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What Experimental Embryology Can Teach Us About the Development of the Extraocular Muscles in Anophthalmia:

At the Interface of Basic and Clinical Sciences

Linda K. McLoon, PhD

Departments of Ophthalmology and Neuroscience, University of Minnesota, Minneapolis

Birth defects affect the health and development of children, particularly because concomitant lifelong disabilities can ensue. Anophthalmia and microphthalmia, birth defects that are important causes of childhood blindness, have a national prevalence of 1.87 per 10 000 live births, and an estimated 780 cases occur annually in the United States.¹ Clinical care for patients with congenital anophthalmia and severe microphthalmia frequently requires surgical reconstruction to restore orbital size and placement of orbital implants,² which are secured by their attachment to the extraocular muscles (EOMs). Thus, if the EOMs are functional, orbital implants can provide movement of and a more natural appearance for the prosthesis. In the study by Bohnsack et al³ in this issue of the *Archives*, the magnetic resonance imaging results of 3 patients demonstrate the spectrum of the morphologic characteristics of EOMs observed in patients with anophthalmia and severe microphthalmia. These results range from an essentially normal configuration (despite the near absence of the globe) to undetectable muscle tissue. The reason for these significant differences is not understood and cannot be predicted based on the ultimate phenotype of the EOMs. Bohnsack et al³ investigated the potential basis for these differences by comparing the timing of eye loss with the formation of the EOMs, using molecular biological and classical embryologic tools in zebrafish and chick embryos. Although this approach may seem unconventional, combining the clinical observation of variable formation of the EOMs in patients with anophthalmia with a basic-science experimental approach directed at answering questions raised by the observations proved to be quite powerful.

GENETICS OF ANOPHTHALMIA AND SEVERE MICROPHTHALMIA

It is useful to consider what is currently known regarding the underlying genetic mutations that cause anophthalmia and microphthalmia and their known potential effects on the development of the EOMs. Prenatal exposure to specific molecules that bind to receptors and cause downstream changes in various signaling factors (eg, retinoic acid)⁴ is associated with eye loss. Approximately 25% to 30% of patients with anophthalmia and/or microphthalmia have been identified to harbor significant chromosomal abnormalities.^{5,6} Understanding of the control of the eye and the formation of the EOMs is made more complex by the large number of distinct gene mutations associated with these malformations and related syndromes.⁷ Single gene mutations identified thus far include an array of development transcription factors, including *SOX2* (OMIM 184429), *PAX6* (OMIM

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Correspondence: Dr McLoon, Department of Ophthalmology, University of Minnesota, 2001 6th St SE, Minneapolis, MN 55455 (mcloo001@umn.edu).

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607108), *CHD7* (OMIM 608892), and *BCOR* (OMIM 300485); these factors interact with each other during development. This makes it difficult to determine whether a given ocular anomaly is a primary gene effect or related to secondary, downstream effects on a different gene product. One such example is the interaction between *SOX2* and *PAX6*, in which a decrease in *SOX2* expression results in a large increase in *PAX6* expression, which, in turn, results in concomitant microphthalmia.⁸ This example underscores the complexity in determining the cause of anophthalmia and related anomalies.

DEVELOPMENT OF THE EOMs

Until recently, little was known regarding the genetic control of the development of the EOMs. The gene that controls the formation of somitic musculature, *PAX3* (OMIM 606597), does not play a role in the formation of the EOMs.⁹ Instead, the formation of the EOMs requires the expression of *PITX2* (OMIM 601542); furthermore, the overall size of an individual EOM is dependent on the *PITX2* gene dosage.^{10,11} However, a complex interaction takes place between different embryonic precursor cells that will form the globe and the EOMs and the molecules that regulate their differentiated fate. The globe is primarily derived from neural and surface ectoderm; however, periocular mesenchyme surrounding the optic vesicle during early development give rise to many mature cell lineages in the eye and the orbit. These include structures composing the aqueous humor outflow tract, the sclera, the corneal endothelium and stroma, the ocular blood vessels, and the EOMs,^{12,13} which are derived from periorbital mesodermal cells. Mixed among the periorbital cells of mesodermal lineage are neural crest cells that migrate secondarily to the region. The ultimate position of the EOMs in the orbit depends on retinoic acid signaling derived from the neural crest cells that migrated into the periorbital region.¹⁴ *PITX2*, the gene responsible for EOM differentiation, is a retinoic acid-responsive gene. In the absence of neural crest cells, no retinoic acid is released. Some muscle tissue develops from mesodermal primordial cells that survive (most die), but the muscle forms in an ectopic location.¹⁵ Neural crest cell migration is, in turn, related to the presence of the developing eye, and in eyeless (ie, *RX*) mutations, the neural crest cells fail to migrate properly.¹⁶ The transcriptional control of these processes is not well understood, and thus far, at least 5 early transcription factors that include *FOXC1* (OMIM 601090), *FOXC2* (OMIM 602402), *PITX1* (OMIM 602149), *PITX2*, and *MYOG* (OMIM 159980) are expressed in distinct patterns during early ocular development; these appear to play a role in the differentiation and organization of the EOMs.¹³

TEMPORAL RELATIONSHIP BETWEEN PHENOTYPE OF EOMs AND ANOPHTHALMIA

The basic question from a clinical standpoint is whether the presence or absence of EOMs could provide insight into the timing of when a particular mutation acts to cause an anophthalmic orbit. Bohnsack et al³ approached the clinical finding of different content and configuration of the EOMs in patients with anophthalmia by examining the temporal relationship between the loss of the developing eye and the final morphologic characteristics of the EOMs. Using modern molecular biological and classical embryologic techniques, they varied the timing of eye loss in zebrafish and chick embryos and subsequently assessed the organization of the EOMs resulting from each embryonic perturbation. By changing 1 variable, ie, the timing of eye loss, they demonstrated that the organization and function of the EOMs had a temporal dependence on when eye loss occurred during development.

To test whether the initial formation of an eye vesicle was needed for the organization of the EOMs, Bohnsack et al³ examined the *rx3/chokh* mutant zebrafish, a fish model for *RX* mutations in humans that result in anophthalmia.¹⁷ The *rx3/chokh* mutant zebrafish never

forms an eye because optic vesicle evagination is prevented.¹⁸ Under this condition, no discrete EOMs were found, although small clusters of disorganized muscle tissue were present. This observation resembled the clinical observation derived from the magnetic resonance imaging results of patient 3 in the study by Bohnsack et al,³ in whom the EOMs were barely identifiable.

To test whether neural crest cells were necessary for formation and normal organization of the EOMs, Bohnsack et al³ surgically removed the optic vesicle in zebrafish embryos before or after neural crest cells had migrated into the periorbital mesenchyme but before differentiation of the EOMs. If the eye was removed prior to neural crest cell migration, EOMs were small and abnormal. If the eye was removed after neural crest cell migration, well-developed EOMs were found. These temporally spaced surgical interventions demonstrated a direct relationship between neural crest cell migration into the periorbital mesenchyme and formation of the EOMs and the time of eye removal and formation of the EOMs. These experimental results were confirmed by specific knockdown of the neural crest transcription factor *SOX10* (OMIM 602229) using morpholino oligonucleotide injections to prevent neural crest cell migration. As with the surgical manipulations, a temporal relationship was observed between the presence of functional neural crest cells and the formation of the EOMs. The phenotype in zebrafish seen after early enucleation or after inhibition of neural crest cell migration resembled that reported by Bohnsack et al,³ namely, that in patient 3, EOMs were barely discernable. The phenotype observed in the zebrafish when enucleation was performed after neural crest cell migration was similar to that seen in patients 1 and 2 from the same study, in whom EOMs were normal in position and in size or normal in position but reduced in size. This temporal correlation between eye removal before or after neural crest cell migration into the periorbital mesenchyme and organization of the EOMs was confirmed in similar experiments performed in chick embryos. The results of these embryologic studies, combined with the observations in human patients, strongly suggest that the variability in development of the EOMs seen in children with anophthalmia is linked to the temporal onset of ocular mal-development and, specifically, to the timing of eye loss relative to the timing of neural crest cell migration.

Ocular developmental teratogens, such as retinoic acid¹⁴ or influenza B virus,¹⁹ as well as a large number of genes, including *CHX10* (OMIM 142993), *GDF6* (OMIM 601147), *OTX2* (OMIM 600037), *RAX* (OMIM 601881), *SOX2*, *PAX6*, *CHD7*, *SIX3* (OMIM 603714), *HESX1* (OMIM 601802), *SHH* (OMIM 600725), and *BCOR*,^{7,20} have been linked to the occurrence of anophthalmia and severe microphthalmia. Severity of the ocular phenotype results in a spectrum of ocular anomalies, from coloboma to anophthalmia, variable even with identical gene mutations.^{21,22} The study by Bohnsack et al³ suggests that careful examination of the organization of the EOMs in children with anophthalmia and severe microphthalmia can provide insight into the timing of disruption of their ocular development, whether teratogenic or genetic. Also, it potentially may provide guidance for genetic screening and, ultimately, for genetic counseling for families with a child who has anophthalmia or severe microphthalmia.

This study also suggests many additional testable hypotheses that can be directly answered through laboratory studies. If configuration of the EOMs (ie, number, size, and location) are a window into the timing of a genetic or teratogenic insult, then focused experiments can be performed to address the potential cause of anophthalmia and how specific gene products control cell fate in early ocular development. For example, what is the timing of onset of the various genes associated with anophthalmia and severe microphthalmia? Which of these genes are expressed in neural crest cells? Does deletion of a gene or reduction in gene dosage result in neural crest cell migration defects and, concomitantly, anophthalmia? Can

neural crest cell migration be restored by the addition of a single missing gene product? Will doing so rescue the formation of the EOMs?

THE INTERSECTION OF CLINICAL PRACTICE AND BASIC SCIENCE

On another level, the article by Bohnsack et al³ is a testimony to the value of integrating clinical observations with basic scientific experimentation. Modern Departments of Ophthalmology exist in a world of managed care and increasing dependence on clinical earnings to cover salaries and operational expenses; economic pressures are strong. In this environment, what is the role of basic and translational science in a clinical department? Also, what are the advantages in ensuring that biomedical researchers (ie, physicians, physician-scientists, and basic-science faculty members) have regular avenues for exchange of ideas and knowledge in clinical departments within medical schools?

Bohnsack et al³ made the important clinical observation that despite a similar phenotype, namely, the presence of anophthalmia, a spectrum of phenotypes exists relative to the morphologic characteristics of the EOMs in this group of patients. Only an investigator with clinical knowledge and a background in classical embryologic approaches could devise this particular hypothesis and the experiments to test it. Clearly, this combination was critical in combining the clinical question with the potential basis for the spectrum of 1 part of the complex developmental anomalies seen in anophthalmia.

Although chance may favor the prepared mind, as Louis Pasteur once opined, it is equally likely that the prepared mind is better able to see patterns and connections and to purposefully design experiments. But the prepared mind also requires the time and the resources to design and to perform the necessary experiments. The study by Bohnsack et al³ is a perfect example of the synergy that results from the collaborative use of clinical and basic-science expertise. It reinforces the value of the physician-scientist and the need to ensure that academic physicians have sufficient protected time to think, to collaborate, to perform experiments, and to address fundamental questions that arise from their clinical observations. For the basic-science faculty member in a clinical department and for the academic physician without specific training in basic-science techniques, the same blending of clinical and basic-science expertise is critical to progression in understanding the causes of and in the development of potential treatments for the many disorders that result in vision loss. This synergy is possible only through conversation, proximity, and some structure that allows for discussion of current problems and their potential solutions on a daily or weekly basis.

Many clinical academic departments in medical schools throughout the United States are moving away from the classic model of faculty members (ie, physicians, physician-scientists, and basic-science faculty members) toward a model that resembles private practice. It is important to consider what we gain, as a discipline and as a society, from the synergistic interactions of faculty members with different types of expertise working together to address clinical problems, as well as what we will lose if we allow those interactions to disappear based on monetary concerns. The question and the struggle remain regarding how best to support and nurture basic-science faculty members and physician-scientists within academic clinical departments, but I contend that the cost is much less than the return on our collective investment.

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