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Correlation of proliferation, TGF-β3 promoter methylation, and Smad signaling in MEPM cells during the development of ATRA-induced cleft palate

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ABSTRACT

Mesenchymal cell proliferation is one of the processes in shelf outgrowth. Both all-trans retinoic acid (atRA) and transforming growth factor-β3 (TGF-β3) play an important role in mouse embryonic palate mesenchymal (MEPM) cell proliferation. The cellular effects of TGF-β are mediated by Smad-dependent or Smad-independent pathways. In the present study, we demonstrate that atRA promotes TGF-β3 promoter demethylation and protein expression, but can cause depression of mesenchymal cell proliferation, especially at embryonic day 14 (E14). Moreover, the inhibition of MEPM cell proliferation by atRA results in the downregulation of Smad signaling mediated by transforming growth interacting factor (TGIF). We speculate that the effects of atRA on MEPM cell proliferation may be mediated by Smad pathways, which are regulated by TGIF but are not related to TGF-β3 expression. Finally, the cellular effects of TGF-β3 on MEPM cell proliferation may be mediated by Smad-independent pathways.

1. Introduction

Cleft palate (CP) is one of the most common birth defects in humans, occurring at a frequency of 1.7:1000 [1–3]. Both gene mutations and environmental effects underlie the complex etiology of CP [4–6]. The identification of genes that cause CP upon mutation has improved, whereas only minor progress has been made in understanding how environmental factors contribute to this defect. Environmental influences can adversely affect the development of the secondary palate by means of genetic variants or epigenetic alterations. Retinoic acid (RA), an oxidative metabolite of vitamin A, is one of the environmental factors for which both deficiency and overdose cause CP in mice and humans [7]. CP can result from reduced mesenchymal cell proliferation immediately prior to the contact of palatal shelves [8–10] combined with the failure of palatal shelf adhesion and fusion [11–14]. However, the mechanism behind CP induced by RA overdose is still unclear, and conflicting viewpoints arise. Because most of these previous studies have been based on genetics, histopathology, embryology, and statistics, a different approach may shed new light on the etiology of CP.

Recently, much focus has been on epigenetics, which is the study of inherited changes in phenotype or gene expression by means of mechanisms that do not alter DNA sequences. Multiple mechanisms operate concertedly in epigenetics, such as histone acetylation, chromatin modification, and most importantly, DNA methylation. In genomic DNA of mammals, CpG islands that possess unmethylated GC-rich regions are found, whereas methylated CpGs in CpG islands of promoter areas repress gene expression [15]. Tissue-specific DNA methylation patterns are precisely programmed during embryogenesis [16,17]. However, failure to establish correct methylation patterns can lead to embryonic lethality [18] or developmental craniofacial malformations [19–23], including CP [24,25]. Kuriyama et al. [26] were the first to observe changes in the methylation status of CpG islands and global DNA methylation in the secondary palates of mice born

Abbreviations: CP, cleft palate; atRA, all-trans retinoic acid; TGF-β, transforming growth factor-β; MEPM, mouse embryonic palate mesenchymal; TGIF, transforming growth interacting factor; RAR, retinoic acid receptor; E0, embryonic day 0; AP, anterior-posterior.

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to mothers exposed to atRA overdose [26]. They proposed that atRA-induced changes in DNA methylation and demethylation status, especially within the CpG islands, may be associated with the manifestation of CP. The methylation patterns underlying secondary palate development and their contribution to CP have attracted research attention only recently. Moreover, identifying genes whose promoters undergo differential methylation during palate development is a significant step toward predicting and deciphering the pathogenesis of CP.

In mammals, TGF-β exists in three isoforms, namely, TGF-β1, TGF-β2 and TGF-β3, which are encoded by three different genes [27]. During development, TGF-β gene activation is regulated to restrict their expression to the correct cell type and developmental stage [28]. The spatially and temporally restricted pattern of gene expression implies that these genes might play important roles in the corresponding physiological processes [29]. At this point (E14), decreased cell proliferation has been observed in the TGF-$\beta3^{-/-}$ mouse palatal mesenchyme [8,9], which indicates the crucial role of TGF-β3 gene in palate development. The cellular effects of TGF-β3 are mediated by Smad-dependent or Smad-independent pathways [30]. Our previous study demonstrated that atRA inhibited MEPM cell proliferation by downregulating Smad signaling in vitro, which is involved in the regulation of TGIF [10]. Moreover, some studies have demonstrated that the transcriptional activation of the TGF-β3 gene is determined by the promoter methylation of its gene [31] and that RA can regulate gene expression through an epigenetic mechanism [32,33]. Nevertheless, details on the expression and methylation patterns of TGF-β3 that may be regulated by RA during palate development are few. Another unclear issue is whether the atRA-inhibited MEPM cell proliferation is related to the TGF-β3 expression or to the downregulation of Smad signaling in vivo. Whether the methylation of the TGF-β3 promoter is related to the downregulation to Smad signaling is a concern that needs clarification. To explore the mechanisms of CP development induced by atRA overdose, we investigated the methylation levels of the TGF-β3 promoter, the expression levels of the members in Smad signaling cascades, and the function of TGIF in atRA-treated embryos and controls.

2. Materials and methods

2.1. Animals and treatment

C57BL/6 mice (12 weeks old and ~25 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). Females were mated with fertile males overnight, and embryonic day 0 (E0) was designated at 8 AM of the day when a vaginal plug was found. Pregnant females at day 10 were randomly divided into two groups. The mice in one group were administered with atRA (Sigma-Aldrich, MO, USA) dissolved in corn oil using gavage at 100 mg/kg, whereas the mice in the other group were given the equivalent volume of the carrier as a control. The mice were killed on E13, E14, and E15, and the palatal shelves were collected and dissected from fetuses using microscissors for further analysis.

2.2. Histochemical staining

Embryonic heads were fixed in 4% paraformaldehyde, dehydrated through an ethanol series, and embedded in paraffin for sectioning by routine procedures. For general morphology, deparaffinized sections (5 μm) were stained with hematoxylin and eosin by standard procedures.

2.3. BrdU incorporation

Pregnant females from E13 to E15 were administered with vehicle (saline) or BrdU (200 mg/kg of body weight; Sigma-Aldrich, MO) by intra-peritoneal injection. Six hours after BrdU administration, the mice were euthanized, and the head of the embryos were excised and fixed in 4% paraformaldehyde for 8 h followed by embedding in paraffin. Coronal sections (5 μm) were used for immunohistochemistry. Briefly, sections were incubated at 37 °C with 3% H_2O_2 for 10 min, followed by denaturation of DNA in 2 N HCl for 30 min and enzymatic pretreatment with Trypsin for 30 min. Sections were blocked for 1 h with 5% goat serum followed by incubation with mouse anti-BrdU antibody (1:800) (ab8039, Abcom, CA, USA) overnight at 4 °C. The sections were incubated with biotinylated anti-IgG secondary antibody (1:50) (Anti-Ig HRP Detection Kit, BD Pharmingen, CA) for 30 min at room temperature (RT), followed by streptavidin-HRP incubation for another 30 min at RT and a brief wash with DAB chromogen substrate solution. The primary antibody was omitted in the negative control.

2.4. DNA methylation analysis

After RA or vehicle-only treatment at E10, controls (n = 4) and RA-treated (n = 4) pregnant females were killed at E14 and the embryos were collected. Palatal shelves were dissected to minimize contamination with tooth buds, quickly washed with phosphate-buffered saline (PBS), and placed in a separate culture dish containing Dispase II (2.4U/ml; Roche Diagnostics). The palates were then incubated for 15 min at 37 °C to separate mesenchyme from epithelium. Genomic DNA was extracted from MEPM using a QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. DNA concentration and purity were determined based on the absorbance at 260 nm and 280 nm. A total of 1.5 μg of genomic DNA from each sample was treated with bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer's instructions. Sequenom MassARRAY platform (CapitalBio, Beijing, China), which was composed of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and combined with RNA base-specific cleavage, was used to analyze TGF-β3-promoter methylation quantitatively (Gen-Bank Accession Number: NM_009368.3). PCR primers were designed using EpiDesigner (http://www.epidesigner.com). For each reverse primer, an additional T7-promoter tag for in vivo transcription was added, whereas a 10mer tag on the forward primer was used to adjust melting temperature differences. We used the following primers based on the reverse complementary strands of TGF-β3-promoter target region 1 (5′-aggaagagagTTTAGAAAGGGTTTAGGAGA TTTGG-3′ and 5′-cagtaatacgactcactatagggagaaggctCATTCCAAACCCCAAAATAA AAC-3′) and TGF-β3 promoter target region 2 (5′-aggaagagagTGGTTGTTTATTTG GAAAGTTTTTT-3′ and 5′-cagtaatacgactcactatagggagaaggctCCAACCTCCATACC TCTATCACTAA-3′′). Mass spectra were obtained by MassARRAY Compact MALDI-TOF (Sequenom, San Diego, CA) and the spectra methylation ratios were generated by EpiTYPER software version 1.0 (Sequenom). To validate the results from the MassARRAY platform (Sequenom), eight samples were chosen at random, including four control and four at RA-treated samples.

2.5. Western blot analysis

MEPM collection was as described in the DNA methylation analysis. Cell lysates were prepared using 2× SDS-lysis buffer supplemented with protease inhibitors (M250, Amresco, Ohio, USA) and phosphatase inhibitors (WB0117, Weiao Biotech, Shanghai, China). Protein concentration was determined using a standard BSA