

Wnt6 influences the viability of mouse embryonic palatal mesenchymal cells via the β -catenin pathway

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Abstract. The embryological stages of palatal shelf elongation and elevation, mainly induced by the proliferation and extracellular matrix secretion of embryonic palatal mesenchymal (MEPM) cells, are essential for normal palatal development. Wingless-related MMTV integration site gene family (Wnt) signaling pathways serve key roles in craniofacial development and palate formation. Recent studies have indicated that Wnt6 participates in embryonic development of the palate, though its exact role in palate development remains unclear. In the present study, to investigate the role of Wnt6 during the stages of palatal shelves elongation and elevation, mouse MEPM cells were cultured from dissected palatal shelves at embryonic day 13.5. Results of an MTT assay and flow cytometric analysis demonstrated that treatment with recombinant Wnt6 increased the viability of MEPM cells ($P < 0.01$) and the proportion of cells in the S and G2/M phases ($P < 0.01$). Meanwhile, Wnt6 activated the β -catenin signaling pathway as indicated by the dual luciferase assay result, and blockade of the WNT/ β -catenin pathway reduced the cytoactivity of Wnt6 in MEPM cells ($P < 0.01$). Collectively, these findings indicate that Wnt6 promotes the vitality of MEPM cells by increasing the S + G2/M-phase cell population, potentially through activation of the β -catenin pathway during palatal shelf elongation and elevation.

Introduction

Cleft palate is among the most common congenital birth defects in humans (1-3), and is caused by a failure in secondary palate formation (4,5). From embryonic days (E) 12.5 to E15.5, the secondary palate is initiated to extend from the maxillary processes to form the palatal shelves (6). At approximately

E13.5, the bilateral palatal shelves elongate and elevate to form the midline edge seam (7), while the size of the palatal shelves is determined by the proliferation and apoptosis of mouse embryonic palatal mesenchymal (MEPM) cells (8). During this period, disturbances in the normal viability or extracellular matrix secretion of MEPM cells can cause cleft palate (9). These two processes are controlled by complex signaling cascades that are induced by a number of growth factors, including members of the wingless-related MMTV integration site gene family (Wnt) (8,10,11).

The Wnt signaling glycoproteins serve critical roles in cell viability, differentiation, migration, polarity, and death, and thereby regulate embryonic development (12-18). On the basis of their downstream signal transduction, Wnts have been divided into ' β -catenin-dependent' and ' β -catenin-independent' signaling proteins. β -catenin-dependent signaling is defined by the stabilization of β -catenin, which enters the nucleus and forms a complex with lymphoid-enhancer-binding factor/T cell factor (TCF) DNA-binding proteins to activate transcription of Wnt target genes (12-16). Meanwhile, β -catenin-independent signaling performed without β -catenin includes a number of less well characterized intracellular pathways (18-20). In previous studies, the Wnt signaling pathways have been suggested to serve important roles in craniofacial development (21,22) and palate formation (8,21,23-25).

Wnt family member 6 (Wnt6), as a member of the Wnt signaling family, has been reported to be essential for the viability of macrophages (26), myoblasts (27) and stromal cells (28), and to be expressed in mouse embryonic palatal tissue from E12.5 to E14.5 (10). Furthermore, the *WNT6-WNT10A* cluster has been identified to exhibit linkage and disequilibrium in cleft palate (29). These previous data indicate that Wnt6 participates in embryonic development of the palate. However, the exact role of Wnt6 in palate development remains unclear.

The purpose of the present study was to investigate the effect of Wnt6 in MEPM cells using the MTT assay, flow cytometry, western blot analysis and reporter gene assay. The results suggest that Wnt6 may regulate the viability of palatal mesenchymal cells through the β -catenin pathway.

Materials and methods

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health

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(Bethesda, MD, USA) (6). The protocol was approved by the Committee for the Ethics of Animal Experiments of Xiamen University, Xiamen, China (permit no. SCXK2013-0001). All surgical procedures were performed under urethane (Thermo Fisher Scientific, Inc., Waltham, MA, USA) anesthesia (1.0 g/kg via intraperitoneal injection), and all efforts were made to minimize suffering.

MEPM cell culture. A total of 60 female and 20 male wild-type CD1 mice (Charles River Laboratories, Inc., Wilmington, MA, USA) of the same strain were housed at an ambient temperature of 22°C with 12-h light/dark cycle and had access to food and water *ad libitum*. Mature male and female mice (6 weeks old, weighing 17–23 g) were mated overnight, and the presence of a vaginal plug the following morning was designated as E0.5. Primary MEPM cell cultures were established from secondary palatal tissue microdissected from E13.5 mouse embryos (11). The dissected palate shelves were treated with 1 U/ml dispase II (Roche Diagnostics, Indianapolis, IN, USA) at 37°C for 15 min (8,30). Following removal of the desquamated epithelia, the tissue was digested with 0.25% trypsin/0.05% EDTA, and then the dissociated cells were cultured for 48 h in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were passaged and seeded at 4 × 10⁵ cells/25 cm² in DMEM/F12 without FBS and incubated at 37°C for 40 min; in such conditions, the epithelial cells adhered more slowly than the MEPM cells. After culturing for 40 min at 37°C, the medium without FBS was replaced with fresh DMEM/F12 including FBS and antibiotics to acquire MEPM cells of high purity. Cells at passage 3 cultured at 37°C for 8 h were used for subsequent assays. Experimental cell groups were established, in which cells were always incubated with additional 100 ng/ml recombinant Wnt6 (Abgent, Inc., San Diego, CA, USA) with or without additional 100 ng/ml recombinant dickkopf WNT signaling pathway inhibitor 1 (DKK1; R&D Systems, Inc., Minneapolis, MN, USA) in DMEM/F12 containing 10% FBS and 1% penicillin/streptomycin at 37°C. In the control cell group, MEPM cells were only cultured with 10% FBS and 1% penicillin/streptomycin in DMEM/F12.

MTT assay. The MEPM cells were transferred to 24-well plates (Corning Incorporated, Corning, NY, USA) at a density of 1.6 × 10⁴ cells/well and cultured at 37°C in DMEM/F12 with 10% FBS for 1–4 days. MTT solution (5 mg/ml) was added to each well and the plates were incubated at 37°C for 4 h. Subsequently, the supernatant was removed and dimethyl sulfoxide 0.5 mg/ml was added to each well to dissolve the formazan crystals. The optical density at 490 nm was quantified using the PowerWave XS2 package at daily intervals over the 4-day assay period (BioTek Instruments, Inc., Winooski, VT, USA). Three independent replicates were performed for each cell group.

Flow cytometry. Following treatment with or without Wnt6 or Wnt6 + DKK1 for 48 h as stated above, the MEPM cells were harvested and fixed in cold 75% ethanol at -4°C overnight,

cells were pelleted and washed twice in 1x PBS at 1,000 × g for 5 min at 4°C. Subsequently, cells were incubated with 50 µg/ml propidium iodide and 5 µg/ml RNAase at room temperature for 30 min. Following supplementation with 50 mg/l propidium iodide and incubation in the dark for 1 h at room temperature, cell cycle analysis was performed using a BD FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and CellQuest Pro 5.1 software (BD Biosciences) with a minimum of 20,000 events for each cell group.

Reporter gene assay. After 48 h of treatment with or without 100 ng/ml recombinant Wnt6 as stated above, the MEPM cells were seeded at 1.6 × 10⁵ cells/well onto 12-well plates, and transient transfections for 5 min at room temperature were performed using Lipofectamine 2,000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were transfected with 1.6 µg of TCF luciferase reporter constructs (Promega Corporation, Madison, WI, USA) (TOPflash or FOPflash, respectively) and 0.08 µg of *Renilla reniformis* gene vectors (Promega Corporation) for normalization. The TOPflash TCF reporter plasmid contains two sets of three copies of the binding site upstream of the thymidine kinase minimal promoter and luciferase open reading frame, while FOPflash contains mutated TCF binding sites and was used as a negative control (31–34). A subset of the cells lacking Wnt6 treatment were also cotransfected with β-catenin pcDNA (1.6 µg; Biocytogen LLC, Beijing, China) as a positive control. Following 48 h of incubation, the luciferase assay was performed using a Dual Luciferase Assay System kit (Promega Corporation) according to the manufacturer's protocol. Relative luciferase activity was reported as the ratio of firefly/*Renilla* luciferase activity.

Western blot analysis. Total protein was isolated from cultured MEPM cells after 48 h of treatment with or without Wnt6 ± DKK1 using RIPA lysis and extraction buffer (Beyotime Institute of Biotechnology, Haimen, China). Protein quantification was performed with a Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The intracellular protein expression levels of β-catenin and β-actin and the Wnt6 protein levels in the cell culture supernatant were assessed. To obtain Wnt6 protein from the supernatant, conditioned culture media were collected and concentrated with Amicon® Ultra-4 Centrifugal Filter Units (10,000 NMWL; EMD Millipore, Billerica, MA, USA), then extracted with a Protein Extraction kit II (Applygen Technologies, Inc., Beijing, China), as described previously (22). Equal amounts of protein (60 µg per lane) were separated on 10% SDS-polyacrylamide gels and transferred onto polyvinylidenedifluoride membranes (Roche Diagnostics). The membrane was blocked in a 6% non-fat milk solution in Tris-buffered saline with 0.5% Tween-20 (TBST) (Roche Diagnostics) at room temperature for 1 h, and then incubated with rabbit anti-Wnt6 monoclonal antibody (Abcam, Cambridge, UK; cat. no. ab154144; dilution 1:200), rabbit anti-β-catenin monoclonal antibody (Abcam; cat. no. ab32572; dilution 1:500) or rabbit anti-β-actin monoclonal antibody (Wuhan Antgene Biotechnology Co., Ltd, Wuhan, China; cat. no. ANT009; dilution 1:800) overnight at 4°C. After rinsing with TBST for three times, the goat

anti-rabbit HRP-conjugated secondary antibody (Wuhan Antgene Biotechnology Co., Ltd., cat. no. ANT020; dilution 1:5,000) was applied to the membranes at room temperature for 1 h. The blot was visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) and the protein bands were analyzed with ImageJ 1.48 software (National Institutes of Health).

Statistical analysis. All quantitative data were presented as the mean $\pm$ standard deviation. Statistical analysis of differences was performed with SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA) $P < 0.05$ was considered to indicate statistical significance. The significance of data was determined by one-way analysis of variance followed by a Bonferroni post-hoc test.

Results

Wnt6 promotes the viability of MEPM cells. The expression of Wnt6 was identified in primary (control) MEPM cells at E13.5 (Fig. 1). To investigate the effect of Wnt6 on MEPM cell viability, recombinant Wnt6 was added to the culture medium, and cell viability was assessed with an MTT assay. It was observed that the viability of MEPM cells treated with Wnt6 was significantly higher than that of control cells between 2 and 4 days after treatment ($P < 0.01$; Fig. 2).

In addition, the results of flow cytometric analysis indicated that the proportion of primary MEPM cells in S + G2/M phases ($29.91 \pm 0.72\%$) was significantly increased by treatment with Wnt6 ($50.93 \pm 0.89\%$; $P < 0.01$; Fig. 3). These findings suggested that Wnt6 promoted the viability of MEPM cells and increased the population of S + G2/M-phase cells.

Wnt6 activates the β -catenin/TCF signaling pathway. A TOP flash luciferase reporter assay was performed to investigate whether Wnt6 activates Wnt/ β -catenin signaling in MEPM cells. Cells treated with Wnt6 or pcDNA- β -catenin exhibited significantly higher baselines of β -catenin/TCF-mediated transcriptional activity when compared with the control cells ($P < 0.01$). In addition, the TOPflash reporter activity did not differ significantly between the Wnt6 and β -catenin groups (TOPflash data not shown) (Fig. 4). Furthermore, the total amount of β -catenin was markedly increased in the Wnt6-treated cells when compared with the control cells (Fig. 1).

Blockade of the WNT/ β -catenin pathway reduces the cytotoxicity of Wnt6. DKK1 binds to low density lipoprotein receptor-related protein 5/6 and is considered to be a general canonical Wnt signaling inhibitor (35). In the present study, DKK1 was used to confirm the effect of Wnt6/ β -catenin signaling on MEPM cell viability. Cells treated with Wnt6 were incubated with or without DKK1. As depicted in Fig. 1, the additional treatment with DKK1 suppressed the expression of β -catenin. In addition, the results in Fig. 2 demonstrated that DKK1 significantly reduced the Wnt6-mediated increase in cell viability from day 2 post-treatment ($P < 0.01$). Furthermore, when the Wnt6/ β -catenin pathway was blocked, the viability of MEPM cells was significantly lower than that of control cells ($P < 0.01$). A notable decrease in the S + G2/M cell proportion was also observed in the DKK1 + Wnt6 group ($20.43 \pm 0.59\%$;

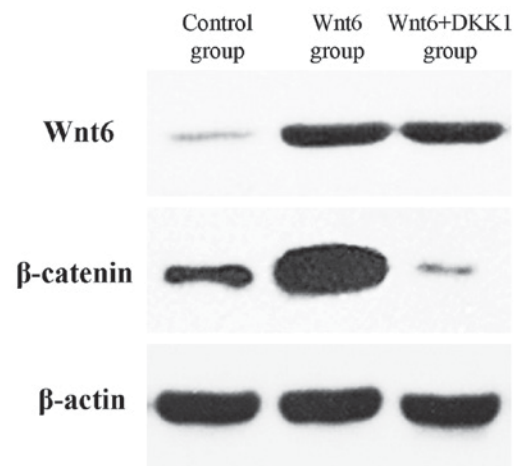


Figure 1. Protein levels of Wnt6 and β -catenin. Following treatment with Wnt6 or Wnt6 + DKK1 for 48 h, the MEPM cells were harvested and western blotting assays were performed. Intracellular β -catenin and β -actin were extracted by cell lysis, and extracellular Wnt6 was extracted from the culture supernatant. Wnt6, wingless-related MMTV integration site gene family member 6; DKK1, dickkopf Wnt signaling pathway inhibitor 1.

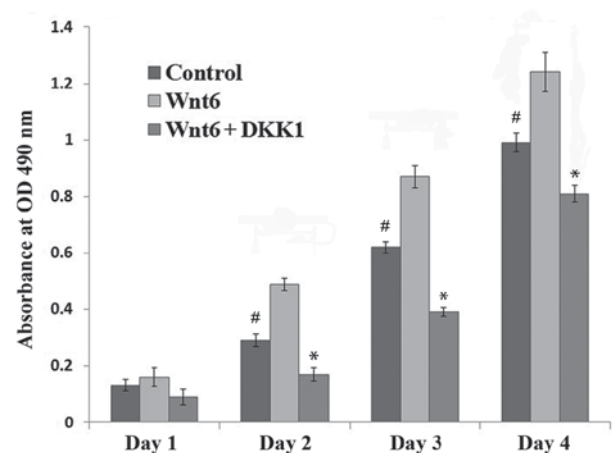


Figure 2. Effect of Wnt6 on the viability of MEPM cells. MEPM cells were treated with or without Wnt6 or Wnt6 + DKK1. The relative absorbance of MEPM cells in an MTT assay was determined at daily intervals over a 4-day assay period. The data were presented as the mean $\pm$ standard deviation ($n = 3$). * $P < 0.01$ vs. Wnt6; # $P < 0.01$ vs. Wnt6 + DKK1 as indicated. Wnt6, wingless-related MMTV integration site gene family member 6; DKK1, dickkopf Wnt signaling pathway inhibitor 1; mouse embryonic palatal mesenchymal.

Fig. 3) when compared with the control and Wnt6 groups ($P < 0.01$), and cell cycle arrest in G0/G1 phase was indicated in the DKK1 + Wnt6 group.

Discussion

Embryonic development of the palate is a temporospatially coordinated process. The secondary palate is initiated to extend from the maxillary processes to form the palatal shelves between E12.5 and E15.5 (6). At approximately E13.5, the bilateral palatal shelves, composed of a core of mesenchyme cells, elongate and elevate toward each other (7). In the present study, to investigate the role of Wnt6 during the stages of palatal shelf elongation and elevation, MEPM cells were cultured from palatal shelves dissected from mouse

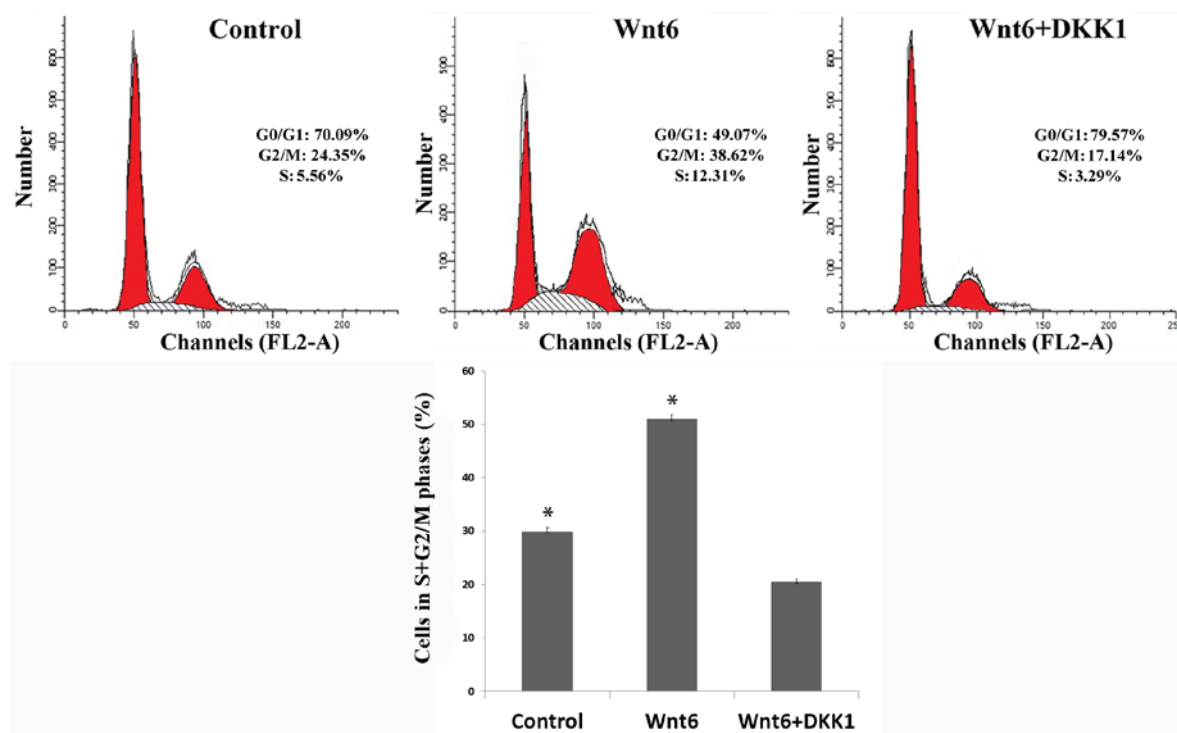


Figure 3. Effect of Wnt6 on the cell cycling of MEPM cells. Flow cytometry analysis of the cell cycle distribution of MEPM cells upon Wnt6 or Wnt6 + DKK1 treatment for 48 h. The data were presented as the mean $\pm$ standard deviation (n=3). *P<0.01 vs. Wnt6 + DKK1. Wnt6, wingless-related MMTV integration site gene family member 6; DKK1, dickkopf Wnt signaling pathway inhibitor 1; mouse embryonic palatal mesenchymal.

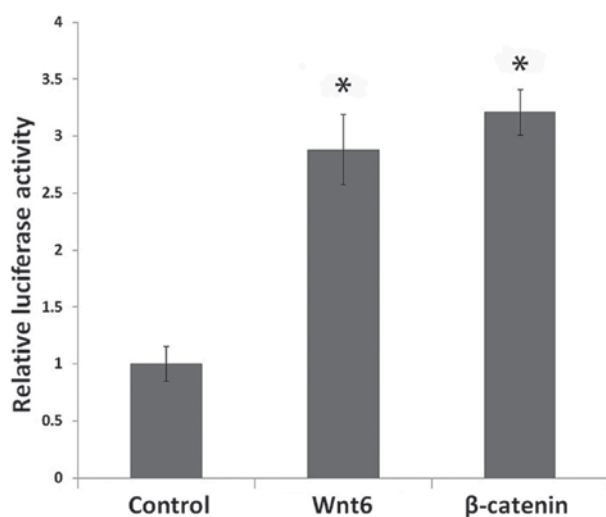


Figure 4. Wnt6 enhances β -catenin transcriptional activity in MEPM cells. Relative luciferase activity following transfection with TOPFlash or FOPFlash reporter plasmid and cotransfection with pcDNA- β -catenin or Wnt6 treatment in MEPM cells. The data were presented as the mean $\pm$ standard deviation (n=3). *P<0.01 vs. control. Wnt6, wingless-related MMTV integration site gene family member 6; mouse embryonic palatal mesenchymal.

fetuses at E13.5. The results of MTT assay and flow cytometry analysis indicated that the viability and S + G2/M population of the MEPM cells was increased by Wnt6. Meanwhile, a dual luciferase assay and analysis with DKK1 identified a pivotal role of β -catenin signaling in the development of MEPM cells. Collectively, the results indicated that Wnt6 promoted the viability of the MEPM cells by increasing the population of

S + G2/M cells. The involvement of Wnt6 in the β -catenin pathway during palatal shelf elongation and elevation was also implicated.

Former studies have already demonstrated that Wnt6 is a necessary cytoactive factor in macrophages (26), myoblasts (27) and stromal cells (28). Wnt6 has also been reported to be expressed in the mouse embryonic palatal tissue from E12.5 to E14.5 (10), and the *WNT6-WNT10A* cluster exhibits linkage and disequilibrium in cleft palate (29). In the present study, the effect of Wnt6 on MEPM cell viability during embryonic palate development was elucidated *in vitro*. Further studies into the effects of Wnt6 knockout on palatogenesis in mice should now be performed to identify potentially associated malformations and regulatory mechanisms.

Numerous different factors are involved in the complex transduction of Wnt signals, though particular cells only respond to specific Wnt factors (36). It has been reported that Wnt6 activates β -catenin signaling in F9 embryonal carcinoma cells and serves an important role in endoderm specification (37,38) and formation (39). Furthermore, Wnt6 restricted heart muscle development in embryonic mesenchymal cells through the β -catenin pathway (40), and the osteoblastogenesis of mesenchymal stem cells was regulated by Wnt6/ β -catenin signaling (41). In the current study, Wnt6 was indicated to stimulate the transcriptional activity of β -catenin, suggesting that it may activate Wnt/ β -catenin signaling to enhance the viability of MEPM cells. Treatment with a recombinant version of the Wnt signaling antagonist DKK1 was also performed to verify the relationship between Wnt6 and β -catenin signaling. The results demonstrated that the increased viability of Wnt6-treated MEPM cells was inhibited by DKK1. This data

confirmed the stimulatory effect of Wnt6 on the viability of MEPM cells. However, as DKK1 is not a specific inhibitor of Wnt6, further studies are required to confirm the exact signal transduction mechanism of the Wnt6/ β -catenin pathway in MEPM cells.

In addition, the present study identified that compromised Wnt6 activity and β -catenin signaling in MEPM cells reduced the cytoactivity of Wnt6 and caused cell cycle arrest in G0/G1 phase when compared with control cells. Thus, conditional inhibition of the β -catenin pathway may disturb the normal cytoactivity of MEPM cells, which may ultimately lead to the development of cleft palate.

It is established that reduced Wnt/ β -catenin signaling is involved in the development of cleft palate (21,23-25). Furthermore, the Wnt/ β -catenin signaling pathway has previously been associated with alterations in the viability and apoptosis of MEPM cells (8). These previous findings and the present data suggest that Wnt/ β -catenin pathway is among the most important signaling cascades for palatal development. However, the exact mechanism regarding the involvement of β -catenin in MEPM cell activity requires further investigation.

In conclusion, to the best of our knowledge, the present results are the first to indicate that Wnt6/ β -catenin signaling is involved in the viability of MEPM cells during the stages of palatal shelf elongation and elevation *in vitro*, thus suggesting that Wnt6/ β -catenin signaling is involved in the process of palatogenesis.

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