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Retinoic acid regulates embryonic development of mammalian submandibular salivary glands ☆

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ABSTRACT

Organogenesis is orchestrated by cell and tissue interactions mediated by molecular signals. Identification of relevant signals, and the tissues that generate and receive them, are important goals of developmental research. Here, we demonstrate that Retinoic Acid (RA) is a critical signaling molecule important for morphogenesis of mammalian submandibular salivary glands (SMG). By examining late stage RA deficient embryos of *Rdh10* mutant mice we show that SMG development requires RA in a dose-dependent manner. Additionally, we find that active RA signaling occurs in SMG tissues, arising earlier than any other known marker of SMG development and persisting throughout gland morphogenesis. At the initial bud stage of development, we find RA production occurs in SMG mesenchyme, while RA signaling occurs in epithelium. We also demonstrate active RA signaling occurs in glands cultured *ex vivo*, and treatment with an inhibitor of RA signaling blocks growth and branching. Together these data identify RA signaling as a direct regulator of SMG organogenesis.

1. Introduction

Saliva lubricates the mouth, aiding in speech, swallowing and digestion, neutralizing acids damaging to teeth, and protecting oral tissues. In mammals saliva is produced in large part by three salivary gland pairs-the submandibular, sublingual, and parotid-the formation and function of which have been well described in recent reviews (Holmberg and Hoffman, 2014; Knosp et al., 2012; Tucker, 2007). Formation of salivary glands during embryogenesis is an active area research, being important both to the field of developmental biology, and also because knowledge of organogenesis can serve as a paradigm for regeneration of adult organs (reviewed in (Patel and Hoffman, 2014)).

Each salivary gland is composed of numerous sac-like acini, where saliva is produced. These are connected to the oral cavity via a branching network of ducts. The acini and ducts, which are epithelial, are surrounded by nerve fibers, blood vessels, fibroblasts, and immune cells, all within the context of extracellular matrix. Current knowledge about formation of mammalian salivary glands is based largely upon studies of organogenesis of the SMG in mouse. Mouse mutations that cause defects in embryonic formation of salivary glands have been instrumental in identifying genes critical for gland formation. *Ex vivo* culture experiments of explanted mouse SMG tissues have yielded information about molecules and tissue interactions that regulate gland formation.

Development of the mouse SMG occurs as a progressive series of events, many of which involve interactions between different tissue types. Early in gland organogenesis, at embryonic day 10.5 (E10.5), a domain of epithelium within the developing mandible gains the ability to induce gland formation when combined with underlying pharyngeal arch mesenchyme (Wells et al., 2013). Genes or molecules responsible for the instructive signal are not known, nor have any markers of this early E10.5 pre-SMG territory been identified. At E11.5, the epithelium thickens to form a placode in the oral epithelium, a morphogenic event that coincides with a switch in instructive capacity from the epithelium to the mesenchyme (Wells et al., 2013). The placode epithelium subsequently invaginates into the underlying mesenchyme forming an initial bud by E12.5, which then branches to become a pseudoglandular structure by E13.5. The epithelium continues to grow and branch, a process dependent upon signals from the mesenchyme (Kratochwil, 1969; Kusakabe et al., 1985; Tucker, 2007; Wells et al., 2013), resulting in a highly branched tree-like structure of epithelial acini and ducts by E17.5. In addition to signals

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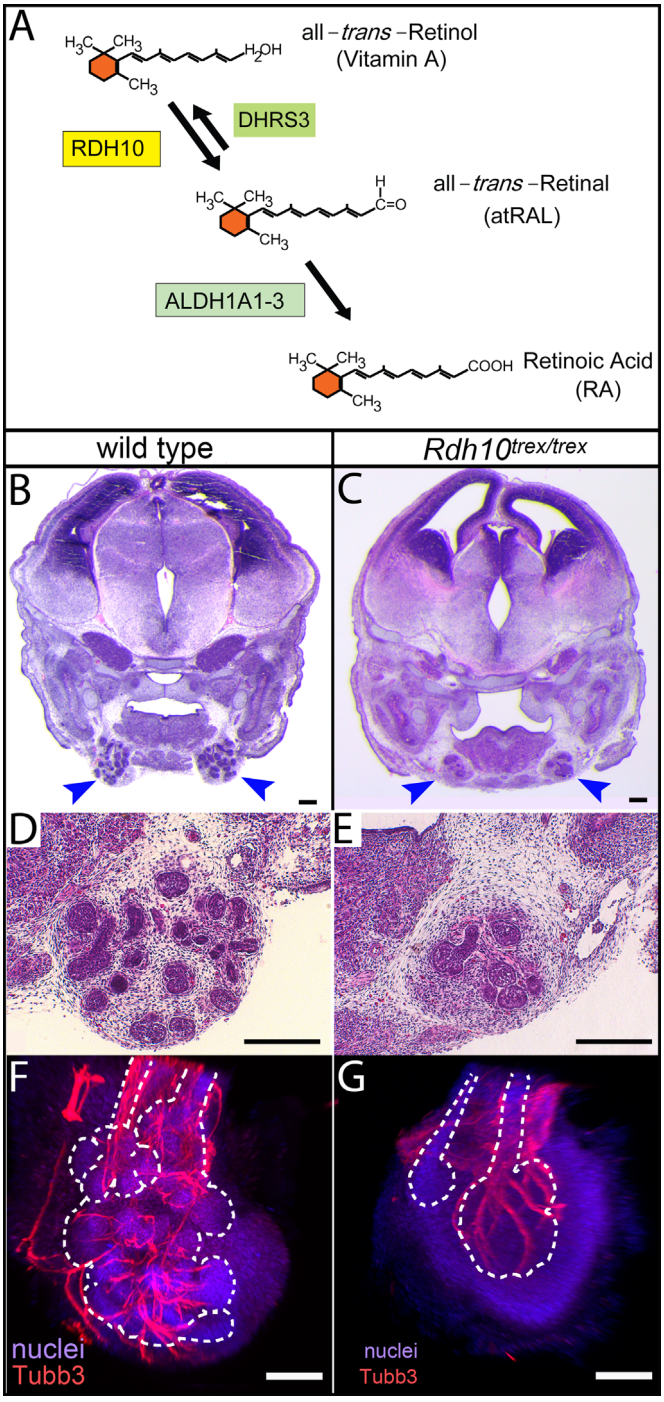

Fig. 1. Deficient production of RA in *Rdh10*$^{trex/trex}$ embryos impairs development of the SMG. (A) Vitamin A is converted into RA via two sequential enzymatic reactions. The first reaction, conversion of all-*trans*-Retinol (Vitamin A) to all-*trans*-Retinal (atRAL) is mediated within an embryo by RDH10. The first reaction is reversible, with the opposite reaction being mediated by DHRS3. The second reaction, the irreversible conversion of the intermediate atRAL to RA, is mediated by three aldehyde dehydrogenases, ALDH1A1, ALDH1A2, and ALDH1A3. Lack of RDH10 function results in severe RA deficiency and embryonic lethality usually prior to E11.5 (Sandell et al., 2007). (B–E) SMG development is impaired in *Rdh10*$^{trex/trex}$ embryos rescued to survive to E14.5 by maternal dietary supplementation with a minimal dose (40 μg) of the intermediate atRAL. Frontal paraffin sections through SMG of wild type and mutant embryos were stained with Hematoxylin and Eosin. SMG of 40 μg atRAL-rescued *Rdh10*$^{trex/trex}$ embryos (C and E) were substantially smaller than those of wild type littermates (B and D). Blue arrowheads indicate SMG. (F and G) Parasympathetic SMG ganglion and nerve development occurs in *Rdh10*$^{trex/trex}$ mutant embryos. Whole mount SMG from wild type (F) and *Rdh10*$^{trex/trex}$ mutant (G) embryos from 40 μg atRAL litters were immunostained for βIII neuronal tubulin (Tubb3), to reveal neurons of the parasympathetic ganglion and nerve, and with fluorescent nuclear stain Red Dot, to reveal all nuclei. For each gland a z-stack of confocal images was collapsed to form a single projection image. White dotted lines represent outline of gland epithelium visualized based on density of nuclear stain. Scale bars = 100 μm.

between epithelium and mesenchyme, there are also important are interactions between the SMG epithelium and the submandibular parasympathetic ganglion and nerve that are critical for development of the ganglion, and for maintaining epithelial progenitor cells in an undifferentiated state (Knosp et al., 2015; Knox et al., 2013, 2010).

Retinoic Acid (RA) is a diffusible small hormone-like molecule generated by a two-step enzymatic oxidation of dietary Vitamin A (Fig. 1A) (reviewed in (Duester, 2008; Niederreither and Dolle, 2008)). Vitamin A, also known as all-*trans*-retinol, is first converted into the metabolic intermediate all-*trans*-retinal (atRAL) via retinol dehydrogenase 10 (RDH10) (Sandell et al., 2007). The