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Modulation of estrogen causes disruption of craniofacial chondrogenesis in *Danio rerio*

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ABSTRACT

Estrogen is a steroid hormone that is ubiquitous in vertebrates, but its role in cartilage formation has not been extensively studied. Abnormalities of craniofacial cartilage and bone account for a large portion of birth defects in the United States. Zebrafish (*Danio rerio*) have been used as models of human disease, and their transparency in the embryonic period affords additional advantages in studying craniofacial development. In this study, zebrafish embryos were treated with 17-β estradiol (E_2) or with an aromatase inhibitor and observed for defects in craniofacial cartilage. Concentrations of E_2 greater than 2 μM caused major disruptions in cartilage formation. Concentrations below 2 μM caused subtle changed in cartilage morphology that were only revealed by measurement. The angles formed by cartilage elements in fish treated with 1.5 and 2 μM E_2 were increasingly wide, while the length of the primary anterior–posterior cartilage element in these fish decreased significantly from controls. These treatments resulted in fish with shorter, flatter faces as estrogen concentration increased. Inhibition of aromatase activity also resulted in similar craniofacial disruption indicating that careful control of estrogen signaling is required for appropriate development. Further investigation of the phenomena described in this study could lead to a better understanding of the etiology of craniofacial birth defects and endocrine disruption of cartilage formation.

1. Introduction

Craniofacial defects are among the most common birth defects in humans, affecting approximately one in one thousand live births (IPDTOC Working Group, 2011). Craniofacial defects such as orofacial clefts impact both the developed and developing world (Oginni and Adenekan, 2012). Though most craniofacial defects are not life threatening, individuals with craniofacial defects often have difficulties with eating, hearing, speech and respiration that can severely decrease quality of life. Both genetic and environmental factors are known to play a role in craniofacial defects in vertebrate embryos (Yu et al., 2009; Clouthier et al., 2010).

Estrogens are a family of nonpolar steroid molecules used in cell-cell communication, synthesized by the enzyme aromatase found in vertebrate embryos including zebrafish (Lassiter and Linney, 2007). Estrogens mediate many crucial processes in both the embryonic and adult stages of vertebrates and can also affect the organism through exogenous sources. Nuclear estrogen receptors (ERα and ERβ) dimerize when bound by estrogen, revealing a DNA-binding domain that induces many genomic effects (Lassiter et al., 2002; Tankó et al., 2008). Non-genomic effects of estrogen are triggered when estrogen binds to GPR-30, a transmembrane G-protein, activating phospholipase C, which in turn initiates a number of signaling cascades (Tankó et al., 2008; Jenei-Lanzl et al., 2010).

Though estrogen is commonly thought of as a sex hormone, it is also used in non-sexual processes including heart development (Allgood et al., 2013), bone formation (Gao et al., 2013), and chondrogenesis (Fujita et al., 2004; Fushimi et al., 2009). ERα and ERβ are present in chondrocytes in many different animals, including rats, monkeys, rabbits, cows, pigs, and humans (Tankó et al., 2008). Chondrocytes can produce estrogen, which protects the chondrocytes against apoptosis and stimulates them to proliferate *in vitro* (Chagin et al., 2006). ERα, ERβ, and GPR-30 are all present in mesenchymal stem cells and during chondrogenesis (Jenei-Lanzl et al., 2010). Human mesenchymal stem cells treated with estrogen in that study formed less cartilage likely due to a lack of extracellular matrix deposited. Additionally, estrogen-related receptors such as ERRγ have also been implicated in chrondrogenesis during embryonic development (Cardelli et al., 2013). Estrogen affects cartilage

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and bone development in a variety of fish species, including fathead minnow, tilapia, and zebrafish (Ng et al., 2001; Warner and Jenkins, 2007; Fushimi et al., 2009).

While some research has been done on the effects of estrogen disturbances on chondrogenesis in young animals, the effects of estrogen disturbances on cartilage development in the embryonic period remains an active area of research. *Danio rerio* (zebrafish) are a well-developed model and have been used extensively to study human disease (Ingham, 2009). The viscerocranial skeleton is well-conserved among vertebrates, so zebrafish can be used to model craniofacial development in humans (Kuratani et al., 1997). In addition, the transparency of zebrafish embryos allows for easy visualization of developmental processes. The use of an aquatic model system expands the applications of this study into the environmental realm, as many developed countries have estrogenic compounds in their waterways, mainly due to extensive use of estrogen-like pesticides and oral contraceptives (Rajapakse et al., 2002).

While others have investigated some of the effects of high estrogen dosage (greater than 5 μM) on craniofacial development in zebrafish (Fushimi et al., 2009), we wanted to determine the effects, if any, of more moderate concentrations of estrogen. We hypothesized that subtle craniofacial defects would occur at concentrations below those that give rise to gross morphological changes. We also hypothesized that inhibition of estrogen production would cause similar subtle changes based on a narrow window of optimal estrogen concentration that others have found (Takano et al., 2008). The present study is the first to quantitatively assess the effects of estrogen on craniofacial chondrogenesis by analyzing the size and shape of cartilage elements in the viscerocranium of zebrafish larvae.

2. Materials and methods

2.1. Fish husbandry and treatment

Adult zebrafish were maintained on a 14/10 h light/dark schedule on a diet of Omega One freeze-dried brine shrimp or live brine shrimp supplemented with TetraMin tropical flakes. Zebrafish embryos were raised in 0.3× Danieau with 0.2 mM phenylthiourea at 28.5 °C (1× Danieau is 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6).

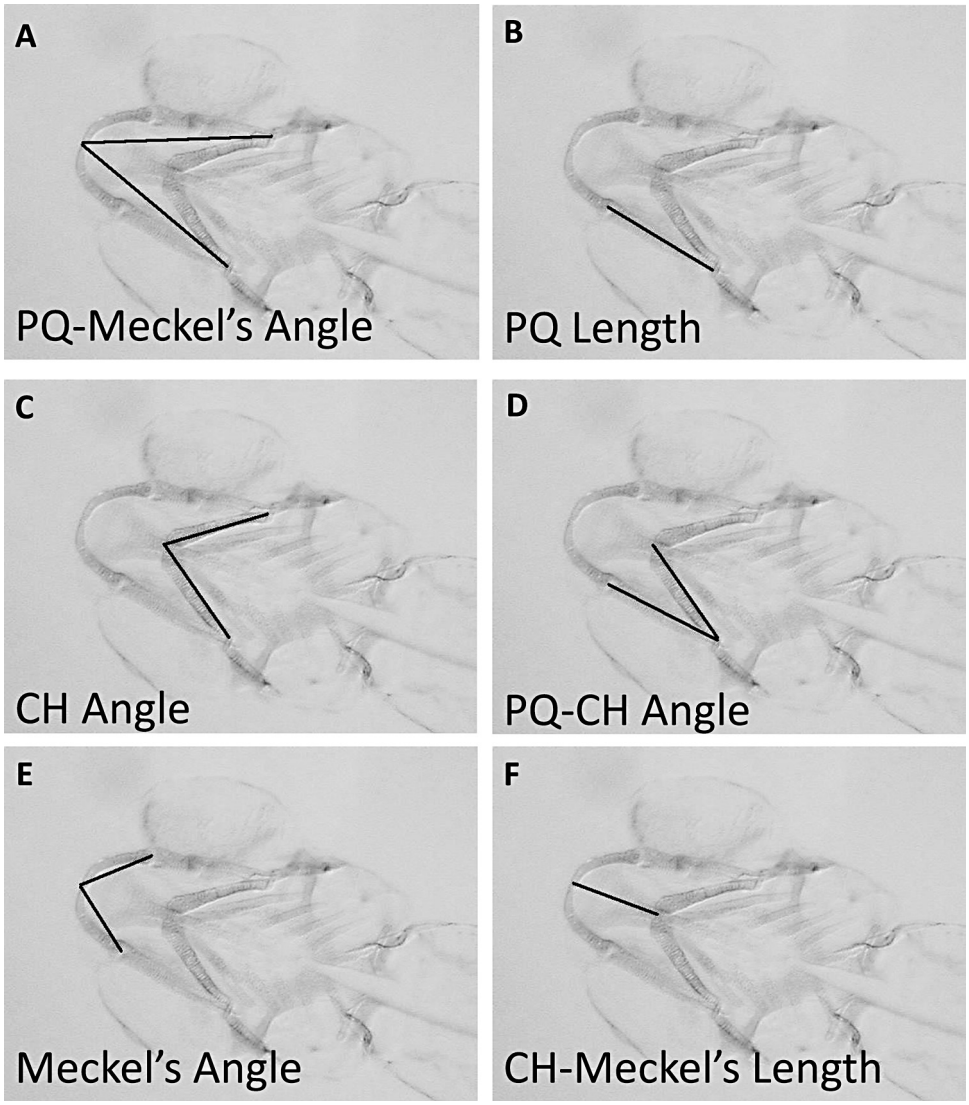

Fig. 1. Angles and lengths measured on Alcian Blue stained zebrafish embryos. The black lines represent the angles and lengths measured on each stained embryo. (A) PQ and Meckel's angle. (B) PQ length. (C) CH angle. (D) PQ and CH angle. (E) Meckel's angle. (F) CH to Meckel Length. PQ = palatoquadral cartilage, CH = ceratohyal cartilage. 50× magnification.