Biochemical and Biophysical Research Communications 458 (2015) 525–530

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Retinoic acid inhibits histone methyltransferase Whsc1 during palatogenesis

Shiying Liu, Norihisa Higashihori*, Kohei Yahiro, Keiji Moriyama

Maxillofacial Orthognathics, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU), 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

ARTICLE INFO

Article history:
Received 19 January 2015
Available online 10 February 2015

Keywords:
Cleft palate
Epigenetic
Histone methyltransferase
Whsc1
Retinoic acid

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.

* Corresponding author
E-mail address: n-higashihori.mort@tmd.ac.jp (N. Higashihori).

http://dx.doi.org/10.1016/j.bbrc.2015.01.148
0006-291X/

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