 14.5, 15.0 and 15.5 for *in vivo* experiments. The palatal shelves were removed and observed under a stereomicroscope to investigate palatal development. For *in vitro* experiments, the palatal shelves were dissected under a stereomicroscope on GD 13.5 and then subjected to rotary culture for 0, 24, 36 or 48 h. The expression of PDGFR-α at different time points was detected by immunohistochemical staining and western blot analysis. Both methods of analysis displayed PDGFR-α expression in mesenchymal and epithelial cells at GD 13.5, 14.5, 15.0 and 15.5, *in vivo* and *in vitro*. The level of PDGFR-α expression peaked on GD 14.5. The expression of PDGFR-α in palatal shelves in *in vitro* rotary culture was consistent with that *in vivo*. Therefore, the novel technique of palatal rotary organ culture presented in the current study could provide a good model for studying the mechanism of pathological palatal fusion *in vitro*. Additionally, the present study further confirmed that PDGFR-α gene expression was associated with the development of palatal shelves.

Introduction

Non-syndromic cleft lip or palate (NSCL/P) is the most common congenital birth defect, with an incidence of 1-2% (1). The interaction between environmental risks and genetic factors is widely accepted as the molecular pathological basis of congenital cleft palate (2). Palate fusion is regulated by a variety of cytokines, signaling molecules and signaling pathways, including transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), Wnt and sonic hedgehog (Shh) signaling pathways (3-6). Previous studies have demonstrated that knocking out the *Tgfb3* gene can inhibit palatal plate fusion in normal mice (7). In the palatal process epithelium, Wnt/β-catenin signaling can control palatal fusion through *Tgfb3*. It serves a bidirectional regulatory role with TGF-β in regulating cell proliferation, differentiation and apoptosis (7,8). In the early stages of embryonic development, exogenous teratogenic agents can affect the normal palate development by altering the expression of a number of signaling factors, including Gli3, FGF, Tbx1 and TGF-β. If the palatal process is not raised during development or the bilateral palatal process cannot be contacted, it will lead to cleft palate on a cellular level (9-12). Retinoic acid (RA) is a derivative of vitamin A and is considered to be a common teratogenic factor (13,14). The function of retinoic acid is mediated through binding to its corresponding membrane receptor, transporting retinoic acid binding protein into the nucleus through the cytoplasm, where it regulates of gene transcription. It has been previous demonstrated that Gli3, FGF, TBX1 and Hoxa2 expression are altered by retinoic acid (15-17) which results in palatal mesenchymal cell apoptosis, resulting in cleft palate.

Gene expression is spatiotemporal during palatal formation (18). Illustrating the temporal and spatial specificity of gene expression during palatal formation, may help to establish the pathological mechanism of cleft palate. Epidemiological studies have previously revealed that platelet-derived growth factor receptor (PDGFR)-α is closely related to growth and development, and its absence may lead to a series of congenital malformations, including NSCL/P (19,20). The PDGFR-α signaling pathway is one of a number of signaling pathways involved in the regulation of craniofacial development, and PDGFR-α plays an important role in human embryonic development, as well as normal physiological activities (21-23). The lack of PDGFR-α expression in neural crest cells can lead to obstruction of the palate and the nasal septum in mouse models, resulting in facial bone structure and cartilage abnormalities (24). The platelet-derived growth factor (PDGF) family consists of two receptor genes, PDGFR-α and PDGFR-β. PDGFR-α is involved in cellular responses, including migration, proliferation and division (24). Mice

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with disrupted PDGF-α signaling display cheek fissures, bone deformities and cleft palate when they are born, whilst those that do not survive typically die by day 10-15 during embryonic development (25).

During normal embryonic palatal development, exposure to drugs or environmental chemicals may result in alterations in palatal fusion that can cause cleft palate (26). There are several *in vitro* models that can be used to assess the pathogenesis of cleft palate, including mouse embryonic primary mesenchyme (MEPM) cell culture (27,28), organ and tissue cultures of palatal shelves (29-32) and whole mouse embryo cultures (33,34). Each of these three models has their own advantages and disadvantages, which should be carefully considered when deciding which model to use to investigate specific experimental questions. MEPM cell culture is commonly used to investigate how MEPM cells proliferate and differentiate. Palatal shelves are composed of MEPM and medial edge (MEE) cells. Organ cultures of palatal shelves allow the study of the interactions between MEPM and MEE cells during palatal fusion (28,35). The whole mouse embryo culture allows the study of the mechanisms underlying palatal development (36,37).

The present study reported a novel method of cultivating palate cells from gestation day (GD) 13.5 mouse fetuses in DMEM/F12 medium containing 10% fetal bovine serum. The palates maintained in rotary organ culture displayed substantial growth and fused similarly *in vitro* as *in vivo* mouse fetuses. Furthermore, the expression of PDGFR-α was assessed and compared between *in vitro* and *in vivo* palatal shelves to further confirm the reliability of the rotary organ culture method.

Materials and methods

Rotary organ culture of palatal shelves. In the present study, electric rotary devices were built based on the methods described by Shiota *et al* (38). The organ culture instrument used was designed based on the principle of the bacterial culture instrument (Labstar 50; Shandong SCENKER Biotechnology Co., Ltd.) used in the clinical laboratory of the Affiliated hospital of Qingdao University (Shandong, China). The rotary table had an inner diameter of 94 mm and culture dishes 3 cm in diameter were placed onto the rotating table for the experiment. The rotary table was able to accommodate a maximum of three dishes at any one time, where the speed of rotation was adjusted to 20-25 rpm (Fig. 1).

In vivo and in vitro development of the C57BL/6J mouse fetus palate. A total of 20 male and 32 female mice were used for the present study (license no. HYXK20180116; Hua Fu Kang Experimental Animal Center). The animals were housed at 22˚C, 70% humidity, with a 12-h light/dark cycle and were provided with pellet food and tap water *ad libitum*. Specific pathogen-free C57BL/6J male mice aged 7-8 weeks (weight, 22-25 g) and female mice aged 6-7 weeks (weight, 17-20 g) were mated. The present study was approved by the Qing Dao University Institutional Animal Care and Use Committee (reference no. AHQU-MAL2018079). The mice were subsequently housed at a male:female ratio of 1:2. The observation of vaginal plugs the following morning was considered day 0 of the embryo (GD 0.5). A total of 26 pregnant mice were euthanized on GD 13.5, and 2 were euthanized on each of GD 14.5, 15.0 and 15.5 by cervical dislocation. Since they were utilized solely for breeding purposes, none of the 20 male mice were harmed for the duration of the present study and were given to researchers from other research groups. Under sterile conditions, palatal explants were dissected under a dissection microscope (magnification, x2) using micro-scissors. The jaw and tongue body were resected from the horizontal left and right corners of the mouth, leaving the bilateral palatal shelf exposed. The head was removed above the eyes by horizontal incision. Briefly, a total of 195 palatal explants were obtained. The procedure used for organ culture was performed as previously described by Shiota *et al* (38). For *in vivo* experiments, 55 palatal explants at four different embryonic stages (GD 13.5, 14.5, 15.0 and 15.5) were collected. A total of six palatal explants from each embryonic stage were fixed in 4% paraformaldehyde overnight at 4˚C for immunohistochemistry (IHC). The remaining 31 palatal explants were used for tissue protein extraction and western blot analysis. On GD 13.5, the 140 palatal explant samples for *in vitro* experimentation were removed and 6 palatal explant samples were placed culture dishes 3 cm in diameter. These palatal explant samples were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 1% penicillin-streptomycin solution (Hyclone; GE Healthcare Life Sciences) and placed in culture dishes on a rotary table (25 rpm) in an incubator at 37˚C with an atmosphere of 95% O_2 and 5% CO_2.

Palatal shelves were cultured *in vitro* for 0, 24, 36 or 48 h, similar to the four different embryonic stages (GD 13.5, 14.5, 15.0 and 15.5), respectively. After 0, 24, 36 or 48 h, the palatal shelves were removed from culture and visualized under a stereomicroscope (magnification, x10; Leica M50; Leica Microsystems GmbH) to image palatal development.

IHC staining. The fetal palatal shelves were fixed in 4% paraformaldehyde for 48 h at 4˚C. The samples were then embedded in paraffin and were subsequently cut into 3-μm thick sections in the coronal orientation. The tissue sections were then deparaffinized with xylene at room temperature and rehydrated using a descending ethanol series. Antigen retrieval was performed by incubating the sections with citric acid buffer (pH 6.0; Beijing Solarbio Science & Technology Co., Ltd.) for 30 min in a microwave at about ~60˚C for antigen retrieval, followed by non-specific peroxidase blocking using peroxidase blocking solution (cat no. ZLI-9310; ZSGB-BIO; OriGene Technologies, Inc.) for 10 min at room temperature. The slides were then incubated with primary rabbit anti-mouse PDGFR-α monoclonal antibody (1:200; cat. no. ab32570; Abcam) overnight at 4˚C. Following the primary incubation, the sections were washed with PBS and incubated with a secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:600; cat. no. ab205718; Abcam) at 37˚C for 1 h. Immunoreactivity was detected using an Histostain™-SP Kits (cat. no. SPN-9001; ZSGB-BIO; OriGene Technologies, Inc.) and counterstained with hematoxylin for 30 min at room temperature. Images were obtained using an Olympus BX60 fluorescence microscope (magnification x100; Olympus Corporation).

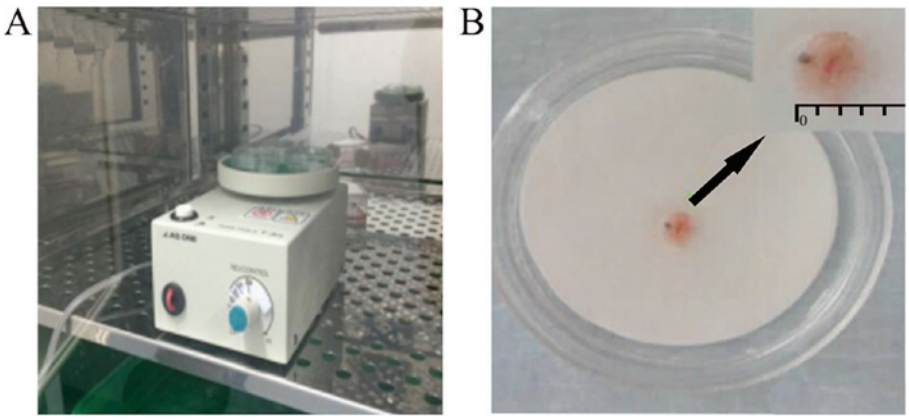

Figure 1. Rotary culture tool. (A) The rotation culture device was placed in a cell incubator. (B) A culture plate, 3 cm in diameter, containing the palate shelves was placed on the rotary culture device table. Scale bar, 1 mm.

Western blotting. Western blot analysis was performed to assess the protein levels of PDGFR-α in palatal explants. Cells from the palatal shelves were harvested at different time points (GD 13.5, 14.5, 15.0 and 15.5 *in vivo* or 0, 24, 36 and 48 h *in vitro*) for protein extraction with mammalian protein extraction reagent (Pierce; Thermo Fisher Scientific, Inc.). The total protein content of the supernatant was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of total protein (~50 μg) were electrophoresed on a 10% SDS-PAGE gel and transferred onto a PVDF membrane (EMD Millipore) in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Inc.) at 15 V for 30 min. The membrane was then blocked for 2 h at room temperature with 5% skimmed milk in Tris-buffered saline containing 0.05% Tween-20. Subsequently, the membrane was incubated 4˚C overnight with the following primary antibodies: Anti-PDGFR-α (1:2,000; cat. no. ab32570; Abcam) and monoclonal anti-GAPDH (1:5,000; cat. no. ab181602; Abcam). Membranes were then incubated with a peroxidase-conjugated secondary antibody (1:5,000; cat. no. ab205718; Abcam) for 1 h at 37˚C. Protein bands were visualized using an ECL1 Detection kit (cat. no. RPN2109; GE Healthcare) according to the manufacturer's instructions. Protein expression was quantified using Gel-Pro Analyzer software (version 3.1; Media Cybernetics, Inc.) with GAPDH as the loading control. The grey level was analyzed using ImageJ v1.8.0 software (National Institutes of Health).

Statistical analysis. Statistical analyses were performed using SPSS software (version 18.0; IBM Corp.). Comparisons were performed using one-way ANOVA followed by Fisher's Least Significant Difference post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of PDGFR-α protein was observed by IHC. At GD 13.5 *in vivo* (Fig. 2A) and after 0 h cultivation *in vitro* (Fig. 3A), PDGFR-α was expressed in the vertical growth of the palatal shelf, primarily in the epithelial region. The palatal mesenchyme was concentrated in the central and lateral regions of the palatal shelf. The expression of PDGFR-α in oral epithelium adjacent to palatal frame was higher compared with that in nasal epithelium. The palatal mesenchyme was concentrated in the central and lateral regions of the palatal shelf (Figs. 2A and 3A). However, little or no PDGFR-α expression was observed in the mesenchyme near the nasal cavity epithelium. Interestingly, no notable expression of PDGFR-α was observed in the apical palatal frame at GD 13.5 *in vivo* (Fig. 2A), but a higher level of expression was found in the apical part after 0 h cultivation *in vitro* compared with at GD 13.5 *in vivo* (Figs. 2A and 3A).

It was observed that PDGFR-α was partially expressed on the surface of palatal epithelium on GD 13.5 (Fig. 3A). With the elevation of palatal frame, PDGFR-α was expressed in the palatal process, oral and nasal palatal epithelial cells on GD 14.5 *in vivo* (Fig. 2B) and after 24 h cultivation *in vitro* (Fig. 3B). The expression of PDGFR-α in palatal mesenchymal tissue on GD 14.5 *in vivo* (Fig. 2B) was more pronounced compared with that *in vitro* (Fig. 3B).

At GD 15.0 *in vivo* (Fig. 2C) and *in vitro* for 36 h (Fig. 3C), a marked reduction in the expression of PDGFR-α was observed compared with GD14.5 *in vivo* (Fig. 2B) or *in vitro* for 24 h (Fig. 3B), respectively. The expression of PDGFR-α in palatal frame epithelium was limited to the middle palatal crest epithelium and its adjacent palatal nasal epithelium. In the mesenchymal tissue, the expression of PDGFR-α was mainly concentrated near the epithelium on both sides of the palatal crest, but the expression level was low in the mesenchyme near the oral epithelium near the palatal frame (Figs. 2C and 3C). It was observed that the expression of PDGFR-α in the triangular region composed of the middle palatal crest epithelium and adjacent nasal palatal epithelium but not in the oral epithelium near the palatal frame (Fig. 2C).

The expression of PDGFR-α in the epithelium and mesenchyme of the palatal shelf was lower at GD 15.5 *in vivo* (Fig. 2D) and after 48 h culture *in vitro* (Fig. 3D) compared with those on GD15.0 (Figs. 2C and 3C). The region of PDGFR-α expression in the mesenchyme was primarily concentrated in the middle of the palatal shelf and expression was weak. There was some PDGFR-α expression in the nasal epithelium adjacent to the palatal shelf, but no expression was observed in the oral epithelium (Fig. 2D).

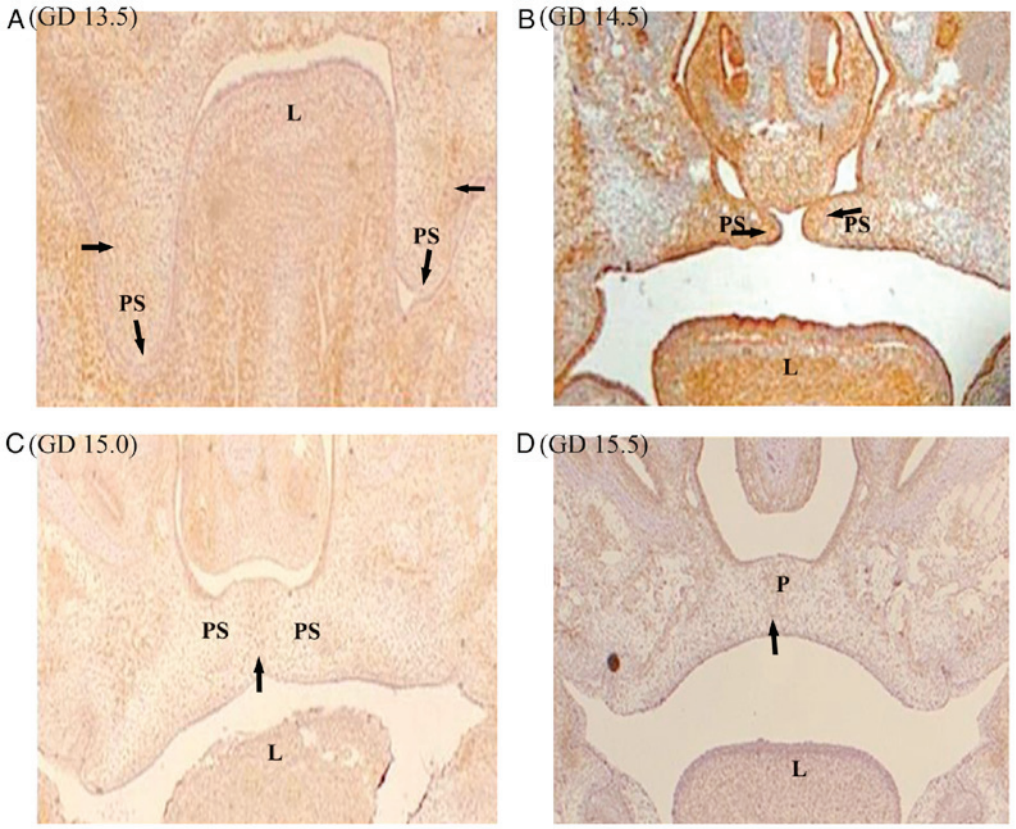

Figure 2. Immunohistochemical staining of platelet derived growth factor receptor-α in the palatal shelves of a mouse embryo at different stages of development *in vivo*, at magnification x40. (A) GD 13.5, (B) GD 14.5, (C) GD 15.0 and (D) GD 15.5. Arrows indicate sites of PDGFR-α expression. GD, gestation day; PS, palatal shelf; L, lingual; P, palate.

The expression of PDGFR-α displayed a continuous and dynamic trend across the different stages of development of the mouse embryonic palate. At 0 h of *in vitro* culture (GD 13.5 *in vivo*), PDGFR-α was primarily expressed in the palatal shelf (Fig. 3A). After 24 h cultivation *in vitro* (GD 14.5 *in vivo*), PDGFR-α gradually covered the oral and nasal epithelium and mesenchymal cells of the palate (Fig. 3B). Starting with GD15.0, the expression of PDGFR-α decreased gradually in the palatal frame, and only in the middle palatal crest epithelium or adjacent nasal epithelium (Fig. 3C). With the fusion of palate in GD15.5, the epithelium of middle palatal crest disappeared and the expression of PDGFR-α was reduced in the palate (Fig. 3D).

Expression of PDGFR-α protein as observed by western blotting. The expression of PDGFR-α protein was analyzed *in vivo* and *in vitro* at different stages of palatal bone development by western blot analysis (Fig. 4). The expression profiles of PDGFR-α following *in vivo* and *in vitro* cultivation exhibited comparable patterns, with peaks observed following 24 h culture *in vitro* and GD 14.5 *in vivo*. The results of western blot and immunohistochemistry exhibited consistent trends.

Observation of in vitro cultured palatal shelves. On GD13.5, the nasal floor structure could be observed in the oral cavity (Fig. 5A). Stereomicroscopy results revealed that after 24 h culture *in vitro*, the bilateral palatal frames gradually gathered in the middle, where the distance was markedly shortened compared with that observed in GD13.5 (Fig. 5A and B). After 36 h culture (Fig. 5C), all 36 pairs of palatal shelves were found to have made contact. Histologically, 22 (61.1%) displayed mesenchymal fusion and 14 (38.9%) displayed epithelial fusion without mesenchymal confluence. After 48 h culture, contact between the palatal shelves was observed in all 36 pairs of palatal shelves. Histologically, 36 (100%) displayed mesenchymal confluence and disappearance of the midline epithelial seam (Fig. 5D).

Discussion

Palate development in mammals is a complex process that involves the vertical formation, rotation and horizontal proximity of the palatal shelf; the formation and degeneration of epithelial ridges in the median palatine process; the adhesion of epithelial cells and the fusion of mesenchymal cells (9,39). A number of previous studies have reported that genetic and environmental interactions contribute to the development of congenital cleft lip and palate (18,40).

PDGFR-α is a cell surface receptor tyrosine kinase for PDGFs (23). The human PDGFR-α genes are located on chromosome 4q12 (41,42). Under physiological conditions, PDGF interacts with its corresponding receptor PDGFR-α to form and activate the ligand-receptor complex. PDGFR-α signaling activation can activate related genes, including *Tbx1*, *SATB2* and *Gli3* (15-17) and a number of signaling pathways, including WNT, TGF, FGF and SHH, resulting in a variety of physiological activities within the cells (23,43-45).

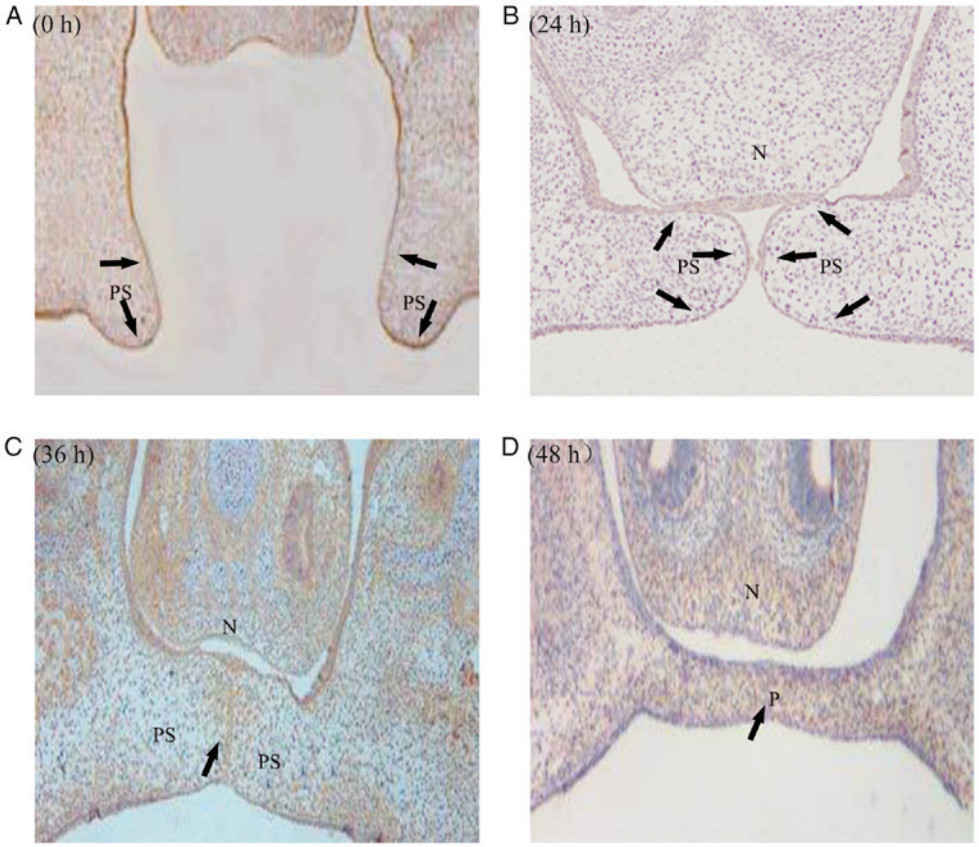

Figure 3. Immunohistochemical staining of platelet derived growth factor receptor-α at different stages of embryonic palate shelf culture *in vitro*, at magnification, x40. (A) 0 h, (B) 24 h, (C) 36 h and (D) 48 h. Arrows indicate sites of PDGFR-α expression. PS, palatal shelf; N, nose; P, palate.

Disruption of PDGFR-α in zebra fish by microRNA 140 was found to cause craniofacial abnormalities, including cleft palate (23,46). Previous studies in PDGFR-α knockout mice have provided evidence supporting a significant role for PDGFR-α in palatal development (47-49). Qian *et al* (50) demonstrated that the correct timing and level of PDGFR-α expression are crucial for embryonic development. Furthermore, it was reported that deletion of PDGFR-α caused developmental defects of multiple endoderm- and mesoderm-derived structures, resulting in a complex phenotype including orofacial cleft, spina bifida, rib deformities and omphalocele in mice (51,52). Results from studies utilizing animal models are not the only evidence that indicates that PDGFR-α plays an important role in cleft palate formation. In a previous study, 102 patients with NSCL/P were examined by DNA genome and PCR sequencing analysis, revealing seven mutations in PDGFR-α in nine patients, totaling to an incidence of 8.8%. An incidence of 8.8% is significantly higher than the mutation rate of the general population (1%; $P<0.01$) (19,20). Therefore, the results obtained in the present study further suggested that PDGFR-α plays an important role in human embryonic palatal fusion.

PDGFR-α is an important regulator of embryonic palatal development (23). However, to the best of our knowledge, there have been no reports detailing the spatiotemporal expression pattern of PDGFR-α in the development of embryonic palatal shelves. In the present study, PDGFR-α was detected in mouse palatal shelves at GD 13.5, 14.5, 15.0 and 15.5, and the expression of PDGFR-α was highest at GD 14.5. These results indicated that PDGFR-α displays a dynamic expression pattern in the development of palatal shelves. The bilateral palatal process began to fuse to form the middle palatal suture, where PDGFR-α expression also began to decrease on GD15.0 and was mainly concentrated in the middle palatal crest epithelium. On GD15.5, complete fusion was observed in the specimens and PDGFR-α expression was largely diminished. The expression patterns of PDGFR-α in the palatal shelves were similar *in vitro* and *in vivo*, suggesting that PDGFR-α serves a role in palatal fusion.

Current methods of cultivating palatal shelves *in vitro* include metal fence culture (51), Trowell culture (32,53) and other stationary culture methods (54,55). In 1990, the palatal suspension culture method was established by Shiota *et al* (38). In this method, the palatal shelves were cultured in a rotating culture device, which displayed a number of advantages over the traditional stationary culture method. Firstly, the method of scissoring the palatal shelves was different. Palatal explants were dissected under a dissection microscope using a microscopic shear and a horizontal incision was made through the oral opening. The upper part of the head was resected by making a second incision parallel to the first incision, at the level of the eyeballs. Palatal explants included palatal shelves and part of the maxillary protrusion. The brain tissue, tongue and lower jaw were removed with microscope forceps, following which the palatal shelves were placed in a horizontal position in a culture dish (46,56). This method described by Shiota *et al* (38) is easier to implement than other methods, largely preserves the integrity of the palatal shelves and ensures the survival rate of the palatal shelves *in vitro* (57).

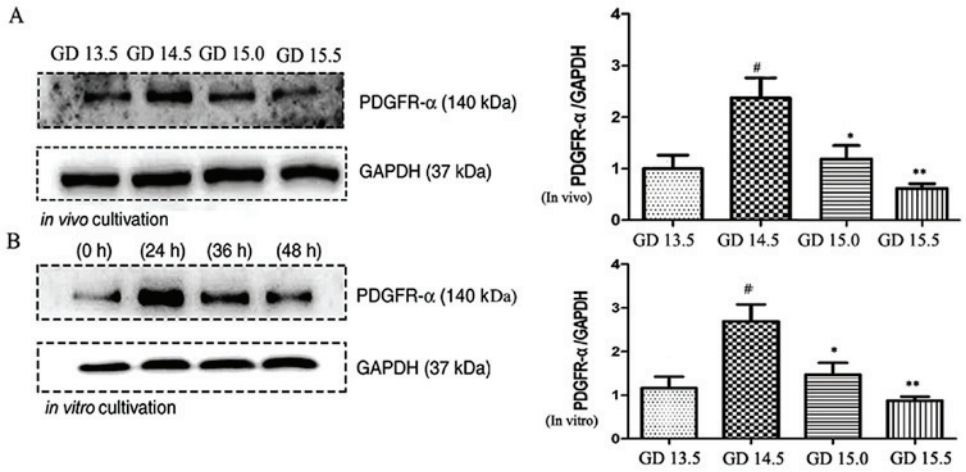

Figure 4. Western blot analysis of total PDGFR-α expression in palatal shelf tissues following *in vivo* and in *vitro* culture. (A) Western blot analysis of PDGFR-α expression during different stages of embryonic palate shelf development *in vivo*. (B) Western blot analysis of platelet derived growth factor-α expression during different stages of embryonic palate culture *in vitro*. Values are shown as the mean ± standard from two independent experiments. $^{\#}$P<0.05 vs. GD13.5; *P<0.05 vs. GD14.5 and **P<0.05 vs. GD15.0. GD, gestation day; PDGFR-α, platelet derived growth factor-α.

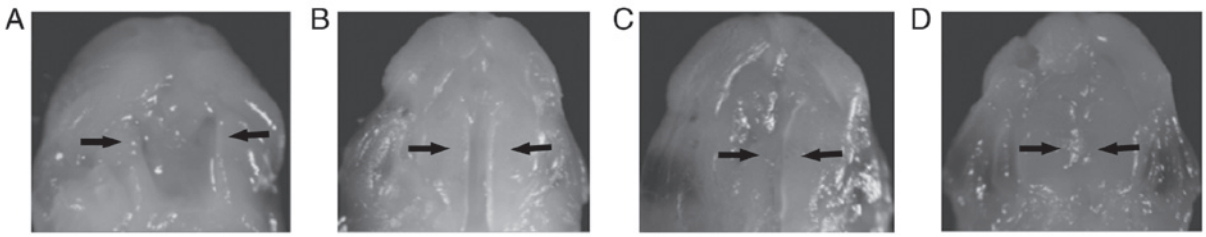

Figure 5. Results of microscopic observation during different stages of embryonic palate shelf development during *in vitro* culture, (magnification x10). The arrows show the palatal process on both sides. (A) Bilateral palatal processes are distributed on both sides of the maxilla at 0 h culture *in vitro*. (B) After 24 h culture in vitro, the bilateral palatal process grew, gathered in the middle compared with 0 h culture, where the distance between bilateral palatal process was reduced. (C) After 36 h culture, the bilateral palatal process continued to grow, and the distance between the two ends was gradually shortened to a suture. (D) After 48 h culture, the bilateral palatal process was completely fused and the space disappeared.

The rotary culture device rotates at a speed of 20-25 rpm, rotating the palatal shelves with the filter membrane in a petri dish at the same rate as the converted rotary culture device. Finally, palatal explants, petri dishes and the rotary culture devices are placed in a cell incubator for culture. The power line is drawn out through the edge of the cell incubator door. The edge of the incubator door has rubber strips, ensuring that the incubator is airtight when the power line passes through (51,58). The palatal explants remain almost suspended due to changing gravity vectors and no single gravity factor was found to influence any dominant direction of growth. This maintains the differentiation and migration of palatal shelf cells, which establishes the three-dimensional culture model *in vitro* of palate shelves in the laboratory (50,58). In addition, the growth, elevation, contact and fusion of palatal shelves can be observed *in vitro* using this method. In the present study, the morphological development and fusion process of palatal shelves in rotary culture were similar to that observed *in vivo*. The spatiotemporal expression of PDGFR-α also confirmed the feasibility of the rotary culture method used in the present study, The level of PDGFR-α protein expression after 24, 36 and 48 h cultivation *in vitro* were comparable with that after GD 14.5, 15.0 and 15.5 *in vivo*.

The method used in the present study was modified from the suspension organ culture originally described by Shiota *et al* (38). Firstly, palatal explants were placed in petri dishes rather than in culture bottles. Secondly, palatal explants and rotary culture devices were cultured in cell incubators instead of bottles, consisted of oxygen, nitrogen and carbon dioxide. The palatal fusion rate of the method used in the current study was 100%, which appeared to completely simulate *in vivo* palatal development, suggesting that the method used in the current study may be easier to implement than the method originally described by Shiota *et al* (38).

The experimental method used in the current study reduced the number of steps required to complete the experiment, but also provided a practical *in vitro* animal model for studying normal palate development, the pathogenesis of cleft palate and screening of teratogenic agents. Furthermore, the present study suggested that PDGFR-α participates in palatal development.

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Availability of data and materials

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

Authors' contributions

WLX contributed to the conception of the study. WLX and GY contributed significantly to analysis and manuscript preparation. GY and NZ carried performed the experiment and designed the figures. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All protocols were approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (IACUC Approval No. QDU 93-016; Shandong, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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