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Retinoic acid inhibits histone methyltransferase Whsc1 during palatogenesis

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ABSTRACT

Cleft lip with or without palate (CL/P) is a common congenital anomaly in humans and is thought to be caused by genetic and environmental factors. However, the epigenetic mechanisms underlying orofacial clefts are not fully understood. Here, we investigate how the overdose of retinoic acid (RA), which can induce cleft palate in mice and humans, regulates histone methyltransferase, Wolf–Hirschhorn syndrome candidate 1 (WHSC1) during palatal development in mice. We treated mouse embryonic fibroblasts (MEFs) with 1 μM all-trans RA and discovered that the global level of H3K36me3 was downregulated and that expression of the H3K36 methyltransferase gene, *Whsc1*, was reduced. The expression level of WHSC1 in embryonic palatal shelves was reduced during palatogenesis, following maternal administration of 100 mg/kg body weight of RA by gastric intubation. Furthermore, the expression of WHSC1 in palatal shelves was observed in epithelial and mesenchymal cells at all stages, suggesting an important role for palatal development. Our results suggest that the pathogenesis of cleft palate observed after excessive RA exposure is likely to be associated with a reduction in the histone methyltransferase, WHSC1.

1. Introduction

Cleft lip with or without palate (CL/P) is a multifactorial disease caused by the interaction of genetic and environmental factors [1]. There are several known candidate genes for CL/P. The best candidates, in which mutations have been reported, are *IRF6*, *SUMO1*, *MSX1*, *FGFR1*, *FGFR2*, *FGF8*, *BMP4*, and *TBX1* [1–3]. Genome-wide association studies have reported several loci strongly associated with CL/P, for example the “gene desert” region on chromosome 8q24, *VAX1* at 10q25, and *VOG* at 17q22 [1]. However, there are also studies that have begun to provide data on environmental risks of CL/P. Maternal smoking; some specific teratogens, for example valproic acid; nutritional factors, such as folate deficiency; and exposure to maternal alcohol consumption have all been suggested as risk factors for cleft palate [1]. Retinoic acid (RA) is the one of the environmental factors for which both deficiency and overdose cause CL/P in mice and humans [4].

The mechanisms by which RA induces cleft palate have been studied from several points of view. At the time of palatal shelf outgrowth, overdose of RA upregulates the cyclin-dependent kinase inhibitor p21 and hypophosphorylates the RB1 protein, resulting in an inhibition of mesenchymal cell proliferation in the palate [5,6]. Moreover, RA inhibits mesenchymal proliferation of palatal shelves through down-regulation of *Bmp2* expression [7]. At the time of shelf elevation, RA prevents tongue withdrawal through down-regulation of *Tbx1*, a candidate gene for DiGeorge syndrome, in which CL/P is one of the phenotypic features. This might physically prevent the elevation of the palatal shelves [8]. Prior to elevation, excess of RA may directly suppress collagen synthesis through binding to retinoic acid response element sites in the α2(I) collagen promoter region, thus hampering extracellular matrix production [9]. During palatal shelf elevation, RA inhibits the synthesis of matrix metalloproteinases in the extracellular matrix (ECM) of the palatal mesenchymal cells and stimulates the expression of tissue inhibitors of matrix metalloproteinases [10]. This might lead to reduced remodeling of the ECM and impaired elevation of the palatal shelves. In the period of palatal shelf fusion, excess of RA reduced the expression of filopodia and chondroitin sulfate proteoglycans in peridermal cells through alteration of

Abbreviations: CL/P, cleft lip with or without palate; CP, cleft palate; RA, retinoic acid; WHSC1, Wolf-Hirschorn Syndrome Candidate 1.

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growth factor signaling, such as that mediated by PDGF and TGF-β3 [11,12]. The filopodia and chondroitin sulfate proteoglycans of medial edge epithelia mediate adhesion, so their reduced expression interferes with palatal shelf adherence. Taken together, these data imply that RA is fundamental for palate development.

There has been increasing evidence that the site-specific methylation of histones is fundamental for gene regulation during development. The effects of histone methylation are complex and depend on both the lysine residue and the methyl mark; H3K4me3, H3K4me2, H3K4me1, H3K9me1, H3K27me1, and H3K36me3 are associated with gene activation, while H3K9me2, H3K9me3, and H3K27me3 are associated with gene repression. However, histone methylation does not only act like an on/off switch; orchestrated methylation states finely control the intensity of gene expression depending on the context of development.

It has been demonstrated that RA regulates gene expression through an epigenetic mechanism during stem cell and cancer cell differentiation. In neuroblastoma cells, RA induced neural maturation through *RET* gene transcriptional activation by increasing H3K4me3 levels at the promoter region and demethylating H3K27me3 at an enhancer region [13]. Furthermore, during RA-induced F9 cell differentiation, *Nr2f1* and *Hoxa5* are transcriptionally activated by RA and display increased levels of the permissive H3K9/K14ac and H3K4me3 epigenetic marks [14]. These studies suggest that RA can regulate gene expression by alteration of histone modifications in different cells.

It has been suggested that some congenital disorders characterized by CL/P are associated with epigenetic disregulation. Mutations of PHF8, a histone demethylase of H4K20me1 and H3K9me1/2, were found in a disorder whose main phenotype was CL/P with mental retardation [15,16]. Haploinsufficiency of KDM6A, a histone demethylase, is associated with cleft palate (CP) with developmental delay [17]. Mice mutant for the histone acetyltransferase, *Moz*, have a similar phenotype to DiGeorge syndrome, itself associated with mutations in *TBX1* [18]. Wolf-Hirschhorn syndrome [19], in which CL/P is seen, is caused by the mutation of Wolf-Hirschhorn syndrome candidate 1 (*WHSC1*), a gene encoding an H3K36me1/me2/me3 methyltransferase [20].

In this study, we investigate how an overdose of RA, which can induce CL/P in mice and humans, regulates WHSC1 during palatal development in mice.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice were mated with males overnight. The following morning, observation of a vaginal plug was designated as embryonic day 0.5 (E0.5). E11.5 pregnant female mice were given gastric intubations of a single dose of all-trans RA (SIGMA, München, Germany) (100 mg/kg body weight). Control animals were given the equivalent volume of corn oil.

Mouse Embryonic Fibroblast (MEF) cells were obtained from E14.5 mouse embryos and grown in DMEM (SIGMA, München, Germany), supplemented with 10% fetal bovine serum (FBS) (Life technologeies, Carlsbad, Canada). After 12 h, the cells were treated with 1 μM RA dissolved in DMSO or DMSO alone.

2.2. Real-time quantitative PCR

Total RNA was isolated from MEF cells and palatal shelves using an RNeasy Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The High-capacity cDNA Reverse Transcription Kit (ABI, Foster, Canada) was used to make cDNA. Real-time PCR analysis was performed in a real-time PCR machine (SDS7300, ABI) using a SYBR-green fluorescence quantification system (Life technologies, Warrington, UK). All data were normalized to the expression of the glyceraldehyde-3-phosphate dehydrogenase gene (*Gapdh*). Primer sequences are given in Supplementary Table S1.

2.3. Western blot

Proteins were extracted from MEF cells and palatal shelves. Western blotting was carried out using standard protocols and antibodies against H3K4me2, H3K4me3, H3K9me3, H3K27me3 (Active Motif 39142, 39160, 39162, 39157, respectively), H3K9me2, H3K36me2, H3K36me3, H4K20me1, Histone H3, WHSC1, and beta-actin (Abcam ab1220, ab9049, ab9050, ab9051, ab1791, ab75359, ab8227, respectively). All the data were normalized to the expression of histone H3 or β-actin.

2.4. Immunofluorescent staining

Mouse embryonic heads were fixed in 4% paraformaldehyde, dehydrated with ethanol, hyalinized with xylene, and embedded in paraffin for sectioning by routine procedures. Coronal sections of embryonic heads were sliced to a thickness of 7 μm. The sections were incubated with antigen retrieval buffer (Tris/EDTA pH 9.0) for 20 min, washed with TBS containing 0.025% Triton X-100 (TBST), and blocked with mouse Ig blocking reagent (Vector Laboratories, Burlingage, U.S.) for 1 h at room temperature. The sections were then washed with TBST and incubated with primary antibody for anti-WHSC1 (abcam, Tokyo, Japan) (1:200). After overnight incubation, bound antibodies were visualized with a secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, U.S.). Nuclei were stained with DAPI.

3. Results

3.1. The global level of H3K36me3 was downregulated by RA treatment in MEFs

There are many reports regarding the pathogenesis of cleft palate by environmental factors [1,4]. Recent findings also demonstrate that metabolic diseases and cancer caused by environmental factors are associated with epigenetic changes in a target gene(s) [21,22]. To examine whether RA affected the global methylation levels of histones in MEFs, we treated MEFs with 1 μM RA or dimethyl sulfoxide (DMSO) vehicle for 24 or 48 h and performed western blot analyses. H3K4me2/me3, H3K9me2/me3, H3K27me3, and H4K20me1 levels were unchanged. The only alteration seen was a reduction in the level of H3K36me3 in RA-treated MEF cells compared with controls after 48 h of treatment (Fig. 1). These data imply that RA globally downregulates the level of H3K36me3 in MEFs.

3.2. Whsc1 mRNA was downregulated by RA in MEFs

The site-specific methylation of histone lysine residues is critical for correct gene regulation. Multiple enzymes can modify the lysine groups of histones by catalyzing the addition or removal of methyl groups. Thus, the global reduction of H3K36 by RA raises the question of whether RA might regulate the catalytic enzymes that modify H3K36me3. To identify which catalytic enzyme(s) was regulated, we treated MEFs with RA or DMSO vehicle over a time course spanning 24–48 h and performed quantitative RT-PCR for H3K36me3 catalytic enzymes (the methyltransferases, *Setd2* and *Whsc1*, and the demethylases, *Kdm4a*, *Kdm4b*, *Kdm4c*, and *Kdm4d*; Supplementary Table 1). After 36 and 48 h of RA treatment, only

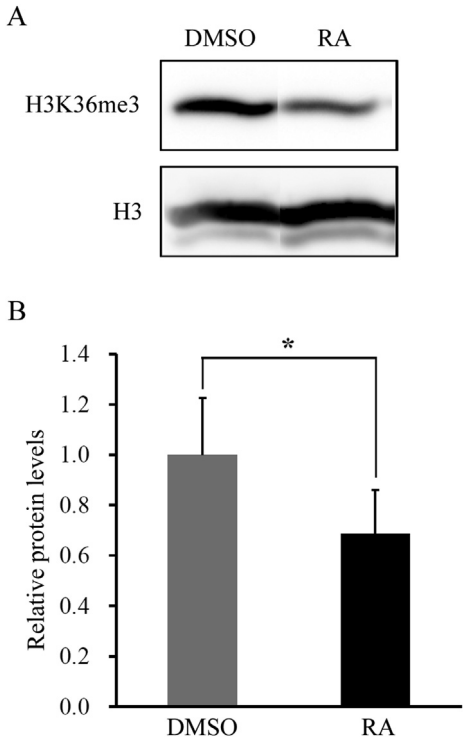

Fig. 1. Protein levels of H3K36me3 in MEFs treated with RA or DMSO for 48 h. (A) Whole cell extracts were analyzed by western blot with the antibodies indicated. (B) The expression level of H3K36me3 relative to histone 3. The DMSO group was set to 1. Error bars indicate S.D. (n = 3). *P < 0.05.

expression of *Whsc1*, a methyltransferase for H3K36me3, showed a significant reduction (Fig. 2). These data indicate that reduction of H3K36me3 in MEFs is caused by downregulation of *Whsc1* expression by RA treatment.

3.3. Excessive RA reduced Whsc1 in palatal shelves

We showed that global downregulation of H3K36me3 states was accompanied by a reduction in *Whsc1*. *WHSC1* is a candidate gene for Wolf-Hirschhorn Syndrome, in which CL/P is one of the main features. *Whsc1* knockout mice develop cleft palate [20]. These observations suggested to us that the pathogenesis of cleft palates induced by RA is likely to be mediated by WHSC1.

To confirm our hypothesis, we first developed a model in which an overdose of RA induced cleft palates in mice. We gave RA (100 mg/kg body weight) to pregnant mice at E11.5 according to the protocol of a previous study [23]. In mothers treated with RA, 100% of harvested embryos had a cleft palate, whereas there were no cases of cleft palate in control mice (Fig. S1).

Next, we extracted the palatal shelves of embryos at E12.5, E13.5, E14.5, and E15.5 following exposure to RA. *Whsc1* mRNA and protein levels were analyzed by quantitative RT-PCR and western blotting. *Whsc1* mRNA expression levels did not show significant changes in palatal shelves at E12.5, but were significantly reduced at E13.5, E14.5, and E15.5 by RA treatment (Fig. 3A). The WHSC1 protein levels in palatal shelves were clearly reduced at all stages after RA exposure (Fig. 3B and C).

3.4. Spatial and temporal expression patterns of WHSC1 in palatal shelves

To analyze the spatial and temporal expression patterns of WHSC1 in palatal shelves during development, we performed immunofluorescence staining on mouse embryonic head sections at different developmental stages (E12.5, E13.5, E14.5, and E15.5). Expression is very prominent in the brain, the vitreous body of the eyes, the epithelium of the nasal cavity, the tongue, the membranous bones developing into the maxilla and the mandible, the dental papilla, and the epithelium of the enamel organ (Fig. S2). Although WHSC1 expression in palatal shelves was relatively weak at E12.5, expression mainly occurred in the epithelium and mesenchyme of the nasal side (Fig. 4A and B). At E13.5, the expression of WHSC1 was reduced in the anterior palatal shelves and only strongly expressed in the epithelium and mesenchyme close to the nasal side. In contrast, the expression increased in the whole of the middle and posterior palatal shelves at E13.5 (Fig. 4C and D). At E14.5, strong expression was seen in the epithelium close to the oral and nasal sides of the anterior and middle palatal shelves, whilst relatively weak expression was seen in whole mesenchyme. In the posterior palatal shelves, the expression in the epithelium was strong and the expression in the mesenchyme mainly occurred in the tip of the palatal process (Fig. 4E and F). At E15.5, the expression of WHSC1 was mainly at points of fusion between the anterior and posterior palate (Fig. 4G and H).

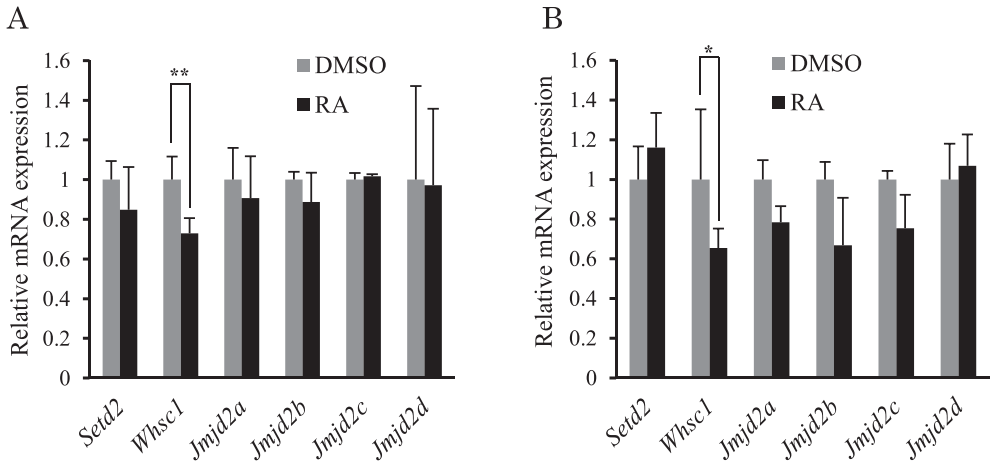

Fig. 2. mRNA levels of H3K36me3 histone methyltransferases and demethylases. (A) Expression of H3K36me3 histone methyltransferases and demethylases of MEFs treated with RA or DMSO for 36 h. Expression was normalized to *Gapdh* and the DMSO groups were set to 1. Error bars indicate S.D. (n = 3). **P < 0.01. (B) The same experiment was performed with MEFs treated for 48 h (n = 3). *P < 0.05.

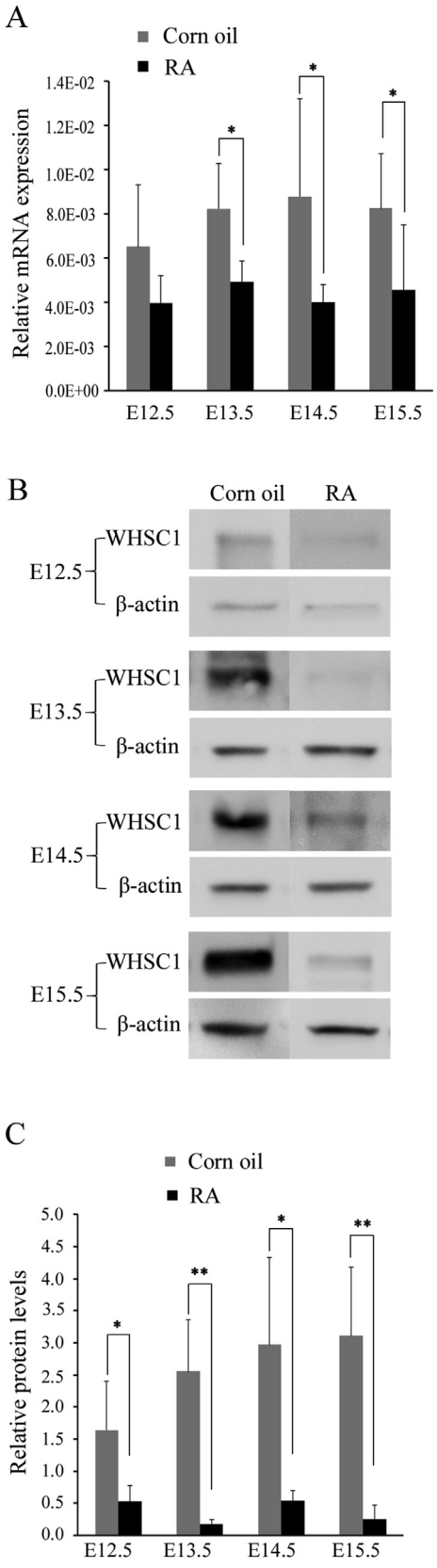

Fig. 3. Induction of Whsc1 in palatal shelves by excessive RA. (A) Expression of *Whsc1* in palatal shelves by excessive RA and corn oil as control. Values were normalized against expression of *Gapdh*. The value of n indicates the number of samples. Error bars represent S.D. *P < 0.05. (B) WHSC1 protein levels in palatal shelves by excessive RA and corn oil control at E12.5, E13.5, E14.5 and E15.5. (C) The expression levels of WHSC1 were normalized to β-actin. Error bars indicate S.D. The value of n indicates the number of samples. *P < 0.05, **P < 0.01.

4. Discussion

CL/P is a common congenital deformity in humans and is thought to be caused by the interaction of genetic and environmental factors. In recent years, gene mutations in epigenetic modifiers have been detected in congenital diseases including CL/P. This suggests that epigenetic regulation is crucial for normal palatal development. However, it is not understood whether environmental factors that contribute to CL/P can affect palatogenesis through epigenetic mechanisms. Here we showed that overdose of RA, which induces CP, downregulates *Whsc1* in palatal mesenchyme. *WHSC1* is a candidate gene for Wolf-Hirschhorn syndrome, of which one of the main features is CL/P. Our results suggest that the pathogenesis of cleft palate observed after excessive RA exposure is likely to be associated with a reduction in the histone methyltransferase WHSC1. This provides a new viewpoint for understanding the mechanisms of cleft palate induced by environmental factors.

4.1. Whsc1 regulation by retinoic acid

We have shown that overdose of RA decreased WHSC1, a histone methyltransferase for H3K36me3, in MEFs and palatal mesenchyme. There are six enzymes that are known to modify lysine 36 of histone 3 in mouse and human. Of those, only WHSC1 was affected by RA, implying that RA may directly induce WHSC1. RA forms complexes with its nuclear receptor, RAR/RXR, and binds to the retinoic acid response element (RARE) in the promoters of target genes, normally leading to upregulation [24]. However, in some cases, RA signaling negatively regulates gene transcription, by both direct and indirect interactions [25]. The RARE is a repeat of RGKTCA (R = A/G, K = G/T) with a spacer of 1–5 bp. Sequence analysis of the mouse *Whsc1* promoter, which lies 5 kb upstream of the transcription start site, revealed a non-repetitive RGKTCA. This implies that RA-mediated *Whsc1* regulation is likely to be indirect. Hudlebusch et al. showed that RA-induced differentiation decreased *Whsc1* expression in neuroblastoma cells. They also showed that the expression of *Whsc1* was decreased along with the differentiation of normal neural cells [26]. These results suggest that decreased expression of *Whsc1* is the result of differentiation, rather than a direct effect of RA signaling.

4.2. Relationship between Whsc1 and palatal mesenchyme proliferation

Several studies of cancer development have shown that WHSC1 is associated with cell differentiation and proliferation. WHSC1 is highly expressed in prostate cancer cell lines, neuroblastoma cells, and normal neural progenitor cells [26,27]. Retinoic acid-induced differentiation of neuroblastoma cells and differentiation of normal neural progenitor cells leads to a decrease of WHSC1 levels [26]. These findings suggest that WHSC1 tends to be expressed in poorly differentiated cells, implying that WHSC1 is required for cell proliferation. During palate development, there is a high proliferation rate from E12.5 to E15.5 [28–30]. In control embryos, WHSC1 protein levels did not change significantly from E12.5 to E15.5, corresponding to the stable high proliferation rate seen in normal embryonic development. In the RA-treated embryos, we propose that RA downregulated *Whsc1* steeply, leading to reduced proliferation, and disrupted development of the palatal shelves.

4.3. Target gene(s) for WHSC1 in palatal development

Immunofluorescence staining showed differential WHSC1 expression along the mediolateral axis of the palatal shelves. The

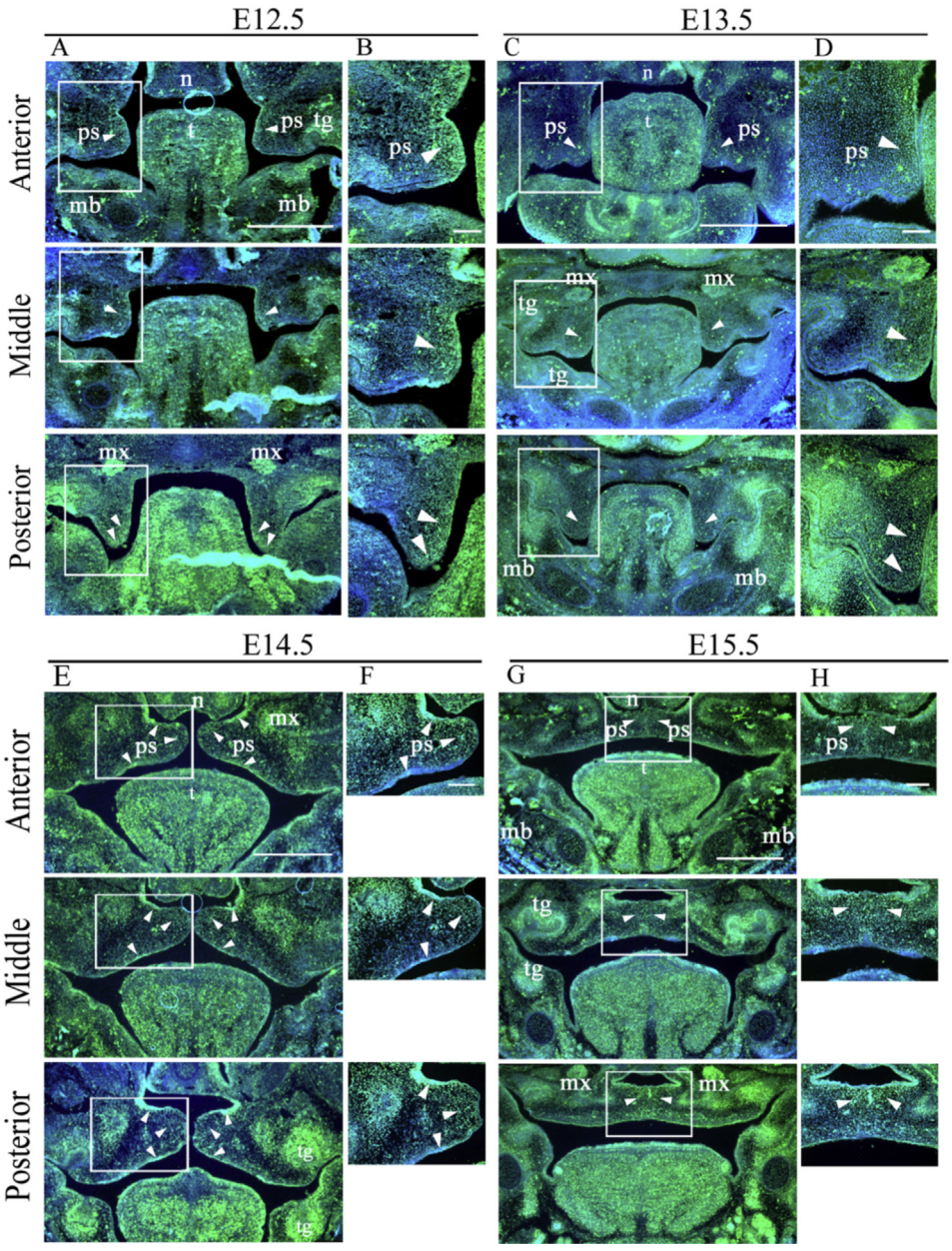

Fig. 4. Expression patterns of WHSC1 in palatal shelves. Immunofluorescence staining was performed with anti-WHSC1 antibody against the embryonic sections at the developmental stage indicated. The green signal indicates WHSC1 expression and blue signal indicates nuclei. Boxed regions were shown at higher magnification. mx, maxilla; mb, mandible; n, nose; ps, palatal shelves; t, tongue; tg, tooth germ; white arrows indicate the expression region of WHSC1. Scale bars = 500 μm; (A, C, E, G); 100 μm; (B, D, F, H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression of WHSC1 mainly occurred in the epithelial and mesenchymal domains adjacent to the medial edge during palatal shelf elevation. Several other proteins also show differential expression along the mediolateral axis of the palatal shelves during palatogenesis. These include Dlx5, Fgf7, Wnt5a, Fzd4, and Ror2 [31,32]. Dlx5 and Fgf7 are expressed in the medial mesenchyme of palatal shelves in a similar pattern to WHSC1. However, the palatal shelves of *Dlx5* knockout mice elevated and fused [31] and mice lacking *Fgf7* did not show obvious palatal abnormalities [33]. The Wnt signaling pathway is known to regulate processes such as cell proliferation, survival, and differentiation during embryonic development. Evidence from genetic screening suggests that *WNT3A*, *WNT5A*, and *WNT11* are associated with non-syndromic CL/P [11]. The expression region of WNT5A and its receptors, FZD4 and ROR2, in the anterior palatal shelves of embryonic mice is similar to that of WHSC1 [32]. In addition, in human bladder and lung cancer tissues, upregulation of WHSC1 expression regulates β-catenin, part of the Wnt pathway, and transcriptionally upregulates *CCD1*, the target gene of the β-catenin/Tcf-4 complex, through H3K36me3 [22]. Therefore, we speculate that WHSC1 might function as a coactivator for the Wnt pathway during palatogenesis.

Isolated cleft palate is strongly associated with other congenital anomalies such as heart defects [34]. Furthermore, syndromes such as Wolf-Hirschhorn syndrome, DiGeorge syndrome and Pilotto syndrome exhibit both cleft palate and heart defects [2,18,35]. These facts imply that there might be a mutual signaling pathway involved in both palatogenesis and cardiogenesis. A recent study has shown that mice mutant for the histone acetyltransferase, *Moz*,

developed a DiGeorge-like syndrome. Furthermore, administration of RA to heterozygous Moz mutant increased the incidence of CP that control mice through downregulation of *Tbx1* expression [18]. This suggests that *Tbx1* could be a target for WHSC1 during palatal development.

The transcription factor, *TWIST1*, is a candidate gene for Saethre-Chotzen syndrome [36] and is important for epithelial–mesenchymal transition (EMT). In prostate cancer, WHSC1 binds to the *TWIST1* locus and leads to an increase of *TWIST1* expression. This increased *TWIST1* expression is thought to promote metastasis along with an increase in other EMT markers [25]. At the time of palatal fusion, attached epithelial cells at each side of the palatal process are transformed into mesenchyme to complete the fusion of the palate. Disruption of EMT also causes CP and CP has been observed in patients with *TWIST1* mutations [36,37]. Taken together, we think that Twist1 may be also a target for WHSC1.

Conflict of interest

None.

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Transparency document

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Appendix A. Supplementary data

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