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Using frogs faces to dissect the mechanisms underlying human orofacial defects

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ABSTRACT

In this review I discuss how *Xenopus laevis* is an effective model to dissect the mechanisms underlying orofacial defects. This species has been particularly useful in studying the understudied structures of the developing face including the embryonic mouth and primary palate. The embryonic mouth is the first opening between the foregut and the environment and is critical for adult mouth development. The final step in embryonic mouth formation is the perforation of a thin layer of tissue covering the digestive tube called the buccopharyngeal membrane. When this tissue does not perforate in humans it can pose serious health risks for the fetus and child. The primary palate forms just dorsal to the embryonic mouth and in non-amniotes it functions as the roof of the adult mouth. Defects in the primary palate result in a median oral cleft that appears similar across the vertebrates. In humans, these median clefts are often severe and surgically difficult to repair. *Xenopus* has several qualities that make it advantageous for craniofacial research. The free living embryo has an easily accessible face and we have also developed several new tools to analyze the development of the region. Further, *Xenopus* is readily amenable to chemical screens allowing us to uncover novel gene-environment interactions during orofacial development, as well as to define underlying mechanisms governing such interactions. In conclusion, we are utilizing *Xenopus* in new and innovative ways to contribute to craniofacial research.

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1. Introduction

In all vertebrates the orofacial region develops from several facial prominences that grow and converge to surround the embryonic mouth. Multiple signals, transcription factors, and epigenetic regulators orchestrate the precise coordination of very complex processes that are required for orofacial development (reviewed in Ref. [1]). Since there are these numerous molecular and morphogenetic events, it is not surprising that defects in the face including cleft lip and palate are the most common birth defect worldwide. *Xenopus* has emerged as an effective model to dissect the mechanisms underlying orofacial defects. Not only is orofacial development well conserved in this species [2], it also offers many advantages over studies in mammals, chick and zebrafish. For example, developmental experiments can be performed easily in free living embryos that are also large in size, develop rapidly, and can be obtained in great numbers simultaneously. Further, the orofacial region is readily visible, unlike the other model vertebrates where head flexure obscures easy viewing of the mouth (Fig. 1A).

Molecular gain and loss of function experiments are routine in *Xenopus* and we have advanced such experiments by developing a method to spatially regulate agents and proteins using "face transplants" [3] (Fig. 1B). This technique has provided a major leap forward in our ability to study the complexity of orofacial development since it allows for region specific loss or gain of function in all the cell types in the face. This has not been possible with promoter driven gene expression in mammals and fish since no single gene is expressed in all the tissues at the same time in the face. Importantly, face transplants allow us to examine the roles of proteins in the whole orofacial region without worrying about non-specific effects or viability problems in the whole embryo. My lab has also recently pioneered a face explant technique (Fig. 1C) that will allow us to study the particular signaling and mechanical influences of cranial structures on orofacial development.

Finally, the embryonic face is a morphologically complex structure and thus we needed a robust method to assess changes in its development. We therefore adapted geometric morphometrics (Fig. 1D) combined with traditional measurements to the larval frog face [4,5]. This quantitative analysis allows us to easily distinguish between the subtle differences in the size and shape of the face during orofacial development. Moreover, the analysis of craniofacial defects arising from synergistic effects of genes and/or environmental factors will be greatly improved by such a method. It can also reveal even slight improvements of an orofacial defect and thus a useful guide in analyzing potential therapeutics.

In summary, using *Xenopus* for studies of orofacial development allows for an innovative multidisciplinary approach; we can perturb and visualize molecular and cellular aspects in the whole embryo and in vitro, using a combination of modern microscopy, molecular assays, and classical embryology. *Xenopus* is therefore an excellent system to connect the molecular mechanisms and the complex three dimensional tissue morphogenesis that is critical for a better understanding of orofacial development. In this review we will summarize how we are using *Xenopus* to dissect the developmental processes underlying human orofacial birth defects such as persistent buccopharyngeal membrane, choanal atresia and median oral clefts. Further, I will discuss how *Xenopus* has become an ideal model for testing gene-environment interactions in orofacial malformations.

2. Embryonic mouth development

2.1. Formation of the embryonic mouth

The mouth forms from a complex series of growth and fusions of the embryonic facial tissues. Its formation creates an opening to the digestive system in all metazoan, without it animals cannot eat. The initial opening between the gut and the external environment is termed the embryonic or primary mouth [6]. Remarkably, despite its obvious importance, there have been few studies that address the molecular and cellular mechanisms required for embryonic mouth formation. This is surprising as one can imagine that abnormalities in the development of this structure could have devastating effects on the formation of the adult mouth. Moreover, can a mouth even be called a mouth without a connection to the digestive tract? Nevertheless, the embryonic mouth is not often considered in studies of orofacial evolution, development and birth defects, and is rarely mentioned in developmental or anatomical textbooks.

The embryonic mouth develops from a region in the extreme anterior along the midline that is devoid of mesoderm [2,6]. From this region, recently termed the Early Anterior Domain or EAD [7], the cement gland and anterior pituitary also originate [2]. Multiple morphological changes that transform the embryonic mouth anlage into an opening that is continuous with the digestive tract have been identified [6]. The first change observed is the dissolution of the basement membrane that separates ectoderm from endoderm in the EAD. This critical step in embryonic mouth development requires the inhibition of Wnt signaling [3]. The Wnt inhibitors, Frzb-1 and Crescent, decrease the expression of extracellular matrix components, laminin and fibronectin. Tabler et al [8] also showed that sonic hedgehog signaling may be upstream of Wnt signaling in regulating basement membrane dissolution. Soon after the basement membrane disappears, the cranial neural crest migrates anteriorly to surround the presumptive embryonic mouth. Guidance of the neural crest to the orofacial region is regulated in part by kinin–kallikrein signaling that originates from the EAD [7]. Importantly, this emphasizes the idea that, in addition to giving rise to the embryonic mouth, the EAD is a signaling center that coordinates the development of the surrounding face. Later, during tadpole stages, the embryonic mouth anlage invaginates to form the stomodeum. This structure is a well conserved hallmark of mouth development since it is reported in almost all metazoans [9–11]. In *Xenopus*, as the stomodeum is forming, there is a burst of cell death in the region. It is unclear why this occurs, but we have speculated that it is necessary to help thin the stomodeal tissue in preparation for perforation [6]. The last and most defining step is the actual perforation of the tissues covering the digestive tube, called the buccopharyngeal membrane. This perforation or rupture occurs at approximately two and a half days in *Xenopus* and by the fourth week of development in humans ([12]). The mechanisms that regulate the process of buccopharyngeal membrane rupture are completely unknown and therefore my lab has begun to explore this process in more depth.

2.2. Defects of the embryonic mouth: persistent buccopharyngeal membrane and choanal atresia

When the buccopharyngeal membrane fails to perforate in humans it causes a defect known as persistent buccopharyngeal membrane [12,13]. This condition on its own is very rare but can also present in conjunction with other congenital syndromes (Table 1A) and cleft palate [14–16]. A persistent buccopharyngeal membrane prevents the fetus from inhaling and swallowing amniotic fluid which is necessary for proper lung and digestive tube development [17,18]. It can also cause a condition where amni-