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Determinants of orofacial clefting II: Effects of 5-Aza-2′-deoxycytidine on gene methylation during development of the first branchial arch

Ratnam S. Seelan, Partha Mukhopadhyay, Dennis R. Warner, Irina A. Smolenkova, M. Michele Pisano*, Robert M. Greene

Department of Molecular, Cellular and Craniofacial Biology, ULSD, University of Louisville, Louisville, KY 40202, USA

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ABSTRACT

Defects in development of the secondary palate, which arise from the embryonic first branchial arch (1-BA), can cause cleft palate (CP). Administration of 5-Aza-2′-deoxycytidine (AzaD), a demethylating agent, to pregnant mice on gestational day 9.5 resulted in complete penetrance of CP in fetuses. Several genes critical for normal palatogenesis were found to be upregulated in 1-BA, 12 h after AzaD exposure. MethylCap-Seq (MCS) analysis identified several differentially methylated regions (DMRs) in DNA extracted from AzaD-exposed 1-BAs. Hypomethylated DMRs did not correlate with the upregulation of genes in AzaD-exposed 1-BAs. However, most DMRs were associated with endogenous retroviral elements. Expression analyses suggested that interferon signaling was activated in AzaD-exposed 1-BAs. Our data, thus, suggest that a 12-h *in utero* AzaD exposure demethylates and activates endogenous retroviral elements in the 1-BA, thereby triggering an interferon-mediated response. This may result in the dysregulation of key signaling pathways during palatogenesis, causing CP.

1. Introduction

Orofacial clefts, which comprise cleft lip with or without cleft palate (CL/P) and isolated cleft palate (CP) are among the most common types of birth defects. In the United States, approximately 6800 babies with palatal clefts are born annually [1,2]. CP arises from abnormal morphogenesis of the embryonic secondary palate. Development of this structure begins with the emergence of ventrally directed oral projections (palatal processes) from the maxillary prominences and their subsequent reorientation and fusion to each other to form the roof of the oral cavity [3]. Defects in growth, reorientation or fusion of the palatal processes *per se* can contribute to CP. Also, since the maxillary process of the first branchial arch (1-BA) gives rise to the secondary palate [4], developmental disruption of the 1-BA can contribute to CP [5–9].

Both genetic and environmental factors can contribute to the development of CP. Among the latter are nutritional deficiencies, exposure to cigarette smoke, alcohol, drugs, pesticides, and microbial infection [4,10–14]. Epigenetic alterations such as aberrant gene methylation represent one means by which environmental insults could cause orofacial defects. Supportive of this premise is the observation that significant CpG island (CGI) and global DNA hypomethylation was observed in retinoic acid-induced CP [15]. Maternal dietary folate deficiency can also cause orofacial clefts [16,17]. This may, in part, be due to altered DNA methylation, as single carbon groups essential for DNA methylation are derived from folate metabolism [18]. More recently, dysregulation of *Wnt9b* due to the aberrant methylation of a nearby IAP transposon has been implicated in the etiology of CLP in A/WySn mice [19].

In mammals, DNA methylation typically occurs at cytosine residues within CpG dinucleotides, and is catalyzed by a family of active DNA methyltransferases (DNMTs) [20,21]. The cytosine analogue, 5-Aza-2′-deoxycytidine (AzaD) is a potent inhibitor of DNA methylation [22] and acts by forming a covalent adduct with cellular DNMT1, thereby inhibiting its activity [22]. This inhibition leads to a progressive loss of DNA methylation with each successive cell cycle [22,23]. AzaD treatment of murine fetuses on gestational day

Abbreviations: 1-BA, first branchial arch; AzaD, 5-Aza-2′-deoxycytidine; CGI, CpG island; CM, complete media; CP, cleft palate; CL/P, cleft lip with or without cleft palate; GD, gestational day; IPA, ingenuity pathway analysis; MCS, MethylCap-sequencing; MEMM, mouse embryonic maxillary mesenchymal; MRE, microRNA recognition element; NGS, next generation sequencing; qRT-PCR, quantitative real-time PCR; TSS, transcriptional start site.

* Correspondence to: Department of Molecular, Cellular and Craniofacial Biology, University of Louisville, ULSD, 501 South Preston Street, Suite 350, Louisville, KY 40202, USA.

E-mail addresses: rsseel01@louisville.edu (R.S. Seelan), p0mukh01@louisville.edu (P. Mukhopadhyay), dwarn01@louisville.edu (D.R. Warner), irina.smolenkova@louisville.edu (I.A. Smolenkova), drmikeky@gmail.com (M.M. Pisano), dr.bob.greene@gmail.com (R.M. Greene).

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(GD) 9.0 has been reported to induce palatal clefts [24,25], suggesting that aberrant DNA methylation could be the underlying cause of this defect. Because development of the secondary palate in mice occurs during GD 12–14, one can hypothesize that AzaD treatment affects the development of the precursor tissue, the 1-BAs, in these fetuses.

We have observed that pregnant mice exposed *in utero* to AzaD on GD 9.5 give birth to newborns that are completely penetrant for CP. The palatal processes in AzaD-exposed fetuses were comparatively smaller and remained unreoriented and unfused relative to those found in control fetuses at GD 15.5. These observations are consistent with the decreased cellular proliferation and increased apoptosis observed in the 1-BA of *in utero* AzaD-exposed fetuses (see accompanying study [26]). Moreover, significant alterations in the expression of numerous genes associated with cellular proliferation and apoptosis were observed in 1-BAs 12-h after *in utero* AzaD-exposure. Also included were genes associated with the Fgf, Wnt, SHH and Tgfβ signaling pathways and transcriptional regulation, all of which are known to play key roles in palatal morphogenesis (see accompanying study [26]). A number of these genes (82%) were found to be upregulated after AzaD exposure, presumably due to the effects of promoter demethylation. However, a preliminary analysis of CpG methylation levels in 10 selected genes exhibiting some of the most pronounced changes in gene expression in AzaD-exposed 1-BAs indicated that CpG methylation levels were not substantially altered when compared to unexposed, control tissues (see accompanying study [26]). To assess in greater depth the demethylating effects of AzaD on the observed changes in gene expression, we sought to identify differentially methylated regions (DMRs) in the DNA isolated from *in utero* AzaD exposed 1-BAs. We employed MethylCap-Seq (MCS), a method that couples isolation of DNA fragments enriched for methylated CpGs with next generation sequencing (NGS). In this study, we analyze the results of MCS analysis and provide plausible scenarios for how gene expression changes in 1-BAs, after *in utero* 12 h AzaD exposure, could contribute to CP.

2. Experimental procedures

2.1. Animals, AzaD dosing and characterization of the cleft palate phenotype

Animal maintenance, developmental staging of embryos, AzaD dosing regimen and identification of the CP phenotype are provided in detail in the accompanying study [26]. Briefly, pregnant mice (ICR strain) were injected with either AzaD (1 mg/kg) (Sigma Chemical Co., St. Louis, MO) or PBS (the vehicle) on GD 9.5 and 1-BA tissues isolated 12-h later for extraction of both RNA and genomic DNA. GD 15.5 control fetuses exhibited a normal secondary palate, whereas all *in utero* AzaD-exposed fetuses exhibited CP.

2.2. MethylCap-Sequencing analysis

Genomic DNA from AzaD- and vehicle-treated 1-BAs was processed using the MethylCap kit (Diagenode Inc., Denville, NJ). Processed samples, eluted with High-elution buffer, were submitted to the Advanced Genetic Technologies Center, University of Kentucky (Lexington, KY, USA) for MiSeq analysis using the Illumina platform (Illumina Inc. San Diego, CA). Sequencing was performed on two biological replicates.

DNA samples for MiSeq analysis were first analyzed on an Agilent High Sensitivity DNA chip to ensure that the size distribution was in the desired range of 200–800 bp. DNA concentrations were then normalized to obtain 100–200 pg/μl in a total volume of 50 μl. Library preparation was performed according to the TruSeq RNA Sample Prep Kit v2 protocol. Briefly, DNA was treated with End Repair Mix for 30 min at 30 °C and then purified using AmpureXP beads. The 3′ ends of the DNA fragments were then adenylated using A-tailing mix (30 min @ 37 °C), followed by ligation to barcoded Illumina adaptors (30 min @ 30 °C). After purification with AmpureXP beads, DNA was then fractionated by electrophoresis through a 2% agarose E-gel (Life Technologies) and fragments of ~500 bp were recovered from the gel and purified using Zymoclean columns (Zymo Research). Fragments were amplified using 18 PCR cycles with Illumina adaptor primers and then purified using AmpureXP beads. The resulting DNA populations were quantified on a Qubit fluorometer and analyzed on the Agilent Bioanalyzer to verify fragment size-distribution. Finally, libraries were quantified using the KAPA "Illumina Library Quantification Kit" and the individual libraries were pooled in approximately equimolar quantities to a final DNA concentration of 5–10 nM in a final volume of 10 μl. The pooled libraries were then loaded into a 500-cycle flowcell on the MiSeq machine.

Sequence reads from both input and methyl-captured DNA samples were aligned to the mm10 (GRCm38) reference genome using Bowtie2 [27] and the resulting .sam files were converted to .bam files using SAMtools [28]. After sorting the .bam files by genome coordinates, duplicate reads were removed using Picard (http://picard.sourceforge.net). Methylation peaks were called using MACS2 callpeak [29] and differential peaks were identified using MACS2 bdgdiff. Finally, gene annotations were added to the differential peaks using the annotatePeaks.pl script in the homer package (http://homer.salk.edu/homer) [30].

2.3. Putative enhancer analysis

The Vista Enhancer Browser (http://enhancer.lbl.gov/; [31]) was used to determine the association of a DMR with a functional enhancer element listed in the browser. The genomic coordinates of the DMR (mm10) were used to extract the DNA sequence from the UCSC Genome Browser (http://genome.ucsc.edu/). Sequences of the enhancer elements (mm9) were either directly obtained from the Vista Enhancer Browser or the genomic coordinates provided in the browser were used to download the sequences from the mm9 assembly of the UCSC Genome Browser. To identify if the DMR sequence matched that of the enhancer, the two sequences were matched against each other using the BLAST (NCBI) program.

2.4. Pathway analysis

In order to establish an overview of different signaling and biological pathways impacted in 1-BA tissues by *in utero* AzaD exposure, Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA) was performed as previously reported [32] on the following sets of genes: (1) the 68 common genes associated with hypomethylation found in both MCS datasets; and, (2) the core set of 17 genes which shared identical hypomethylated DMRs from the two MCS datasets.

2.5. AzaD treatment of mouse embryonic maxillary mesenchymal (MEMM) cells

MEMM cells were prepared as previously described [33]. Briefly, cells were seeded in three 8 × 10 cm dishes at 2×10^6 cells/10 ml of Complete Media (CM). AzaD (5 μM), prepared in CM, was added to the media the following day and the culture was maintained at 37 °C for 6 days, with replenishment of AzaD (5 μM) every two days. Untreated control cells were cultured in parallel with media (CM) replenishment every two days. Cells were harvested for RNA analysis, as described below.