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REVIEW

Genetics of congenital eye malformations: insights from chick experimental embryology

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Abstract
Embryological manipulations in chick embryos have been pivotal in our understanding of many aspects of vertebrate eye formation. This research was particularly important in uncovering the role of tissue interactions as drivers of eye morphogenesis and to dissect the function of critical genes. Here, we have highlighted a few of these past experiments to endorse their value in searching for hitherto unknown causes of rare congenital eye anomalies, such as microphthalmia, anophthalmia and coloboma. We have also highlighted a number of similarities between the chicken and human eye, which might be exploited to address other eye pathologies, including degenerative ocular diseases.

Introduction

Much of our current knowledge on how vertebrate embryos develop derives from studies performed in the embryo of the chicken (*Gallus gallus domesticus*). Back in the fourth century BC, Aristotle was the first to observe, describe and interpret the changes that an embryo undergoes inside its egg (Needham 1959). Since then, the chick embryo has been an invaluable tool in establishing basic embryological concepts such as, for example, tissue competence, or enabling the discovery of key developmental factors, including nerve growth factor (NGF) and sonic hedgehog (Shh). To the same extent that the chick has been the embryologist's organism of choice, the eye has been the frequent organ of focus. In both cases, the reason is likely to be the same: the chick as a whole and the eye as an organ are easily accessible and amenable to experimental manipulations and culture conditions. It follows that classical embryological studies using the chick eye have established much of our current knowledge on how this organ develops and provided clues towards understanding the basis of human congenital eye malformations. Despite this important "historical" role, the chick embryo has currently lost some ground, at least as a model system for understanding eye diseases. This has been in favour of other species, such as the mouse or the zebrafish, which are more amenable to genetic manipulations. Should we be using the chick more often as a paradigm to gain insights into human genetic eye malformations and other ocular diseases?

There are a number of excellent and recent reviews that answer this question and advocate well for the advantages (without forgetting the disadvantages) of the chick as a model to study eye development and its disorders. The reader is referred to them for more information (i.e. Vergara and Canto-Soler 2012; Wisely et al. 2017). In this perspective article, we have simply attempted to make the case that revisiting some of the past experiments in chick embryos may be useful to predict yet undiscovered causes of congenital eye malformations. To this end, we have selected a few examples among those studies that have linked eye malformations with relevant eye tissues' cross-talks, where we believe that the chick embryo has been a suitable model system for their understanding.

Congenital eye anomalies

The most common group of congenital eye malformations include microphthalmia (significant reduction in the axial length of the globe), anophthalmia (complete absence of the ocular globe) and coloboma (heterogeneous conditions

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characterized primarily by failure of optic fissure closure, but that can also affect the iris, etc.) or, as they are collectively known, MAC. MAC are interrelated and rare anomalies that can occur in isolated form or associated with other malformations in complex syndromic conditions. They can lead to severe visual deficits, and affect the size of the eye and/or visual axis, and can account for up to 11% of infant blindness in developed countries (Williamson and FitzPatrick 2014). MAC are generally caused by biallelic, hemizygous or heterozygous mutations—either hereditary or de novo—in evolutionarily conserved eye developmental genes, although environmental factors have also been reported (Williamson and FitzPatrick 2014). To date, at least 100–200 candidate genes responsible for microphthalmia and anophthalmia have been identified. These are mostly transcription factors that are expressed in the brain or eye (lens/retina) gene regulatory networks (Beccari et al. 2013; Cvekl and Zhang 2017), such as SOX2, OTX2, VSX2/CHX10, BCOR, STRA6, RAX, and FOXE3, key components of cell-to-cell communication, or genes involved in retinal progenitors' proliferation (Richardson et al. 2017; Williamson and FitzPatrick 2014). The list of genes causing human coloboma is also relatively large and depicts a "coloboma gene network", comprising transcription factors and signalling molecules largely related to ventral eye patterning (Gregory-Evans et al. 2004; Richardson et al. 2017). Despite these notable advances, only a proportion of MAC patients receive accurate molecular diagnoses, indicating the existence of additional causative genes and of possible alternative developmental mechanisms responsible for ocular malformations. The identification of yet unknown genes responsible for MAC may come directly from the currently used genome sequencing approaches. However, new candidates may also be found among molecules involved in eye tissue interactions, the discovery of which may have been due in large part to experiments performed in chick embryos.

Linking eye tissue interactions occurring in chick embryo with human eye malformations

In all vertebrates, eye formation starts with the specification of the eye field within the anterior neural plate. In birds, as in mammals, the neural plate folds to form the neural tube. Concomitantly, cells of the eye field, which initially occupy a medial position in the anterior neural plate (Fernandez-Garre et al. 2002), become displaced with an as-yet-unexplored mechanism and protrude from the rest of the neural tube, under the influence of the abutting axial meso-endoderm. The result of this morphogenetic event is the formation of two lateral protuberances, known as optic vesicles. These vesicles are composed of a pseudostratified and apparently homogeneous neuroepithelium, which, upon thickening of the overlying ectoderm into the lens placode, begin to infold to generate two bi-layered optic cups (Hilfer 1983). Optic cup morphogenesis also involves the formation of a transient opening along the ventral retina, termed the optic fissure, which will close after the ingression of the peri-ocular mesenchyme (POM) that gives rise to the retinal vasculature.

Thus, the eye forms due to the contribution of different tissues, the behaviour of which is coordinated by mutual signalling and inductive events (Fig. 1). The developmental anomalies resulting from failure of these tissues to communicate caught researchers' attention as early as the beginning of the twentieth century when chick eye tissues or the eye as a whole began to be cultured or grafted onto the flank of host embryos.

Retinal–POM interactions

Grafting approaches were further aided by the identification of natural chick mutants, such as "Creeper" that displays "phocomelic" limbs and microphthalmic and colobomatous eyes. By grafting Creeper or wild-type (wt) donor optic vesicles onto the flank of wt host embryos, Kenneth Gayer (1942) postulated whether wt vesicles would acquire a Creeper phenotype, or if the phenotype of Creeper vesicles could be ameliorated. Indeed, he noted that wt vesicles, isolated from the surrounding POM, developed coloboma together with abnormalities in the sclera and retinal pigmented epithelium (RPE). On the other hand, sclera defects of grafted Creeper vesicles improved, but the coloboma trait remained. Given our current knowledge, these results are quite fascinating because they provided the first evidence that retina–POM interaction is fundamental to fissure closure, as recently demonstrated using similar grafting experiments in zebrafish (Gestri et al. 2018). Sequencing of the Creeper genome recently revealed a mutation in the *Indian hedgehog* (*Ihh*) gene (Jin et al. 2016). *Ihh*, a member of the hedgehog (Hh) family of signalling proteins, is highly expressed in the mesenchyme associated with different organs, including the POM. Genetic inactivation of the *Ihh* gene in mouse causes abnormalities in the pigmentation of the RPE and in the condensation of the mesenchyme that is required for sclera formation (Dakubo et al. 2008). Thus, $Ihh^{-/-}$ mice recapitulate the ocular phenotype observed in the Creeper chick embryos, with the exception of the fissure coloboma. Notably, Hh family members are involved in optic fissure formation (Morcillo et al. 2006; Wang et al. 2005) and their expression patterns are conserved across chicken, mouse and human (Bakrania et al. 2010; Schimmenti et al. 2003). Thus, the most likely explanation for this difference is that other factors expressed in the POM, but not in the flank mesenchyme, are the cause of the coloboma observed in the

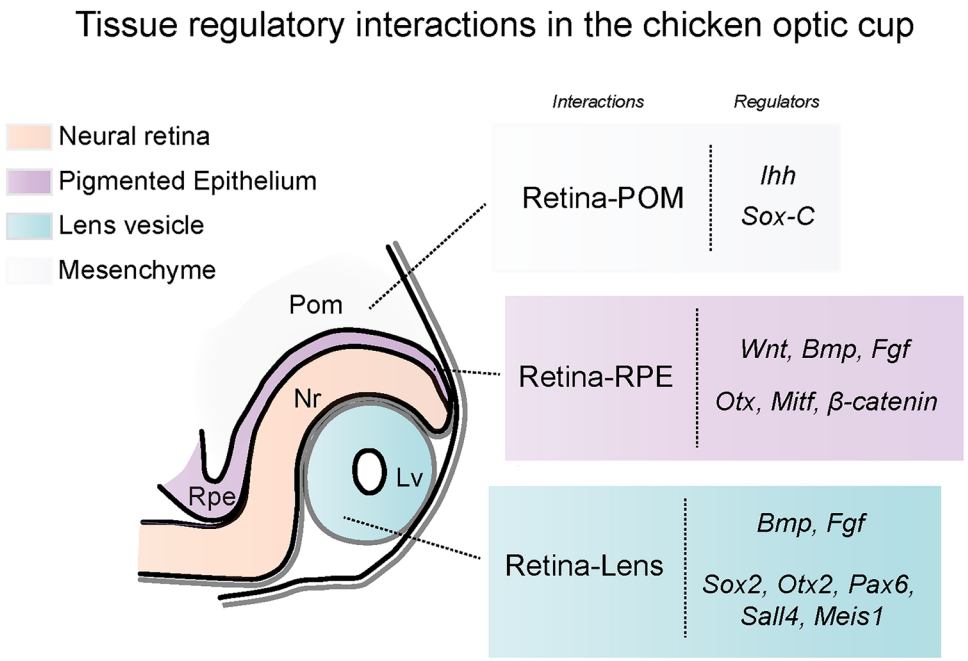

Fig. 1 Tissue regulatory interactions in the chicken optic cup. The scheme represents inductive and regulatory interactions among eye tissues that have been characterized during optic cup development in the chicken. The main eye territories (*Nr* neural retina, *Rpe* retinal pigmented epithelium, *Lv* lens vesicle, and *POM* periocular mesenchyme) and the different tissue interactions are colour coded. The few regulators of relevant tissues' interactions, either signalling molecules or transcription factors, discussed in the text are indicated in the appropriate coloured box

Creeper eye. In zebrafish, knock-down of *sox11* and *sox4*, two transcription factors of the SoxC subfamily expressed in the POM, results in ocular coloboma (Wen et al. 2015). Thus, searching for downstream targets of SoxC genes, using the easily dissectible chick POM, might aid in the identification of additional causes of coloboma. Some of them might be linked to the control of Ihh signalling given that, in zebrafish *sox4* morphants, *ihh* expression seems up-regulated, and its abrogation seems to counteract the coloboma of *sox4* morphants (Wen et al. 2015).

Retinal–lens interactions

The apposition and growth coordination between the lens ectoderm and the optic vesicle neuroepithelium is another example of tissue interaction that has been extensively studied in chick embryos (Coulombre and Coulombre 1964). Notably, disruption of this interaction causes abnormal eye development. Surgical elimination of the lens ectoderm before optic vesicle invagination, but not after optic cup formation, has been shown to prevent optic vesicle formation (Hyer et al. 2003) as well as correct distinction between the neural retina and the RPE territories (Hyer et al. 1998). These and other related experiments led to the long-standing belief that lens development depends on the retina and vice versa, with both cooperating towards further eye formation. Inter-epithelial filopodia connecting the two tissues and signalling factors are the mechanisms that have been proposed to mediate this cross-talk [(Chow and Lang 2001; see also; Adler and Canto-Soler 2007) for a critical review of the topic]. Nevertheless, the idea of retina–lens interdependence has been challenged by the recent observation that mammalian ES-cultured cells can independently generate organized eyes in the absence of a lens (Eiraku et al. 2011; Nakano et al. 2012) and that acquisition of a cup shape is an intrinsic property of the retinal neuroepithelium (Nicolas-Perez et al. 2016). Still, microphthalmia with variable phenotypic severity is often the result of mutations in genes expressed in the lens but not in the retina, such as FOXE3 (Williamson and FitzPatrick 2014), indicating that the lens influences retinal growth. The implantation of beads soaked in specific secreted factors—a technique that can be readily applied to chick embryos—allowed the identification of a number of molecules that support retinal growth in the absence of the lens ectoderm; these include, for example, members of the BMP, FGF and Wnt family [reviewed in (Adler and Canto-Soler 2007)]. Yet, there is still little information on the possible contribution of components of their signalling cascade to microphthalmia and anophthalmia. This is a field that might merit further investigation. Indeed, mutations in *SALL4* have been recently found in microphthalmic individuals (Ullah et al. 2017). SALL4, a transcription factor of the SAL family previously associated with a form of Duane syndrome (Okihiro/Duane-radial ray syndrome), is a downstream effector of BMP signalling. This raises the possibility that alterations in other components of this complex pathway may explain unresolved cases of microphthalmia. It is also worth mentioning that many of the molecularly diagnosed cases of human anophthalmia and microphthalmia are associated with mutations in genes such as *SOX2, OTX2* or *PAX6*, which play multiple roles in the development of both the lens and the retina, as uncovered

by a number of studies in chick embryos [see; (Adler and Canto-Soler 2007) for a review]. Thus, other regulatory genes expressed in both tissues might be good candidates to explain some forms of anophthalmia and microphthalmia. Among them, the *Meis1* gene seems an attractive candidate. Comparative transcriptomic analysis of wt and *Meis1* mutant mice has shown that the corresponding transcription factor controls the expression of a large number of molecules linked to human microphthalmia and expressed either in the lens, the retina or both (Marcos et al. 2015).

Retinal–RPE interaction

Both the neural retina and the RPE derive from a common set of precursors that form the optic vesicle neuroepithelium, but their genetic programs become highly divergent as soon as the vesicle begins to infold. As a result, the neural retina and the RPE acquire very different morphologies, organization and properties. Initial studies in chick embryos suggested that the restriction of *Otx2* expression to the presumptive RPE region is key to this divergence (Bovolenta et al. 1997), and were thereafter confirmed by the analysis of *Otx* mouse mutants (Martinez-Morales et al. 2001). Subsequent studies have shown that RPE specification further requires the activity of different isoforms of the Mitf transcription factor and of Wnt/βcatenin signalling (Fuhrmann et al. 2014), whereas Pax6 seems involved in maintaining RPE identity (Raviv et al. 2014). Notably, studies in birds have established that either Otx2 or Mitf alone (Martinez-Morales et al. 2003) as well as the combined activity of Otx2 and Wnt/βcatenin signalling (Westenskow et al. 2010) or BMP and Wnt (Steinfeld et al. 2017) are sufficient to *trans*-differentiate the neural retina into retinal pigmented cells by activating the expression of genes of the melanogenic cascade. This shows that the retina is competent to acquire RPE characteristics. The opposite is also true. For example, implantations of beads soaked in FGF ligands in the proximity of the future chick RPE converts the tissue into an inverted neural retina, initiating its neurogenesis (Martinez-Morales et al. 2005; Vogel-Hopker et al. 2000). Notably, in his early grafting experiments, Gayer (1942) observed that when wt-grafted optic vesicles developed a coloboma, partial "retina duplications in the outer layer" with "an inverse arrangement of the retinal strata" were always present. Besides underscoring the RPE potential of acquiring a neural retina fate, this observation highlights the relevance of the RPE in optic fissure closure. Failure of neural retina or RPE specification often also culminates in the development of anophthalmia or microphthalmia, as shown by genetic manipulation of key transcription factors in different vertebrate species, including the chick (Tsukiji et al. 2009; Wang et al. 2016). Therefore, additional genetic causes for MAC should be searched among the genes controlling the development of either one of these two tissues. The gene regulatory network controlling neural retinal development is fairly well understood, even in humans (Hoshino et al. 2017). In contrast, our knowledge of RPE specification seems more limited, prompting the question of whether Otx, Mitf, Wnt/βcatenin and perhaps Pax6 are indeed sufficient for RPE specification. Embryological manipulations in chick embryos coupled with transcriptomic studies might help to answer this question.

Linking chick eye studies with other ocular diseases

The chicken and the human eyes differ in several aspects, but also share a number of features that make the chick a suitable model to study not only MAC but also a number of human ocular diseases (Wisely et al. 2017). For example and in contrast to the mouse, the chick eye includes a true corneal Bowman's layer. This layer is involved in corneal wound healing and, therefore, the chick represents a suitable model for both understanding how healing occurs and finding potential treatments to improve this process. Like the human, the chick eye has a stable blood–aqueous barrier and controls the content of the aqueous humour through trabecular filtration (Wisely et al. 2017). Thus, the chick is well suited to study ocular pressure and related diseases, including glaucoma, a leading cause of blindness worldwide. Moreover, chickens and humans are diurnal animals and, therefore, their retinas are enriched in cones, the photoreceptors that mediate light vision. Cone photoreceptors are particularly enriched in the fovea, the central spot in our retina that allows high-acuity vision. The chick retina does not have a proper fovea, but contains an equivalent cone-enriched high-acuity area. A very recent study has shown that the specification of this area depends on a local and strong expression of *Fgf8* associated with the absence of retinoic acid (RA), achieved by a spatially controlled expression of its degrading enzymes. Reduction of *Fgf8* expression or manipulation of RA activity changes all the properties of the high-acuity area, including the specification of cone photoreceptors (da Silva and Cepko 2017). Notably, there is a highly conserved spatial distribution of Fgf8 and RA regulatory enzymes in the human embryonic foveal region (da Silva and Cepko 2017), suggesting a possible conserved function. If this is the case, these molecular components could be exploited as molecular targets to fight against cone photoreceptor degeneration, which is a common feature of retinitis pigmentosa, age-related macular degeneration and other retinal dystrophies that collectively affect millions of people worldwide. Given that these are pathologies with still few effective cures (Letelier et al. 2017), preventing cone death would be a substantial step forward for many patients

(Bovolenta and Cisneros 2009) and further studies on the chick high-acuity area might be critical in this respect.

Conclusions

Embryological manipulations in chick embryos paved the way for many years of research aimed at understanding how the vertebrate eye forms. This research uncovered important signalling interactions among tissues and has dissected the functions of critical genes. With our current knowledge, many of these studies should be seen as invaluable sources of inspiration to search for new or poorly studied aspects of vertebrate eye development. The result might be the identification of unexpected players in eye development and, perhaps, of additional causes of inherited eye malformations. A related example is the recent identification of the receptor for the cleaved and active form of the complement component C3 (C3a) as a regulator of patterning, proliferation and survival of the optic cup neuroepithelium (Grajales-Esquivel et al. 2017). Of note, neutralization of C3aR function with specific antibodies causes microphthalmia and coloboma in treated chick embryos (Grajales-Esquivel et al. 2017).

Looking backwards to go forward is a possible approach to further understand eye disorders, but certainly not the only one. The specific characteristics of the chick eye, its experimental advantages and the increasingly successful use of editing techniques to modify the chick genome (Gandhi et al. 2017; Williams et al. 2018) all call for further use of the chick as a useful and efficient model to address eye diseases.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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