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Activation of Notch1 inhibits medial edge epithelium apoptosis in all-trans retinoic acid-induced cleft palate in mice

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ABSTRACT

Administration of all-trans retinoic acid (atRA) on E12.0 (embryonic day 12.0) leads to failure of medial edge epithelium (MEE) disappearance and cleft palate. However, the molecular mechanism underlying the relationship between atRA and MEE remains to be identified. In this study, atRA (200 mg/kg) administered by gavage induced a 75% incidence of cleft palate in C57BL/6 mice. Notch1 was up-regulated in MEE cells in the atRA-treated group compared with the controls at E15.0, together with reduced apoptosis and elevated proliferation. Next, we investigated the mechanisms underlying atRA, Notch1 and MEE degradation in palate organ culture. Our results revealed that down-regulation of Notch1 partially rescued the inhibition of atRA-induced palate fusion. Molecular analysis indicated that atRA increased the expression of Notch1 and Rbpj and decreased the expression of P21. In addition, depletion of Notch1 expression decreased the expression of Rbpj and increased the expression of P21. Moreover, inhibition of Rbpj expression partially reversed atRA-induced MEE persistence and increased P21 expression. These findings demonstrate that atRA inhibits MEE degradation, which in turn induces a cleft palate, possibly through the Notch1/RBPjk/P21 signaling pathway.

1. Introduction

As a common maxillofacial malformation, cleft palate frequently occurs as a consequence of genetic and environmental factors [1]. Several steps are involved in secondary palate formation, including vertical growth of the palate, elevation, contraction and fusion, and any interruption of these processes can result in a cleft palate. After palate contact, two cell layers, the MES (medial edge seam), form and undergo reduction to a single cell layer. Finally, the MES disappears and a confluent palate forms. If the MES failed disappear, cleft palate develops [2].

As a morphogen of vitamin A, all-trans retinoic acid is recognized as a strong teratogen that can induce congenital malformations, including cleft palate and skeletal defects [3]. Two types of cleft palate have been attributed to different intragastric doses and to the stage of administration of atRA in pregnant mice. For example, the administration of 100 mg/kg atRA on E10.0 results in smaller palatal shelves with failed elevation. In contrast, the administration of 200 mg/kg atRA on E12.0 leads to the development of normal-sized palatal shelves that never fuse [4].

Epithelial-mesenchymal transformation (EMT), programmed cell death (PCD) and cell migration have been proposed as mechanisms for MES dissolution [5]. TGF-β, Shh, BMP, and Wnt signaling pathways have all been considered to contribute to palatal morphogenesis [2]. TGF-β3, which plays an essential role in palatogenesis, has been extensively studied [6]. It has been proposed that the MES independently but sequentially undergoes cell cycle arrest, cell migration, and apoptosis following the treatment of isolated MES cells with TGF-β3 [7]. Recent studies have demonstrated that TGF-β-mediated IRF6 is necessary for palatal fusion in mice and that TGF-β signaling is mediated by Tak1, Smad4 and Trim33 during the development of the palate [8,9]. As a conserved

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pathway, Notch signaling plays an important role in cell proliferation and differentiation. Activation of Notch signaling results in the release of NICD and binding to a number of transcription factors [10]. Recent studies have indicated that Jagged2-Notch1 signaling is involved in the development of the embryo palate [11]. Furthermore, TGF-β3 has been shown to partially regulate Jagged2 during MEE disappearance and palatal fusion [12].

However, the role of Notch signaling in atRA-induced MEE elimination during palatal fusion has not been studied in detail. In the present study, we investigated the role of Notch1 in atRA-induced cleft palate, and we found that atRA mediated MEE retention, in part via the Notch1/Rbpj/P21 signaling pathway.

2. Materials and methods

2.1. Mice

C57BL/6J mice were used in this study. One female mouse was mated with one male mouse in the morning. E0 was determined when a vaginal plug was detected. Pregnant females were administered 200 mg/kg all-trans retinoic acid (atRA, Sigma-Aldrich, St. Louis, MO), while the control group received the same dose of vector corn oil. Embryos were dissected on E13.5, E14.5, E15.0 and E15.5 and prepared for subsequent experiments. Permission was obtained from the Ethics Committee for Animal Experiments of Sun Yat-sen University for all animal experiments (IACUC: DB-15-0302).

2.2. Antibodies

Anti-Notch1 (ab52627), anti-Rbpj (ab180588), anti-P21 (ab109119) and anti-Ki67 (ab15580) antibodies were purchased from Abcam (Abcam, Cambridge, Britain). Anti-GAPDH (2118s) and anti-E-cadherin (3195) antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA).

2.3. Histological staining and TUNEL assay

After fixation in 4% paraformaldehyde for 24 h, embryos and cultured palatal shelves harvested from each group were dehydrated in an alcohol series and then embedded in paraffin. For immunohistochemical staining, 3 μm-thick continuous sections were deparaffinized and hydrated stepwise. The sections were then placed in a microwave for 20 min for antigen repair. Goat serum was used for antigen blocking after nonspecific peroxidase blocking in 3% hydrogen peroxide solution. After antigen blocking, the sections were incubated with the primary antibody at 4 °C for 12 h, and then with the secondary antibody at 37 °C for 30 min. The tissues were subsequently stained with 3, 3′-diaminobenzidine (DAB) and hematoxylin according to standard procedures. For immunofluorescence staining, Alexa 594-conjugated antibody was used. For the TUNEL assay, an apoptosis detection kit (Roche) was used following immunofluorescence staining. Images were captured using an Axio Imager Z2 (Carl Zeiss) microscope and analyzed using Image-Pro Plus 6.0 and Adobe photoshop CS6 software.

2.4. Palate organ culture and RNA interference

Palatal shelves from E13.5 fetuses were used for the palate organ culture. Briefly, palatal shelves were gently microdissected from the maxilla and placed on sterile MF-Millipore membranes (Merck Millipore) in cold Hanks buffer (Invitrogen, Carlsbad, CA). The two palatal shelves were then forced to contact one another at the medial edges, and the palates were cultured in BGJb medium (Invitrogen) supplemented with 100 U/ml penicillin-streptomycin (Gibco). Notch1 and Rbpj siRNA synthesized by RIBOBIO (Ribobio, China) were used to transfect the cultures using RNAiMAX (Invitrogen) diluted in OptiMEM (Invitrogen) at 100 nM according to the standard protocol. 12 h after transfection, the cultures were

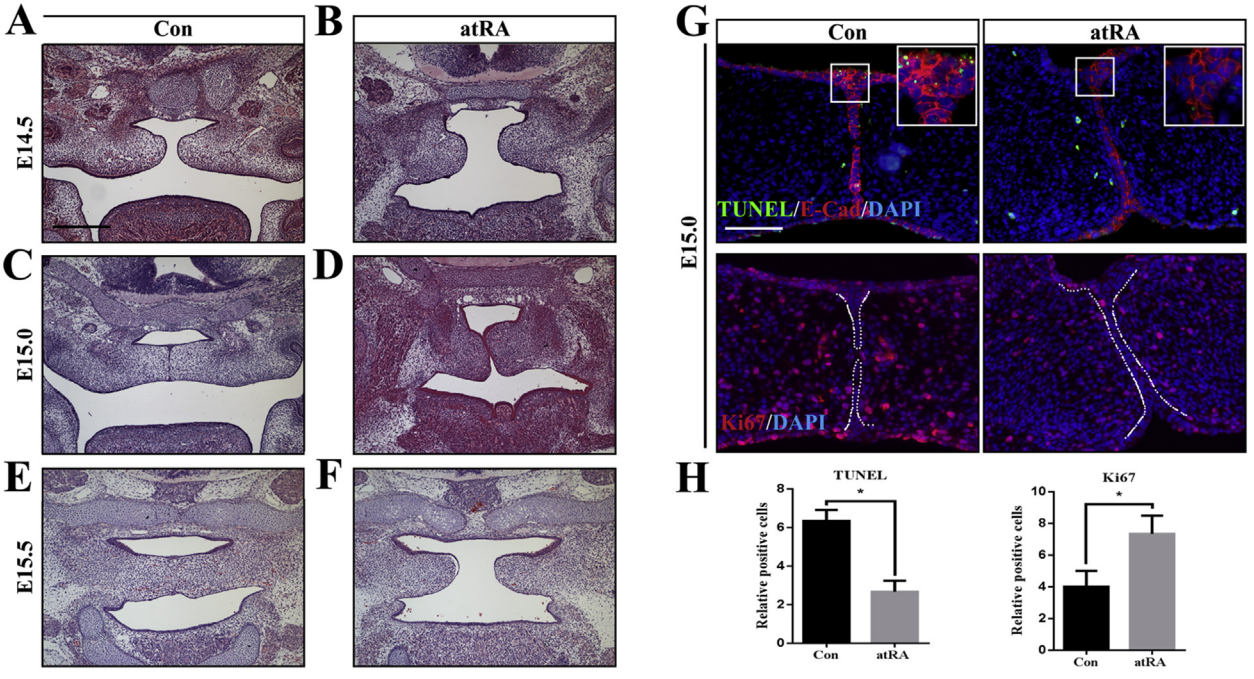

Fig. 1. atRA exposure on E12.0 induced MEE persistence and cleft palate in mice. (A) H&E staining showing that E14.5 palatal shelves of control and atRA-exposed groups (B) were elevated in the horizontal position. (C) At E15.0, the palatal shelves of control fetuses were in contact and fused in the middle region, but fusion failure was observed in the atRA-exposed group (D). (E) At E15.5, a compact palate formed in the control group, while in the atRA-treated group, a cleft palate formed (F). (G) TUNEL assay showing apoptosis in MEE cells in the control and atRA-treated groups. Ki67 was measured to determine proliferation in MEE cells in the control and atRA-treated groups. (H) Quantification of TUNEL-positive cells and Ki67-positive MEE cells in the control and atRA-treated groups. (n = 3, $^*p < 0.05$). Scale bar: 20 μm from A-F, 50 μm in G.