



Transcriptome sequencing analysis of the role of β -catenin in F-actin reorganization in embryonic palatal mesenchymal cells

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Background: Palatogenesis is a highly regulated and coordinated developmental process that is coordinated by multiple transcription factors and signaling pathways. Our previous studies identified that defective palatal shelf reorientation due to aberrant mesenchymal β -catenin signaling is associated with Filamentous actin (F-actin) dysregulation. Herein, the underlying mechanism of mesenchymal β -catenin in regulating F-actin cytoskeleton reorganization is further investigated.

Methods: Firstly, β -catenin silenced and overexpressed mouse embryonic palatal mesenchymal (MEPM) cells were established by adenovirus-mediated transduction. Subsequently, we compared transcriptomes of negative control (NC) group, β -catenin knockdown (KD) group, or β -catenin overexpression group respectively using RNA-sequencing (RNA-seq), and differentially expressed genes (DEGs) screened were further identified by quantitative real-time polymerase chain reaction (qRT-PCR). Finally, *in vivo* experiments further verified the expression change of critical molecules.

Results: We discovered 184 and 522 DEGs in the knockdown and overexpression groups compared to the NC group, respectively (adjusted $P < 0.05$; |fold change| > 2.0). Among these, 106 DEGs were altered in both groups. Gene Ontology (GO) enrichment analysis relating to biological functions identified cytokine-cytokine receptor interaction, and positive modulation of cellular migration. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment assessment indicated that these DEGs were chiefly linked by the regulation of signaling receptor activity and chemokine signaling pathways. Quantitative real-time polymerase chain reaction (qRT-PCR) results showed that the similar expression trend of serum amyloid A3 (*Saa3*) and CXC-chemokine ligand 5 (*Cxcl5*) possibly involved in cytoskeletal rearrangement with RNA-seq data. Experiments *in vivo* displayed that no significant expression change of *Saa3* and *Cxcl5* was observed in β -catenin knockout and overexpressed mouse models.

Conclusions: Our study provides an expression landscape of DEGs in β -catenin silenced and overexpressed MEPM cells, which emphasizes the important role of processes such as chemotactic factor and cell migration. Our data gain deeper insight into genes associated with F-actin reorganization that is regulated by β -catenin either directly or by another route, which will contribute to further investigation of the exact mechanism of mesenchymal β -catenin/F-actin in palatal shelf reorientation.

Keywords: Palatogenesis; β -catenin; F-actin reorganization; RNA-sequencing (RNA-seq); palatal mesenchymal cells

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Introduction

Cleft palate is among the most frequently occurring congenital developmental malformations, arising from genetic and environmental risk factors (1,2). In mammals, secondary palatal shelves (PSs) are mainly comprised of mesenchyme originated from the cranial neural crest surrounded by a thin embryonic epithelium (3). Development of the secondary palate is complex and involves multiple steps during mammalian embryogenesis encompassing growth, reorientation, and fusion of PSs, wherein each step is tightly regulated and coordinated by extensive networks of growth factors and transcription factors (4,5). Although numerous genetic studies in mutant mouse models have greatly enhanced our knowledge about the molecular mechanisms associated with cleft palate, the exact molecular mechanism underlying palatogenesis remains elusive (2,4,6-8).

Palatal shelf elevation results from an endogenous factor that remodels mesenchyme, but the source of the factor remains unknown (9-11). A recent study reported that actin cytoskeleton aligned substantially in palatal mesenchymal cells of elevating PSs (9). Furthermore, mouse embryos lacking Sperm Antigen With Calponin Homology And Coiled-Coil Domains 1 Like (*Specc1l*), a type of cytoskeletal protein that interacts with both actin and microtubules, have been found to exhibit delayed shelf elevation (12). Speed and directionality defects have been detected found in *Specc1l* mutant mouse embryonic palatal mesenchymal

(MEPM) cells (13). Thus, delayed palatal shelf elevation is possibly related with defective mesenchymal remodeling due to actomyosin disorganization.

The Wnt signaling pathway evidently has a significant function during embryonic development, including that of bone, hair follicles, and teeth (14-18). Wnt/ β -catenin signaling in the dental epithelium and mesenchyme were displayed to affect odontogenic fate (15,16). Specific activation of Wnt/ β -catenin signaling in oral epithelium gives rise to initiation of tooth, while stabilization of mesenchymal β -catenin signaling inhibits sequential tooth formation (15,16). Furthermore, specific inactivation of low density lipoprotein receptor-related protein 6 (*Lrp6*), a critical receptor of Wnt signaling, results in serious craniofacial developmental defects, including cleft palate (18). β -catenin not only acts as a transducer that regulates canonical Wnt/ β -catenin signaling but also is a crucial component that couples with actin-cytoskeleton (19). Several studies have validated the involvement of β -catenin in palatogenesis (20-22). Previous research focused mainly on the underlying mechanism of epithelial β -catenin in palatogenesis. They revealed that epithelial β -catenin participates in palatal fusion by controlling the expression of transforming growth factor- β 3 (*TGF β 3*) (20). Although early research reported that the cleft palate phenotype occurred when Wnt/ β -catenin signaling was activated in palatal mesenchyme (21,22), further investigation has never been pursued. To clarify the precise mechanism, our team established a β -catenin knockout mouse model. Our previous study demonstrated that mesenchymal-specific omission of β -catenin via *Sox9*CreER transgenic allele resulted in disrupted palatal elevation by affecting actin polymerization (23). Our recent study revealed that elevated palatal mesenchymal β -catenin signaling through specific activation and pharmacological approaches using lithium chloride (LiCl) gave rise to defective PS reorientation by impairing F-actin reorganization (24). The data indicated that mesenchymal β -catenin/F-actin was involved in shelf reorientation. Despite the essential roles of β -catenin and F-actin during palate development, the molecular mechanism of mesenchymal β -catenin during palate development and the interaction between them remain poorly understood.

Here, we investigated the relationship between β -catenin and F-actin using β -catenin silenced and overexpressed MEPM cells using the adenovirus-mediated RNA interference (RNAi) technique. We performed RNA-seq to identify differentially expressed genes (DEGs)

Highlight box

Key findings

- Our study provides an expression landscape of DEGs in β -catenin silenced and overexpressed MEPM cells, which emphasizes the important role of processes such as chemotactic factor and cell migration.

What is known and what is new?

- Our previous studies identified that defective palatal shelf reorientation due to aberrant mesenchymal β -catenin signaling is associated with F-actin dysregulation.
- Our data provide novel insights into downstream target genes involved in cytoskeletal rearrangement regulated by β -catenin.

What is the implication, and what should change now?

- The present study deepens the knowledge of the molecular mechanism of β -catenin/F-actin in shelf reorientation. We will work towards screening and verifying other key genes associated with cytoskeletal rearrangement regulated by β -catenin.

compared to the negative control (NC) group as well as DEGs in both knockdown and overexpression groups. Then, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses identified the biological functions and signaling pathways (especially in relation to actin cytoskeleton) for the DEGs. We also determined the downstream genes related to F-actin rearrangement controlled by β -catenin signaling, and the RNA-seq data was validated by qRT-PCR with individual genes. Finally, β -catenin conditional knockout or overexpression mouse models were utilized to substantiate the above potential molecules. Collectively, these results will broaden the current knowledge about the β -catenin signaling in elevation process and provide possible insight regarding the downstream targets involved in cytoskeletal rearrangement regulated by β -catenin. We present the following article in accordance with the ARRIVE reporting checklist (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-5772/rc>).

Methods

Mouse lines and cell isolation and culture

We procured *Cttnb1*^{F/F}, *Sox9*CreER, and *Cttnb1*^{ex3f} transgenic mice from the Jackson Laboratory. The generation of *Sox9*CreER; *Cttnb1*^{F/F} and *Sox9*CreER; *Cttnb1*^{ex3f} mice followed previously explained protocols (23,24). Midday of the day of mice's vaginal plug detection was marked as Embryonic Day 0.5 (E0.5). The pregnant mice were intraperitoneally injected with tamoxifen (T5648, Sigma, St. Louis, MO, USA) which was corn oil infused at the dose of 0.2 mg/g body weight on E10.5. The desired pregnant females were euthanized and their embryos were obtained. Animal experiments were performed under a project license (No. WCHSIRB-D-2020-426) granted by the Ethics Committee of West China Stomatological Hospital of Sichuan University, in compliance with Chinese guidelines for the care and use of animals. A protocol was prepared before the study without registration.

Primary MEPM cells were acquired from E13.5 mouse PSs as described previously (24). The collected palatal samples were treated with Dispase II (1 U/mL, Sigma) to separate underlying mesenchyme from palatal epithelium. After harvesting the palatal mesenchyme, trypsin was applied to dissociate the cell suspension after repeated pipetting. Cells were seeded on flasks and grown

in Dulbecco's modified Eagle medium (DMEM)/F12 media augmented with 10% fetal bovine serum (FBS). The following analyses were performed, when MEPM cells were cultured to the third passage.

Adenovirus transfection

Following the manufacturer's manual, MEPM cells were propagated in culturing plates at 1×10^5 cells/well density. After reaching a confluence of 60–80%, the required volume of adenoviruses at the multiplicity of infection (MOI) indicated in the pilot experiment were transfected into MEPM cells (Hanbio Biotechnology, Shanghai, China). Cells were randomly grouped into an empty adenovirus group (vector adenovirus transfected cells), a knockdown group (adenovirus silencing β -catenin transfected cells), and an overexpression group (adenovirus overexpressing β -catenin transfected cells). After a 4–8-hour period of transfection, the culture medium carrying adenoviruses was replaced with fresh medium for further incubation. Cells were harvested for subsequent experiments at 48–72 hours after transfection. Inverted fluorescence microscopy (Olympus, Tokyo, Japan) was applied to observe the transfection efficiency.

RNA-sequencing analysis

Total RNA extracted from MEPM cells was submitted for RNA sequencing. Sequencing was performed on the BGISEQ-500 platform (BGI, Shenzhen, China). After low quality reads were removed from the filtered raw sequencing data, clean reads were obtained. Then, with the help of reference gene and genome, these reads were aligned using the Bowtie2 and HISAT toolboxes, respectively. The fragments/kilobase of transcript/million mapped reads (FPKM) method was applied to analyze each gene's expression level. The DEGs between the NC group and knockdown group or overexpression group were screened using NOISeq method. Genes that checked the requirement of fold change ≥ 2 and divergence probability ≥ 0.8 were considered DEGs. After identifying DEGs, GO assessment was performed to reveal the DEGs' biological role in our study. All DEGs were mapped to GO terms in the database (<http://www.geneontology.org/>), and the Bonferroni correction was applied to adjust P values. KEGG pathway analysis was used to understand the underlying pathways of the DEGs. The Q value ≤ 0.05 was selected to be significantly enriched.

qRT-PCR analysis

The total RNA of MEPM cells was eluted with the help of an RNA Extraction Kit (DP419, Tiangen, Beijing, China), then the RNA was quantified and the purity was assessed by NanoDrop Ultra-micro Spectrophotometer (Thermo Fisher, Waltham, MA, USA). For generating complementary DNA (cDNA), reverse transcription with the Primescript RT kit (Takara, Shiga, Japan) was carried out, followed by qPCR via TB Green[®] Premix Ex Taq[™] II kit (Takara) on the LC480 platform (Roche, Basel, Switzerland). Relative expression of genes was determined by normalizing the target genes expression to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The $2^{-\Delta\Delta C_t}$ method was adopted for analyzing the relative changes. Detailed primer sequences that were used in this investigation are provided in the supplemental materials (Table S1).

Western blot assays

Immunoblotting was carried out according to the previous protocol (23). Radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail was utilized for preparing lysate. The buffer comprised 150 mM NaCl, 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris (pH 7.4). Antibodies utilized for this experiment were as follows: the rabbit monoclonal antibodies to β -catenin (1:1,000; ab32572, Abcam, Cambridge, MA, USA), rabbit monoclonal antibodies to GAPDH (1:2,000; ab181603, Abcam). The signals were detected by electrochemiluminescence (ECL) western blotting reagents (epizyme), and bands were captured with the ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA, USA). Band intensity was quantified with Photoshop Histogram Analysis. The experiment was performed in triplicates and for statistical evaluation, Student's *t*-test was applied.

Immunofluorescence staining

Samples were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. For detecting the immunofluorescence intensity, palatal shelves' coronal sections were stained with the primary antibodies against Saa3 (ab231680, Abcam), Cxcl5 (ab248173, Abcam) as previously mentioned (24). Sections were imaged using Olympus IX73 microscopes.

Statistical analysis

The experiments were carried out at least thrice for statistical analyses and demonstrated as mean \pm standard deviation (SD). Quantification of mean fluorescence intensity of proteins between groups was performed. The related protein's fluorescence intensities were normalized against the area of PSs. Green/red single channel fluorescent protein images were analyzed for the fluorescence intensity over the palatal shelf area with Image J (National Institutes of Health, Bethesda, MD, USA). Data analysis was carried out by Student's *t*-test via SPSS 22.0 (IBM Corp., Armonk, NY, USA), where statistical significance represented by the P value <0.05.

Results

Detection of β -catenin silencing and overexpression effect in MEPM cells

After 48–72 hours of transfection, green fluorescence in MEPM cells were clearly observed under a fluorescence microscope, which indicated successfully transfected cells (Figure 1A). The qRT-PCR demonstrated a notably decreased β -catenin messenger RNA (mRNA) expression in β -catenin knockdown mice than in NC mice (P<0.05, Figure 1B). Western blot revealed that, in comparison with the NC group, the protein level of β -catenin was markedly decreased and enhanced by β -catenin knockdown and overexpression correspondingly (P<0.05, Figure 1C). These results indicated successful inhibition and activation of β -catenin in MEPM cells.

Differential expression analysis of genes

Differential expression analysis revealed a notable difference in gene expression between groups. The total number of DEGs discovered was 184 in the NC versus knockdown groups, involving 115 up- and 69 down-regulated genes (Figure 2A). Our results revealed 522 DEGs between the NC and overexpressed group, among which 317 genes were highly expressed and 205 genes had markedly reduced expression (Figure 2A). By comparing the differential genes among them, we found that there were 106 differential genes in common. With further selection and analysis of DEGs, a cluster heat map revealed 41 DEGs between knockdown or overexpressed group and NC group, which

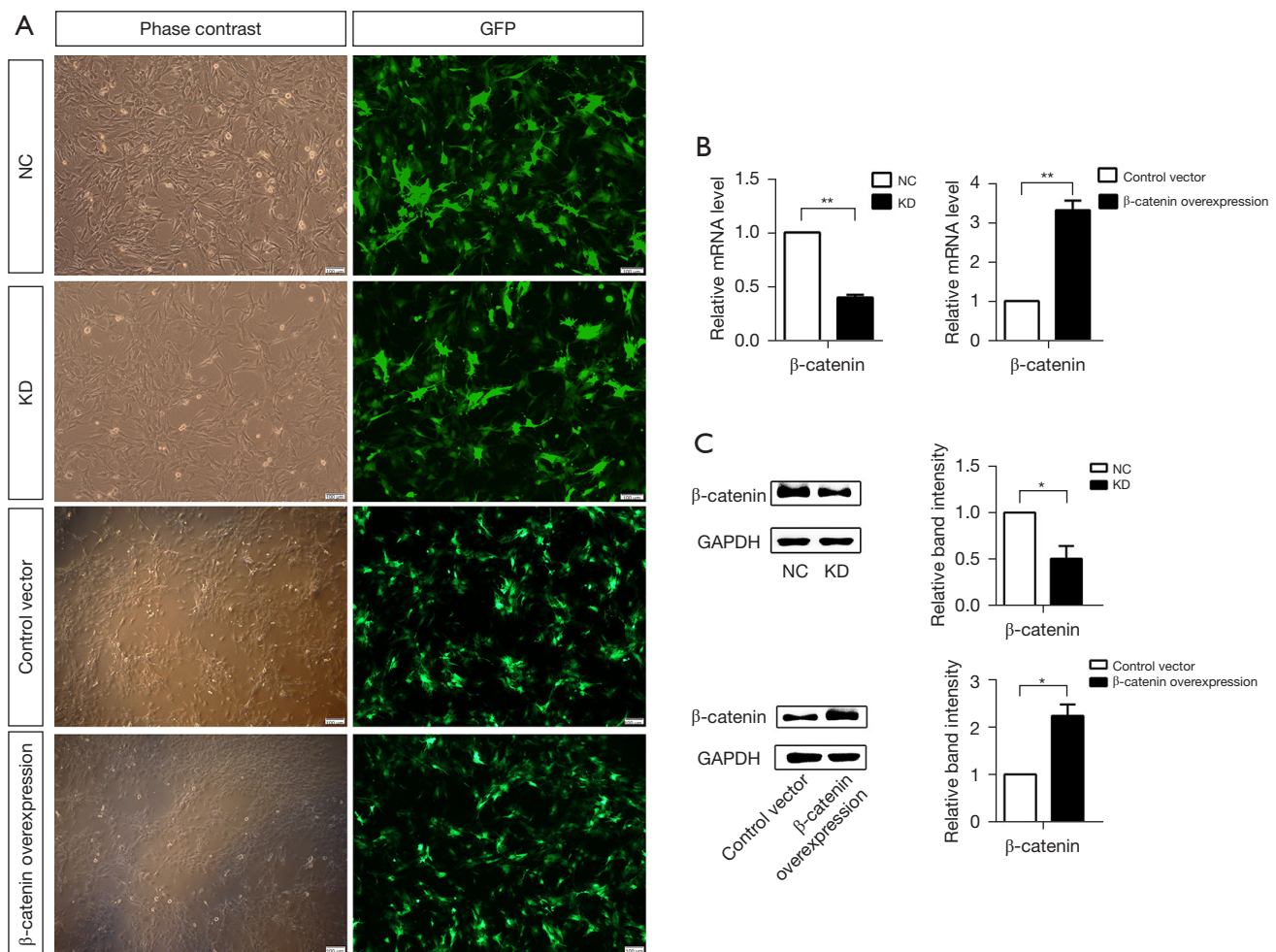


Figure 1 Transfection efficiency detection of β -catenin in MEPM cells. (A) Fluorescence intensity of GFP indicating transfection efficiency after transfection. (B) The relative expression levels of β -catenin after silencing and overexpressing β -catenin in MEPM cells were detected by qRT-PCR. (C) Western blot analysis was performed to determine β -catenin protein levels after knockdown and overexpress of β -catenin in MEPM cells. Data are presented as mean \pm SD. * $P < 0.05$, ** $P < 0.01$. Scale bar, 100 μ m. GFP, green fluorescent protein; NC, negative control; KD, knockdown; MEPM, mouse embryonic palatal mesenchymal; qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation.

might be downstream target genes regulated by β -catenin (Figure 2B). Among them, the following 4 genes: *Saa3*, *Cxcl5*, *Cxcl1*, and *Steap4* are currently the most obvious candidate genes. Moreover, we constructed a protein-protein interaction (PPI) network of the DEGs (Figure S1). It was revealed that *Cxcl1*, *Cxcl5*, Collagen Type I Alpha 1 (*Col1a1*), and Fibroblast Growth Factor 7 (*Fgf7*) were closely associated with β -catenin, which indicated that they had the potential to be downstream targets of β -catenin in MEPM cells. The results above suggested that β -catenin might regulate F-actin reorganization in MEPM cells by

mediating these genes.

GO and KEGG analysis of DEGs

Biological functions including cellular components, biological process, and molecular functions of total DEGs were revealed through GO enrichment analysis. It was revealed that DEGs had significantly enriched functions, including signaling receptor activity and positive cell migration regulation, and ossification (Figure 3A,3B). The probable and crucial signaling pathways linked with

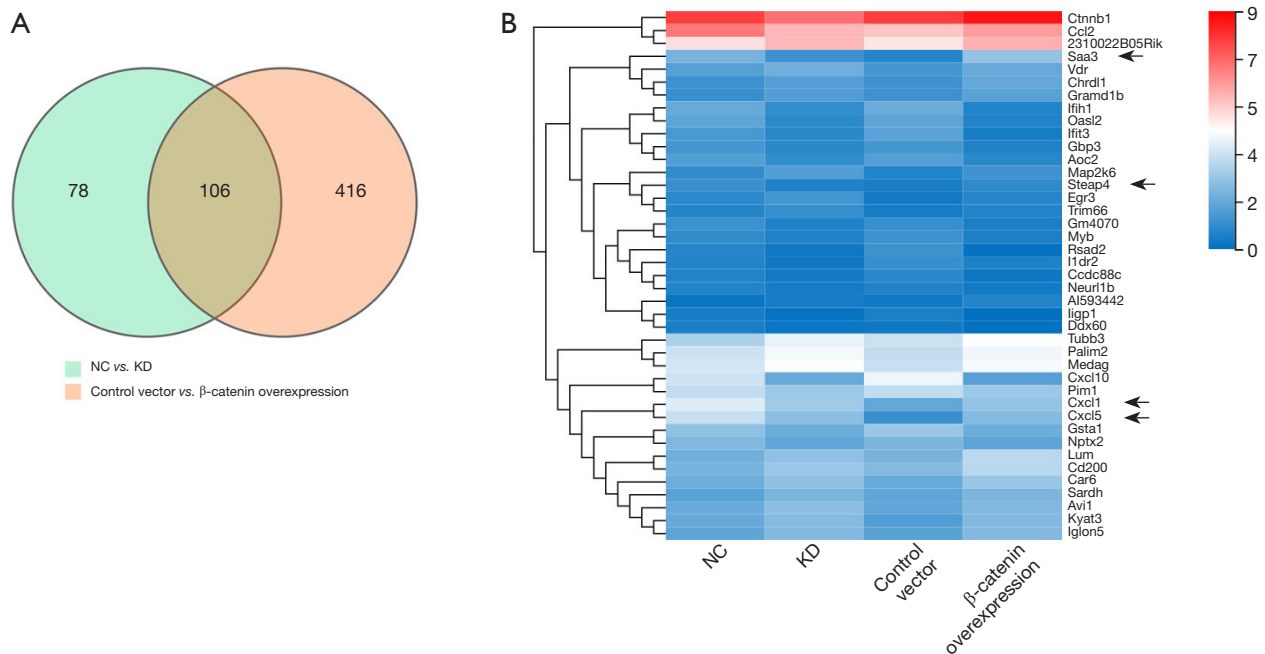


Figure 2 Comparison of significantly DEGs between NC and knockdown or overexpressed groups. (A) The number of DEGs in β -catenin knockdown or overexpressed group compared to NC group. (B) Cluster heat map of DEGs between NC and knockdown or overexpressed groups. Red represents highly expressed DEGs, blue depicts poorly expressed genes. Highlighted with arrows are the four most differentially expressed genes, *Saa3*, *Cxcl5*, *Cxcl1* and *Steap4*. NC, negative control group; KD, β -catenin knockdown group; DEGs, differentially expressed genes.

DEGs were determined with KEGG enrichment analysis. The DEGs were highly enriched in signaling pathways, for instance, cytokine-cytokine receptor interaction, interleukin-17 (IL-17), and chemokine signaling pathways (Figure 3C,3D). The above data suggest involvement of β -catenin in the regulation of cell migration and chemokine of MEPM cells to regulate F-actin reorganization through the above-mentioned genes.

Validation of RNA-Seq results by qRT-PCR

To confirm the sequencing results and the DEGs screened by RNA-seq data analysis, furthermore, the expression level of 2 DEGs (*Saa3* and *Cxcl5*) was assessed using qRT-PCR. The *Saa3* and *Cxcl5* genes might be potentially associated with cytoskeletal rearrangement (21,22). Furthermore, both of these genes showed high expression levels and significantly expressed differences among knockdown and expression groups. The results of qRT-PCR showed that *Saa3* and *Cxcl5* were uniquely decreased in β -catenin silenced MEPM cells compared to the NC group, whereas

Saa3 and *Cxcl5* exhibited significantly upregulated mRNA expression level in β -catenin overexpressed MEPM cells (Figure S2). The qRT-PCR and the RNA-seq data were consistent and had similar expression trend, which indicated that the RNA-seq results were reliable.

Validation of the expression of *Saa3* and *Cxcl5* in the E13.5 *Sox9CreER*; *Ctnnb1*^{F/F} and *Sox9CreER*; *Ctnnb1*^{ex3f} palatal mesenchyme

To further determine the possibility that either *Saa3* or *Cxcl5* is a downstream target of mesenchymal β -catenin to modulate actin cytoskeleton alignment for PS reorientation, the *Sox9CreER*; *Ctnnb1*^{F/F} and *Sox9CreER*; *Ctnnb1*^{ex3f} mouse models we had generated previously were utilized. Unfortunately, different from predictions based on *in vitro* experiments, immunofluorescence staining showed no significant difference in the developing PS mesenchyme between the knockout or overexpressed mouse embryos and control littermates (Figures 4,5). Thus, either *Saa3* or *Cxcl5* is not the target of mesenchymal β -catenin to regulate

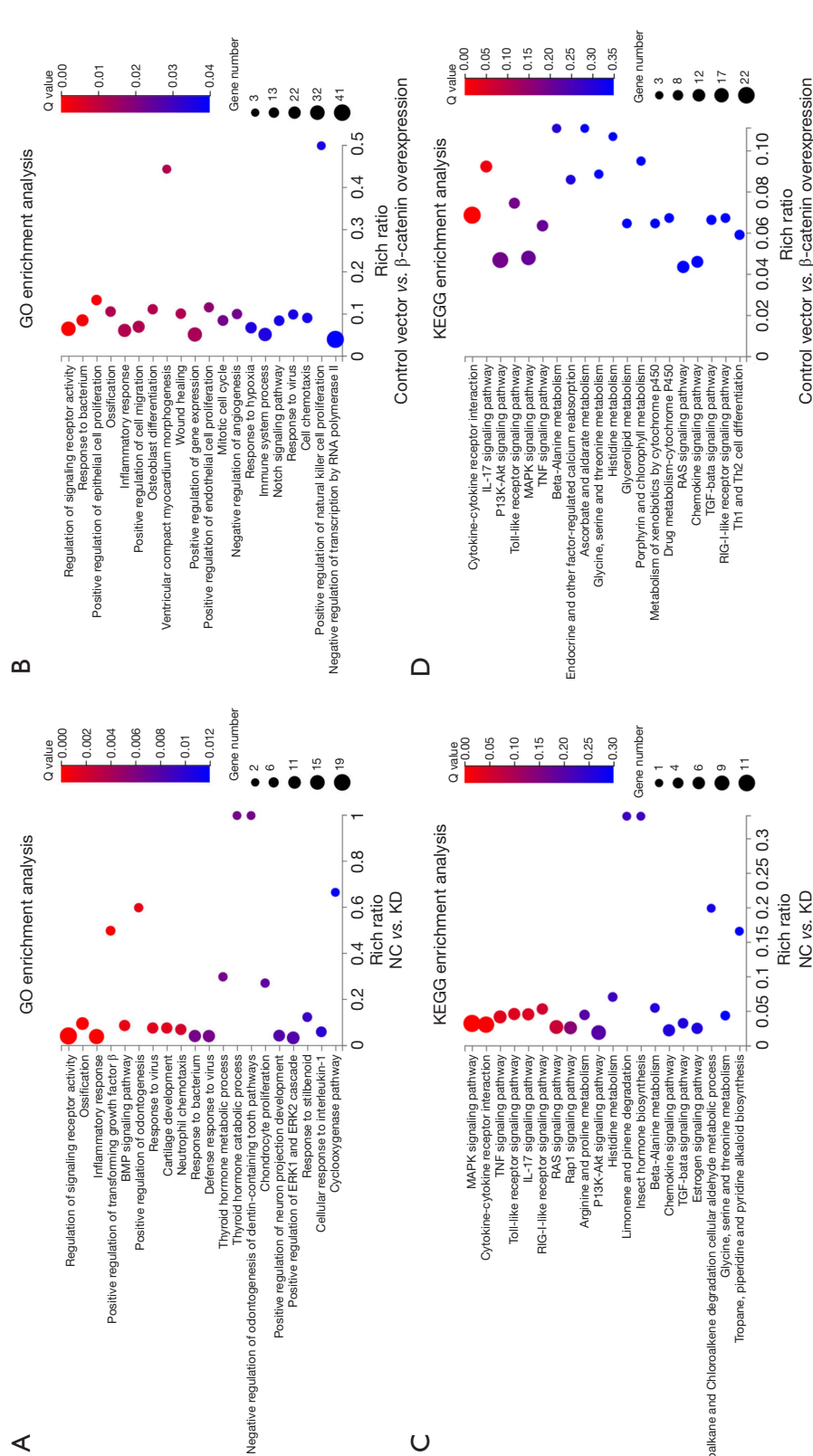


Figure 3 Multiple biological functions and pathways were regulated after silencing and overexpressing β -catenin in MEPM cells. Downstream enrichment analysis of DEGs was conducted to obtain significantly enriched GO biological functions and KEGG pathways. MEPM, mouse embryonic palatal mesenchymal; DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; NC, negative control group; KD, β -catenin knockdown group.

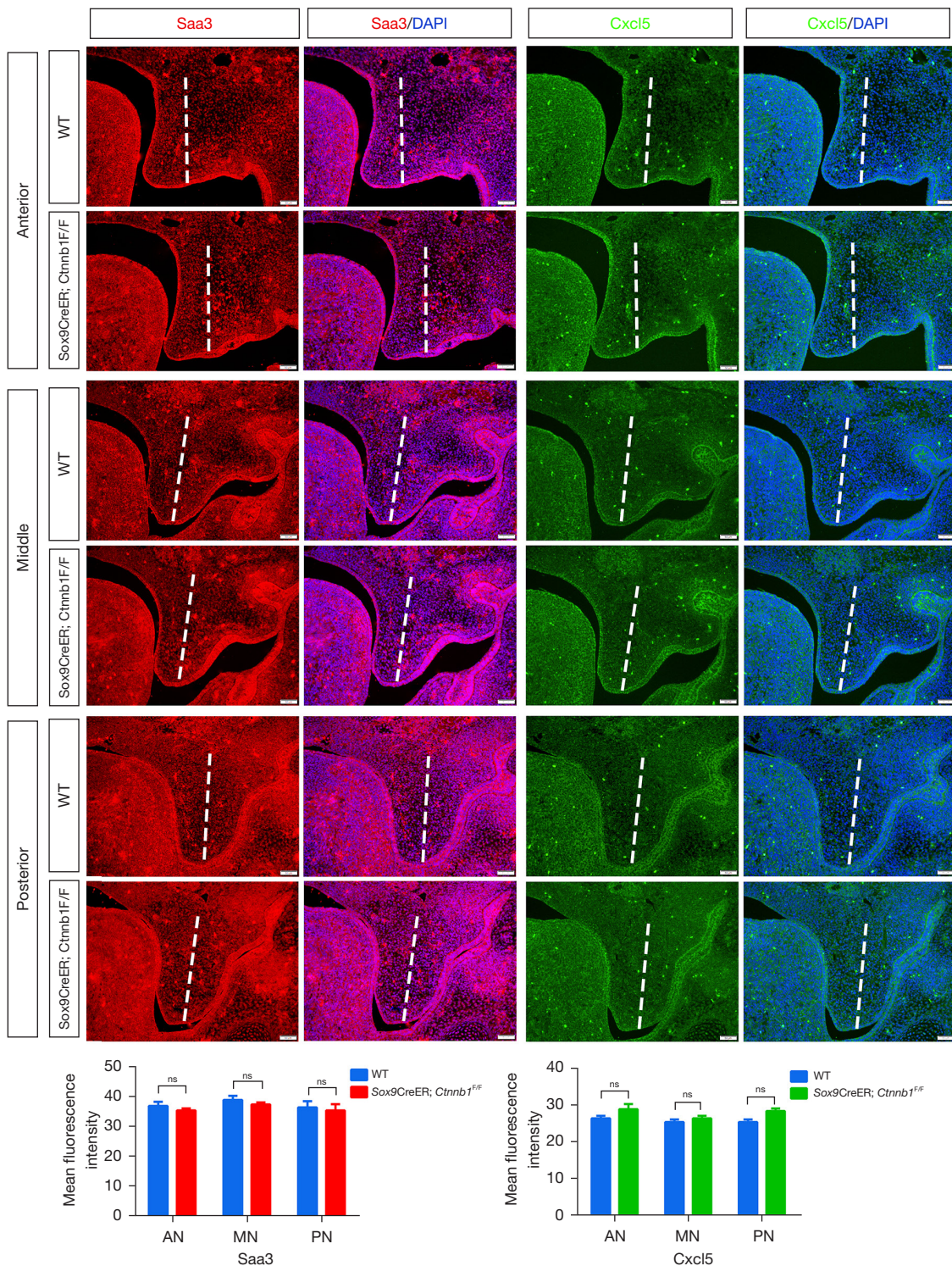


Figure 4 Expression of *Saa3* and *Cxcl5* in the PS mesenchyme in *Sox9CreER; Ctnnb1^{F/F}* mutant embryos. Immunofluorescence staining displayed that the expression levels of *Saa3* and *Cxcl5* in the wild-type PS mesenchyme was similar to that in *Sox9CreER; Ctnnb1^{F/F}* mesenchyme. The nasal region of the PS mesenchyme distinguished by the white dashed lines. Error bars represent SD. ns: $P > 0.05$. Scale bar, 50 μm . WT, wild type; PS, palatal shelf; DAPI, 4',6-diamidino-2-phenylindole; AN, nasal half of the anterior region; MN, nasal half of the middle region; PN, nasal half of the posterior region.

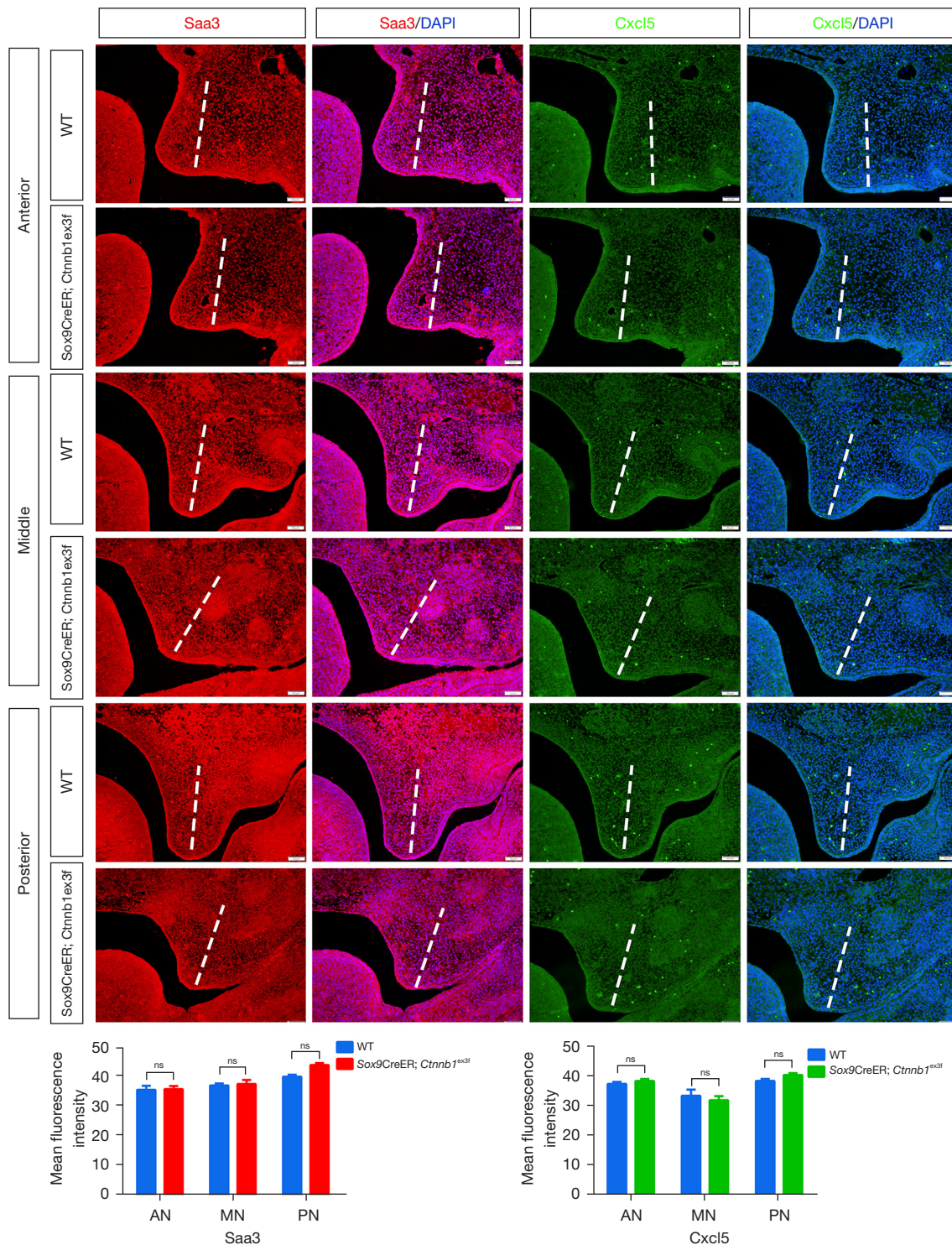


Figure 5 Expression of *Saa3* and *Cxcl5* in the PS mesenchyme in *Sox9CreER; Ctnnb1^{ex3f}* mutant embryos. Immunofluorescence staining displayed that the expression levels of *Saa3* and *Cxcl5* in the wild-type PS mesenchyme was similar to that in *Sox9CreER; Ctnnb1^{ex3f}* mesenchyme. The nasal region of the PS mesenchyme distinguished by the white dashed lines. Error bars represent SD. ns: $P > 0.05$. Scale bar, 50 μm . WT, wild type; PS, palatal shelf; DAPI, 4',6-diamidino-2-phenylindole; AN, nasal half of the anterior region; MN, nasal half of the middle region; PN, nasal half of the posterior region.

palate development. Further investigation to determine the definite mechanism that β -catenin/F-actin is coordinated in the palatal mesenchyme are required.

Discussion

The highly regulated, multi-step morphogenetic process of palate development requires the coordinated participation of extensive growth factors and transcription factors; aberration of any of these factors could lead to the development of cleft palate (4,7,8). A cleft palate phenotype has been reported in a mouse model deficient or stabilized for β -catenin in the PS mesenchyme (21,22). However, the mesenchymal β -catenin mechanisms responsible for palate formation remain to be unexplored. To reveal the underlying mechanisms, we established mouse models carrying deficient or stabilized for β -catenin in the palate mesenchyme (23,24). Our previous studies showed that mesenchymal specific inactivation or stabilization of β -catenin disrupted shelf elevation by regulating F-actin polymerization and resulted in a cleft palate defect, indicating that mesenchymal β -catenin participated in palate development by controlling the actin cytoskeleton (23,24). Here, we performed RNA-Seq to determine the specific downstream molecules relating to F-actin rearrangement regulated by β -catenin, enhancing our understanding of the definite molecular mechanism of mesenchymal β -catenin in clefting of the secondary palate.

Mammalian PSs are predominately composed of the underlying mesenchyme, accompanied by surrounding epithelium. Multiple studies have investigated genes associated with cleft palate using RNA-seq with murine and human samples (25-28). Previously, RNA extraction and sequencing was performed to explore downstream molecular targets using whole PSs tissue excised (25,28). However, β -catenin signaling could be detectable in the epithelium and mesenchyme of the palate during its formation, particularly in the epithelial cells. This investigation mainly focused on the molecular mechanism of palatal mesenchymal β -catenin to regulate F-actin reorganization. If RNA extracted from whole PSs tissue was used to perform RNA-Seq, the β -catenin signaling in palatal epithelium might confuse the actual effect of mesenchymal β -catenin signaling on cleft palate formation. It is difficult to separate mesenchyme intactly without intermingling of palatal epithelium. Thus, silenced and overexpressed MEPM cells for β -catenin were used to explore downstream molecules that are directly and indirectly controlled by

β -catenin signaling. Of note, considering primary MEPM cells senesced after several passages, which is not suitable for stable transfection, we adopted transient transfection by adenovirus.

The screened DEGs are mainly involved in biological functions such as signaling receptor activity regulation, ossification, and positive modulation of cell migration. It is suggested that β -catenin might regulate signaling receptor activity and cell migration of MEPM cells. Numerous studies have demonstrated the importance of directional cell migration in shelf elevation (9,13,29). Both *Wnt5a* and *Specc11* mutant mouse embryos have been found to exhibit abnormal palatal shelf elevation and a cleft palate defect (13,29). *Wnt5a* can function as a chemotactic factor and alter cell migration ability in the palate mesenchyme. In line with the role of *Wnt5a* in regulating directional cell migration, defective cell speed and guidance were also observed in *Specc11* mutant MEPM cells (13). It indicated that a possible role of β -catenin in cell migration by regulating certain DEGs or F-actin rearrangement. The pathways involved mainly included cytokine-cytokine receptor interaction, the mitogen-activated protein kinase (MAPK) signaling pathway, the chemokine signaling pathway, and the phosphatidylinositol-3-kinase (PI3K)-protein kinase B (Akt) signaling pathway. Among them, the chemokine signaling pathway and the PI3K-Akt signaling pathway were possibly associated with F-actin reorganization. For instance, MEPM cells deficient for *Specc11* rescued defective collective movement through activation of PI3K-AKT signaling (13,30).

We identified special genes regulated by β -catenin between groups and showed that among these β -catenin target genes, *Saa3* and *Cxcl5* were downregulated and upregulated accordingly in β -catenin silenced and overexpressed MEPM cells. It has been reported that *Saa3* is a key player in F-actin reorganization and impaired cell migration (31). Furthermore, Verdoni *et al.* demonstrated that a close relationship between *Cxcl5* and cytoskeleton rearrangement (32), which suggested the involvement of *Saa3* and *Cxcl5* in F-actin reorganization. Based on above results, we speculated that mesenchymal β -catenin exerts an impact on palatal shelf reorientation, which mediates potential molecules *Saa3* or *Cxcl5* to regulate F-actin cytoskeleton reorganization. To verify that the predicted molecules by RNA-Seq are targets of the β -catenin to regulate F-actin rearrangement, further *in vivo* mouse model validation was implemented. Unexpectedly, our *in vivo* results were inconsistent with the *in vitro* results,

and we observed that both *Sox9*CreER; *Ctnnb1*^{F/F} and *Sox9*CreER; *Ctnnb1*^{ex3f} embryos had no significant difference of *Saa3* and *Cxcl5* in palatal mesenchyme, arguing against the possibility that *Saa3* or *Cxcl5* was the target of β -catenin in regulating F-actin rearrangement for PS elevation. The possible reason for this discrepancy is that the *in vitro* cell culture environment cannot fully mimic *in vivo* conditions and cell-cell communication. Thus, future work is needed to find out whether other protein mediators are involved in the elevation process, and the relationship between β -catenin and above molecules.

Conclusions

This present study represents a comprehensive analysis of gene expression profiling of DEGs in MEPM cells due to the silencing and overexpression of β -catenin. The DEGs functional enrichment revealed that β -catenin might be a potential regulator of chemotactic factor and cell migration in MEPM cells through multiple genes, such as *Saa3* and *Cxcl5*, which were enriched in chemokine signaling pathway and the PI3K-Akt signaling pathway. Our results indicated that β -catenin may regulate F-actin cytoskeleton reorganization in the palatal shelf mesenchyme by mediating potential downstream molecules *Saa3* or *Cxcl5*, thus controlling palatal shelf reorientation. Nevertheless, further *in vivo* experiments validated that other potential genes led to F-actin reorganization regulated by β -catenin, instead of *Saa3* or *Cxcl5*. Collectively, the data presented in our study provide novel insights into downstream target genes involved in cytoskeletal rearrangement regulated by β -catenin, and deepen the knowledge of the molecular mechanism of β -catenin-regulated shelf reorientation.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-5772/rc>

Data Sharing Statement: Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-5772/dss>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-5772/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. WCHSIRB-D-2020-426) granted by the Ethics Committee of West China Stomatological Hospital of Sichuan University, in compliance with Chinese guidelines for the care and use of animals.

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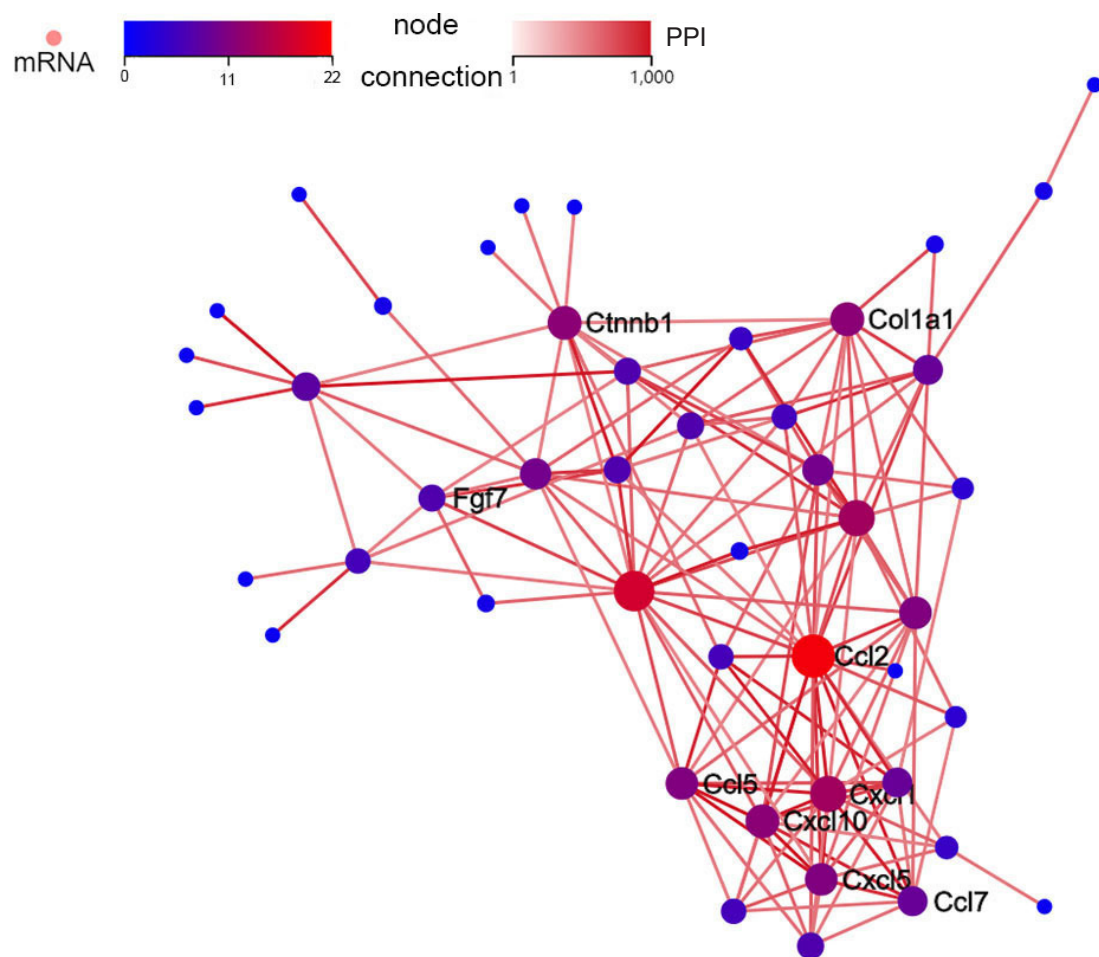


Figure S1 PPI network of the DEGs in MEPM cells. PPI, protein-protein interaction; DEGs, differentially expressed genes; MEPM, mouse embryonic palatal mesenchymal.

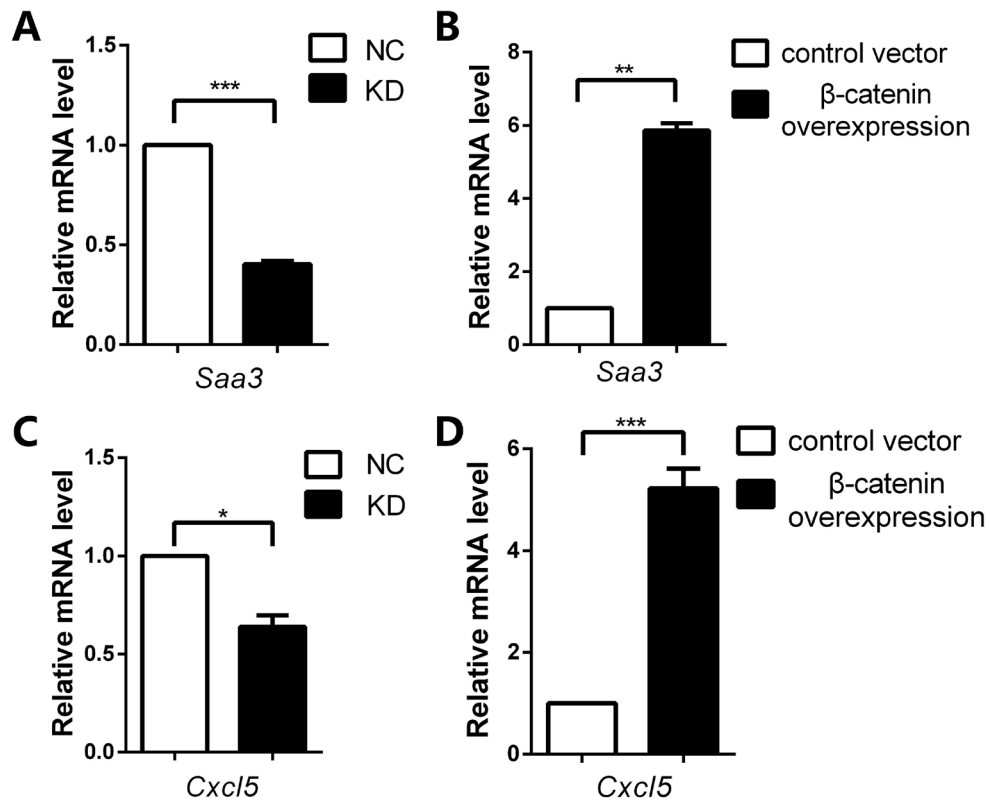


Figure S2 Validation of RNA-Seq results by qRT-PCR. (A,C) *Saa3* and *Cxcl5* were uniquely decreased in β-catenin silenced MEPM cells compared to NC group, (B,D) while *Saa3* and *Cxcl5* exhibited significantly upregulated mRNA expression level in β-catenin overexpressed MEPM cells. Data are presented as mean ± SD. *P<0.05, **P<0.01, ***P<0.001. NC, negative control; KD, knockdown; MEPM, mouse embryonic palatal mesenchymal; qRT-PCR, quantitative real-time polymerase chain reaction.

Table S1 The detailed primer sequences were as follows

Gene	Sequence (5'-3')
<i>β-catenin</i>	F: AGGAATGAAGGCGTGGCAACATAC
	R: GGCACCAATGTCCAGTCCAAGATC
<i>Saa3</i>	F: AGAGAGGCTGTTCAGAAGTTCA
	R: AGCAGGTCGGAAGTGGTTG
<i>Cxcl5</i>	F: TCGTGTGTTTTGCTTAACCG
	R: CTTCCACCGTAGGGCACTG
<i>GAPDH</i>	F: GGTTGTCTCCTGCGACTTCA
	R: TGGTCCAGGGTTTCTTACTCC