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Epithelial Morphogenesis: The Mouse Eye as a Model System¹

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Abstract

Morphogenesis is the developmental process by which tissues and organs acquire the shape that is critical to their function. Here, we review recent advances in our understanding of the mechanisms that drive morphogenesis in the developing eye. These investigations have shown that regulation of the actin cytoskeleton is central to shaping the presumptive lens and retinal epithelia that are the major components of the eye. Regulation of the actin cytoskeleton is mediated by Rho family GTPases, by signaling pathways and indirectly, by transcription factors that govern the expression of critical genes. Changes in the actin cytoskeleton can shape cells through the generation of filopodia (that, in the eye, connect adjacent epithelia) or through apical constriction, a process that produces a wedge-shaped cell. We have also learned that one tissue can influence the shape of an adjacent one, probably by direct force transmission, in a process we term inductive morphogenesis. Though these mechanisms of morphogenesis have been identified using the eye as a model system, they are likely to apply broadly where epithelia influence the shape of organs during development.

1. INTRODUCTION

Though the timescales are rather different, biology and geology both define morphogenesis as the study of mechanisms of shape acquisition. Whether the subject is tissue forms or landforms, the goal is to understand the forces of nature that guide the process. For the biologist, the study of morphogenesis is mostly a study of embryonic development (though regeneration is an option). Biologists interested in morphogenesis frequently combine the tools of developmental biology and cell biology. This gives them the means to establish the relationship between the molecular events at the single cell level and the consequence of

¹This chapter is dedicated to the memory of Yoshiki Sasai, an exceptional scientist whose work contributed critically to many fields of enquiry, including the one reviewed here.

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those events for organismal shape. Though there will be unifying mechanisms of morphogenesis at the cellular scale, the huge complexity of tissue shapes in different species and different organs teaches us that in morphogenesis, details and subtlety matter (an interesting example is the subtle changes in beak shape that allow “Darwin’s finches” to effectively exploit an ecological niche; Mallarino et al., 2011).

Though there are many worthy subjects of study in morphogenesis, in this chapter, we focus on the mouse eye as a model system. Eye morphogenesis in the mouse is very complex and therefore interesting. There are also many tools that can be applied to the study of eye development and so even in these early stages of our understanding, progress has been quite rapid. As a nonessential organ (at least for laboratory mice), the eye also has practical advantages as a subject of study. Finally, perhaps more than any other organ, the shape of the eye is critical for function. When the sensory stimulus is as unforgiving as the photon (just ask anyone over 40 years), the optical characteristics of the tissues and their relative shape and position have to show “inimitable perfection” (Darwin, 1859).

2. EYE MORPHOGENESIS

The basic features of eye morphogenesis that occurs during midgestation in the mouse are shown in Fig. 1. In this chapter, we define two movements of epithelial structures—invagination and evagination—according to the polarity of the epithelium. We define invagination as a coordinated epithelial cell movement in the basal direction and an evagination as an epithelial cell movement in the apical direction. Thus, at E9.5 in the mouse, the optic vesicle (Fig. 1A, ov) is formed through an invagination of the diencephalic neural tube. This invagination brings the distal epithelium of the optic vesicle (the future retina) in close apposition to the embryonic surface ectoderm that will form both the lens and corneal ectoderm. As optic vesicle invagination occurs, the periocular mesenchyme that lies between the optic vesicle and surface ectoderm is excluded. Inductive signaling events (Charlton-Perkins, Brown, & Cook, 2011; Grocott, Tambalo, & Streit, 2012; Gunhaga, 2011; Lang, 2004; Shaham, Menuchin, Farhy, & Ashery-Padan, 2012) result in the formation of the lens placode (Fig. 1B, lp), a thickened domain of the surface ectoderm that will undergo invagination to form the lens pit (Fig. 1C, lpt). Invagination of the lens pit is coordinated with the formation of the optic cup, a bilayered epithelium that ultimately forms the neural retina and retinal pigmented epithelium (RPE). According to our polarity-based definition of epithelial morphogenesis, the presumptive retina undergoes an evagination and the presumptive RPE undergoes an invagination as the optic cup forms. By E11.5 (Fig. 1D), the lens pit has closed to form the lens vesicle (lv) and the surface ectoderm has separated to form the presumptive corneal ectoderm. At this point in development, the basic layout of the major tissues of the eye is established.

3. DYNAMIC CHANGES IN THE ACTIN CYTOSKELETON DRIVE MORPHOGENESIS

It is now well accepted that regulation of cell migration and cell shape via modulation of the actin cytoskeleton is central to morphogenesis. Central to modulation of the actin cytoskeleton are the Rho family GTPases, molecular switches that transfer information in

the cell and gain specificity through a series of effector proteins with a limited set of downstream molecular targets (Bishop & Hall, 2000). The GTPases exist in active (GTP bound) and inactive (GDP bound) states and are regulated in several ways. The Rho GTPase-activating proteins (Tcherkezian & Lamarche-Vane, 2007) and the guanine nucleotide dissociation inhibitors (Dovas & Couchman, 2005) are negative regulators while the Rho guanine-nucleotide exchange factors (RhoGEFs; Rossman, Der, & Sondek, 2005; Schmidt, Diriong, Mery, Fabbriozio, & Debant, 2002) act positively. These are frequently expressed with tissue-specific expression patterns and can themselves be regulated. The Rho family GTPases can regulate morphology, adhesion, differentiation, proliferation, and vesicle trafficking (Heasman & Ridley, 2008; Linseman & Loucks, 2008; Nobes & Hall, 1994, 1999; Villalonga & Ridley, 2006). In some of the first assessments of Rho family GTPase activity, it was noted that cultured cells produced different types of actin and different cell shapes, depending on which active GTPase was expressed. Cdc42 activation resulted in the formation of filopodia, Rac1 in the formation of lamellipodia and RhoA the formation of contractile actin and stress fibers (Jaffe & Hall, 2005). These findings were important for the developmental biologists interested in morphogenesis, because they provided a starting point for the investigation of these pathways *in vivo*.

Based on the experimental analysis that will be summarized below, it is clear that a complex series of physical and signaling interactions between the epithelial and mesenchymal components of the developing eye are required to shape a normal structure. The major theme emerging from this review is that even morphogenetic movements that appear very simple are mechanistically quite complex and require input from many pathways. In addition, it is often the case that each pathway contributes just a little to the overall movement. A further level of complexity emerges from the interaction between adjacent tissues. Though we cannot, as a field, claim to have solved the problem of eye morphogenesis, already many mechanisms have been uncovered. What are these mechanisms? Below, we describe what is known, more-or-less in ontological order. Many of the mechanisms so far identified are directly or indirectly linked to modulation of the actin cytoskeleton.

3.1. What is the function of placode formation in epithelial morphogenesis?

The formation of placodes—regions of thickened epithelium—is associated with the morphogenesis of many structures. Extensive studies on placode specification have been reported (Bailey & Streit, 2006; Streit, 2008), but the mechanisms of epithelial thickening have received less attention. The strong association between placode formation and morphogenesis further suggests that this type of epithelial thickening might be a prerequisite for epithelial bending.

In the case of the lens placode that forms between E9.0 and E9.5 in the mouse, there are several mutant lines in which placodal thickening is compromised. These include the germ line Pax6 mutation present in the *Small eye* mouse (Hill et al., 1991), regulatory mutations that lead to diminished placodal Pax6 expression (Dimanlig, Faber, Auerbach, Makarenkova, & Lang, 2001), as well as conditional deletion of Pax6 in the lens placode (Ashery-Padan, Marquardt, Zhou, & Gruss, 2000). Pax6 has a central role in lens development—it is both necessary (Hill et al., 1991) and sufficient (Altmann, Chow, Lang, & Hemmati-Brivanlou,

1997; Chow, Altmann, Lang, & Hemmati-Brivanlou, 1999) for formation of the lens structure from the placode stage—and so it can be difficult to determine how closely this broadly active transcription factor is associated with the morphogenesis machinery. Despite this, it has been shown, with placodal deletion of Pax6, that there is a loss of expression of Shroom3 (Plageman et al., 2010), an actin scaffold protein with an important role in changing the shape of epithelial cells (Lee, Le, & Wallingford, 2009; Nishimura & Takeichi, 2008; see discussion below). This contrasts with placodal deletion of Sox2, another transcription factor involved in lens development. In the placodal Sox2 deletion, though there are consequences for lens development (Smith, Miller, Radice, Ashery-Padan, & Lang, 2009), Shroom3 expression is retained and placodal invagination initiated (Plageman et al., 2010). Though it is likely that Pax6 regulates many genes after lens induction, these data link the transcription factor to Shroom3 expression and the morphogenesis machinery (Fig. 2).

Some well-known signaling pathways also contribute to formation of the lens placode. When the fibroblast growth factor (Fgf) signaling response is compromised in the preplacodal ectoderm of the mouse, placodal thickening is reduced (Faber et al., 2001), a result confirmed first by mutation of the Fgf receptor adapter protein Frs2 α (Gotoh et al., 2004) and later by conditional Fgf receptor deletion (Garcia et al., 2011). Defective Bmp signaling also results in changes to placode formation. Both Bmp4 and Bmp7 germ-line mutant mice exhibit a failure of lens placode formation (Furuta & Hogan, 1998; Wawersik et al., 1999). The defects in lens morphogenesis and changes in epithelial F-actin distribution that arise when the Bmpr1a and Acvr1 receptors are conditionally deleted postinduction (Rajagopal et al., 2009) suggests that this signaling pathway may be quite closely linked to the morphogenesis machinery. Given existing data (Gamell et al., 2008; Konstantinidis, Moustakas, & Stournaras, 2011; Lee-Hoeflich et al., 2004), it is quite likely that there are several levels at which Bmp signaling can regulate cell shape via modulation of the actin cytoskeleton.

The interaction between the presumptive lens and presumptive retina is important for subsequent morphogenesis. In part, this is because of the inductive signaling that takes place, but is also because a physical association is important for coordinated shape formation in the presumptive lens and retina. The importance of the presumptive lens–retina interface is illustrated by experiments that have examined the role of the extracellular matrix (ECM) and more specifically of fibronectin (FN), in lens morphogenesis. In a placode-conditional Pax6 mutant mouse, ECM deposition between the lens and retina is diminished, suggesting that Pax6 directly or indirectly regulates ECM deposition (Huang et al., 2011). The limited ECM at the presumptive lens–retina interface may contribute to the failure of lens pit invagination in the Pax6 mutant. In addition, when FN1 is conditionally deleted throughout the mouse embryo, there is a failure of both placode thickening and lens pit invagination (Huang et al., 2011). Though this mutant may have many signaling and structural defects, these data suggest that placode thickening may be a prerequisite for invagination. These data are also consistent with descriptive analyses of lens placode thickening, suggesting that an increase in cell density coupled with static cell volumes (McKeehan, 1951) results in thickening. Additional experimental studies attempting to restrict the outward expansion of presumptive lens placodal cells through physical means resulted in ectopic thickening and/or

precocious invagination (Steding, 1967; Wakely, 1984). These data further support the hypothesis that placodal thickening is a prerequisite for invagination. In purely structural terms, this might well make sense: just as a deeper beam can better resist the force of gravity, a thicker epithelium can better generate the forces required for invagination.

3.2. Extrinsic force transmission via filopodia

Events in morphogenesis require precise cell positioning and guidance that can be provided by filopodia. This function can be found in migratory tissue sheets, such as dorsal closure in *Drosophila* (Martin-Blanco, Pastor-Pareja, & Garcia-Bellido, 2000), ventral closure in *Caenorhabditis elegans* (Williams-Masson, Malik, & Hardin, 1997), and wound healing (Wood et al., 2002). Filopodia also feature in developing vasculature (Gallo & Letourneau, 2004), neuronal projections (De Smet, Segura, De Bock, Hohensinner, & Carmeliet, 2009), and the *Drosophila* tracheal system (Wolf, Gerlach, & Schuh, 2002). Studied primarily in fibroblasts and neurite growth cones, filopodia are defined as probing cellular protrusions that aid in migration and extension and that contain bundles of parallel actin filaments (Faix & Rottner, 2006; McClay, 1999; Passey, Pellegrin, & Mellor, 2004; Raich, Agbunag, & Hardin, 1999). Their length differs depending on their tip adhesion; those that are anchored at their tips by cadherins tend to be shorter (Partridge & Marcantonio, 2006; Raich et al., 1999; Wood et al., 2002), whereas the integrin-dependent focal adhesion tips tend to be longer (Partridge & Marcantonio, 2006).

The Rho family GTPase Cdc42 has many functions in the cell, but it is critical for the formation of filopodia (Nobes & Hall, 1995; Ridley, 2006). *In vitro*, it does so through binding in the active state with the ubiquitously expressed effector protein N-WASP (Banzai, Miki, Yamaguchi, & Takenawa, 2000; Egile et al., 1999; Kurisu & Takenawa, 2009; Miki, Sasaki, Takai, & Takenawa, 1998), a Wiskott–Aldrich syndrome gene-family member. N-WASP subsequently activates the actin nucleator Arp2/3, and as a result, fast growing barbed-end actin filaments extend the plasma membrane to form protrusive filopodia. The formation of Cdc42-stimulated filopodia in N-WASP-deficient fibroblasts revealed an alternative pathway for filopodia formation (Snapper et al., 2001). This was later discovered to require the diaphanous-related formins (DRFs; Peng, Wallar, Flanders, Swiatek, & Alberts, 2003), a group of actin nucleators that function downstream of the Rho GTPases. Furthermore, it has been found that Rif forms filopodia through the action of a DRF member, mDia2. A third filopodiogenesis pathway requires the effector protein IRSp53 interacting with either Mena (Krugmann et al., 2001) or Eps8 (Disanza et al., 2006). IRSp53 and Eps8 are also involved in signal transduction, whereas IRSp53 is a substrate for the insulin receptor (Yeh, Ogawa, Danielsen, & Roth, 1996) and Eps8 is a substrate for other tyrosine kinase receptors (Fazioli et al., 1993).

Investigation of filopodia in morphogenesis began with the secondary mesenchymal cells of the sea urchin *Arbacia punctulata* and the sand dollar *Echinarchniu sparma* echinoderm species as early as 1969 (Tilney & Gibbins, 1969). This was later followed by investigation of their contractile function in the tunic cells of the ascidian *Botryllus schlosseri*, where they were found to change cell shape and regulate cell migration (Izzard, 1974). Here, filopodia were observed to be long (reaching lengths of up to 200 μm). Laser ablation studies of the

filopodia arising from secondary mesenchymal cells in the sea urchin suggested that they had a contractile function and aided in generating the forces required for invagination of the archenteron during gastrulation (Hardin, 1988). A role for filopodia in connecting epithelium to mesenchyme was found in the examples of the developing avian lung (Gallagher, 1986), and in the dental mesenchyme making contact with the overlying dental epithelium (Fukumoto et al., 2006). Their role in branching morphogenesis was revealed in avian lung development as well as in *Drosophila* tracheal development (Samakovlis et al., 1996). Their role in wound healing was found through the series of studies conducted in the cornea (Crosson, Klyce, & Beuerman, 1986; Pfister, 1975), skin (Pang, Daniels, & Buck, 1978; Vasioukhin & Fuchs, 2001), and then in *Drosophila* dorsal closure (Millard & Martin, 2008; Wood et al., 2002), the latter of which recapitulated a number of embryonic tissue closure mechanisms. The contractile function of filopodia and the specific myosins in the varying filopodia have recently been deciphered (Berg & Cheney, 2002; Chauhan et al., 2009; Jacinto et al., 2002; Moores, Sabry, & Spudich, 1996; Sousa & Cheney, 2005; Tokuo & Ikebe, 2004; Wagner, Barylko, & Albanesi, 1992; Zhang et al., 2004).

Ida Mann's book on human eye development (Mann, 1950) described "protoplasmic processes" that extended between the epithelia of the presumptive lens and retina. Later, the McAvoy group showed, by TEM, that these processes formed a junction-like structure at the tip where they contacted the presumptive retina (McAvoy, 1980). These structures were shown to be actin-rich and devoid of tubulin, suggesting that they were filopodia (Chauhan et al., 2009). A mutant mouse in which the Rho family GTPase Cdc42 was deleted in the lens placode exhibited an absence of interepithelial filopodia further confirming their identity. This mutant also indicated that most of the filopodia originate in the cells of the lens placode and lens pit. A series of mutant mice, including the Cdc42 placode conditional mutant, a germ line mutant for the Cdc42 effector IRSp53, and a conditional mutant for focal adhesion kinase, revealed a spectrum of filopodial loss and an inverse correlation with interepithelial distance. In the Cdc42 conditional mutant where all filopodia were lost, about one-third of the lens pit invagination depth was also lost, suggesting that these structures served to transmit forces between presumptive retina and lens as a means of refining the process of morphogenesis. This suggestion was consistent with the observation that these filopodia contained active phosphomyosin II and were actively contractile. Filopodia-deficient mutants, not surprisingly, show changes in the shape of the lens pit. However, they also show changes in the shape of the adjacent retinal cup (Fig. 3), the structure to which they connect, probably via integrin-mediated adhesion. These nonautonomous epithelial shape changes provide some evidence that one of the functions of these filopodia is to transmit forces from one epithelium to another. Drawing a parallel with the classical concept of inductive fate change, we refer to this phenomenon as inductive morphogenesis. This analysis indicates that the forces required to drive morphogenesis can be extrinsic (Fig. 2).

3.3. Intrinsic force generation through apical constriction and cell elongation

When an epithelium invaginates, the cells within the epithelium must, on average, adopt a wedge-shaped morphology. There is now much evidence at both the cellular and molecular levels that this shape change is primarily a result of a reduced apical area that is the result of a process referred to as apical constriction (AC). For the transition between the lens placode

and lens pit (Fig. 1) an analysis of cell shape showed that both apical constriction and cell elongation accompanied invagination (Hendrix & Zwaan, 1974). Below, we describe some of the molecular mediators of and epithelial cell elongation and AC and indicate how their activities are integrated.

3.3.1 Apical constriction—Many of the significant advances in our understanding of AC mechanisms have come from analyzing invertebrate morphogenesis including *Drosophila* gastrulation and dorsal closure. During gastrulation, coordinated AC along the ventral embryonic axis drives mesodermal invagination (Leptin, 2005). Mesodermal cells undergo AC through the contraction of an apically positioned network of actomyosin that spans the apical cortex (Dawes-Hoang et al., 2005; Martin, Kaschube, & Wieschaus, 2009). Assembly and contraction of myosin filaments are facilitated through the phosphorylation and activation of myosin regulatory light chain and phosphorylation and deactivation of myosin phosphatases (Bresnick, 1999). The pathway leading to these phosphorylation events in *Drosophila* gastrulation are triggered by a well-understood cascade of events that commences with the mesodermal-specific expression of G protein-coupled receptors that apically recruit a guanine exchange factor (DRhoGEF2). DRhoGEF2 activates the Rho-GTPase, Rho1 and in turn stimulates phosphorylation by activating the serine-threonine kinase Rok (Sawyer et al., 2010). Once active, contraction occurs in a step-wise manner consisting of pulses interspersed by periods of relaxation or apical surface stabilization (Azevedo et al., 2011; Blanchard, Murugesu, Adams, Martinez-Arias, & Gorfinkel, 2010; David, Tishkina, & Harris, 2010; Martin et al., 2009). A molecular connection bridging actomyosin to junctional complexes are also necessary to draw the apical circumference inward and when disrupted the degree of AC is attenuated (Dawes-Hoang et al., 2005; Roh-Johnson et al., 2012; Sawyer, Harris, Slep, Gaul, & Peifer, 2009).

AC within cells of the lens placode is remarkably similar to that observed in *Drosophila* gastrulation. During lens placode invagination, the vertebrate equivalents of the RhoGEF2-Rho1-Rok pathway of myosin activation are significant contributors. Pharmacological studies inhibiting Rock1/2, the vertebrate homolog of Rok, myosin, or actin dynamics all disrupt lens invagination presumably through the inhibition of AC (Borges, Lamers, Forti, Santos, & Yan, 2011; Plageman et al., 2011). Genetic deletion of RhoA or the RhoA-GEF Trio, functional counterparts of RhoA, and DRhoGEF2, respectively, inhibits AC in the lens and decreased apical levels of activated myosin, indicating a strong molecular conservation of this pathway between species (Chauhan, Lou, Zheng, & Lang, 2011; Plageman et al., 2011). Shroom3, a Rock1/2 and actin-binding cytoskeletal protein, initially identified as a protein required for neural tube AC and morphogenesis, is also necessary for lens placode AC (Haigo, Hildebrand, Harland, & Wallingford, 2003; Hildebrand & Soriano, 1999; Plageman et al., 2010).

Shroom3 appears to play a central role in AC during lens placode invagination. First, its expression domain is regionally restricted to the cells which are undergoing AC. This is likely due in part to lens placode-specific transcription factors, many of which have restricted expression, an idea supported by the observation that Shroom3 expression is dependent upon Pax6 (Plageman et al., 2010; Fig. 2). Second, Shroom3 is sufficient to induce AC and facilitate the assembly of myosin into sarcomeric-like structures in the apical

junctions of cultured epithelial cells (Hildebrand, 2005). Third, the absence of Shroom3 causes a reduction in the apical distribution of both F-actin and myosin in the lens placode (Plageman et al., 2010). Its ability to recruit F-actin is likely dependent on the Mena-family of actin modulators which themselves play a role in AC (Plageman et al., 2010; Roffers-Agarwal, Xanthos, Kragtorp, & Miller, 2008). Given that the function of Shroom3 was also shown to be dependent on RhoA, Rock1, and myosin activity, we posit that Shroom3 acts as a nexus of AC activity in the lens by organizing and/or recruiting most if not all of the necessary components.

Like invertebrate AC, the association of actomyosin with the apical junctions is essential for lens placode AC but unlike *Drosophila* mesodermal cells, much of the actomyosin in the lens placode is found in the circumferential belt positioned near the apical junctional complex (Lang, Herman, Reynolds, Hildebrand, & Plageman, 2014). In a somewhat surprising degree of conservation, actomyosin filaments spanning the apical cortex are indeed present in a subset of lens placodal cells and appear to immediately precede AC. Like invertebrate actomyosin filaments, those in lens placode epithelial cells are connected to deformations in the bicellular junctions (Lang et al., 2014) suggesting that they are contractile. Bicellular deformations and AC are both prevented by the myosin inhibitor blebbistatin (Lang et al., 2014). Further analysis demonstrated that the cadherin-binding modulator of Rho-GTPases, p120-catenin, is required for this junctional displacement and AC in the lens (Lang et al., 2014). This suggests that p120 may be playing a significant role in mediating the connection of actomyosin filaments and/or the circumferential actomyosin belt to the apical junctions. In turn, these mediators are likely critical for the generation of intrinsic forces that can bend an epithelium (Fig. 2).

3.3.2 Cell elongation—In cultured cells, the Rac1 GTPase stimulates the formation of lamellipodia (Jaffe & Hall, 2005). This unique type of cellular process is a consequence of the particular effector molecules that this GTPase activates and in turn, the type of actin complexes that are formed (Jaffe & Hall, 2005). One well-known Rac1 effector is the Wiskott–Aldrich syndrome protein family verprolin homolog WAVE (Kurusu & Takenawa, 2009; Miki, Suetsugu, & Takenawa, 1998) which, through upstream activation of the p21-activated kinase (PAK) effector and binding to the actin nucleator Arp2/3, encourages polymerization of the Y-branched actin networks that give lamellipodia and membrane ruffles (Burridge & Wennerberg, 2004). It has also been shown that active PAK1 has an additional role in membrane transport of WAVE2 to the leading edge for lamellipodia formation (Takahashi & Suzuki, 2009). Interestingly, it has been shown using fluorescence resonance energy transfer microscopy in cultured cells that RacGTP levels are the highest at the leading edge of a migrating cell (Kraynov et al., 2000), and that integrin–matrix interactions may play a role in localized Rac1 activity.

When *Rac1* is conditionally deleted from the lens placode, the constituent cells exhibit reduced length (Chauhan et al., 2011). This is likely to be the *in vivo* manifestation, in the epithelial cell type, of the activity that forms lamellapodia in migrating cultured cells. From the point of view of tissue structure, it may be that this type of cell elongation is important for epithelial bending in that it extends the length of the levers that can be used to generate force.

3.3.3 Rac1, RhoA pathways integrate to control cell shape in the lens placode

—It is clear that in some settings, the Rac1 and RhoA actin modulation pathways are mutually antagonistic (Machacek et al., 2009; Xu et al., 2003). In the invaginating lens placode the basic phenotypic response, where lens pit curvature is reduced in RhoA mutants and increased in Rac1 mutants (Chauhan et al., 2011), also suggested this might be the case in *vivo*. An assessment of cell shapes further showed that, as would be expected, RhoA was required for AC (Chauhan et al., 2011). However, in addition, RhoA mutant lens placode cells also showed increased length. In a reciprocal set of responses, Rac1 mutant placode cells showed reduced length, as would be expected, but also showed increased AC (Chauhan et al., 2011). How can these findings be explained mechanistically?

Using independent markers of the RhoA and Rac1 pathway responses, it was shown that in the *RhoA* mutant lens placode, there were indications of Rac1 pathway gain of function. For example, the Arpc2 actin nucleation complex subunit was significantly over-represented basally in the placodal *RhoA* mutant. This could explain why a *RhoA* mutant epithelial cell had become longer than normal. Similarly, in *Rac1* mutant placode cells, phosphomyosin was over-represented at the cell apex. This suggested that the *Rac1* mutant placodal cell had a RhoA pathway gain of function and was an explanation for the increased AC and the tighter curvature of the lens pit in this mutant. These observations suggest that during lens placode invagination, RhoA and Rac1 are mutually antagonistic. Furthermore, the data suggest their activities are balanced in a way that gives fine control of cell height and apical width (Chauhan et al., 2011). Since, the ratio of these two cellular dimensions controls the angle that an epithelial cell occupies, in aggregate, Rac1 and RhoA mutual antagonism regulates epithelial curvature (Fig. 2).

There are many examples of a dramatic change in epithelial curvature over just a few cell diameters, and so it is also possible that the Rac1 to RhoA activity ratio can also be controlled locally, presumably by the signaling responses known to regulate these GTPases (Raftopoulou & Hall, 2004). These are likely to be general mechanisms for regulating epithelial morphogenesis given the ubiquity of the Rho family GTPases. Studies have shown the mechanism of RhoA–Rac1 mutual antagonism can be mediated through either p190Rho-GAP (Nimnual, Taylor, & Bar-Sagi, 2003), the kinase *sticky* (D’Avino, Savoian, & Glover, 2004), or FilGAP (Ohta, Hartwig, & Stossel, 2006). It remains to be determined whether these mechanisms apply to morphogenesis of the eye.

4. SHAPING OF THE OPTIC CUP

Analysis of the role of GTPases and the AC machinery in the invaginating lens pit has made a strong case that the forces that drive epithelial bending are generated intrinsically through cell shape change and extrinsically, via filopodia that connect the invaginating lens placode to the presumptive retina. This arrangement raises the question of whether there is morphogenetic induction—a change in shape dependent on the shape of the adjacent epithelium—that is polarized or reciprocal.

4.1. Does the optic cup pull on the invaginating lens?

Several lines of evidence indicate that the optic cup influences the shape of the lens pit. In several lines of mutant mice, where interepithelial filopodia are reduced in number, there is a reciprocal relationship between the number of filopodia and interepithelial distance. In other words, as the number of filopodia reduces, so does the depth of lens pit invagination. This suggests quite strongly that the optic cup pulls on the lens pit. There is some subtlety, however, in that even in a mouse in which all filopodia are absent, only about one-third of the lens pit invagination depth is lost (Chauhan et al., 2009). This is consistent with a model in which the lens placode has intrinsic force generation (through cell shape change) and that the extrinsic force generation through connecting filopodia is a fine-tuning mechanism (Fig. 2).

4.2. Does the invaginating lens influence the shape of the optic cup?

Recent experiments in which optic cups are developed in culture from ES cells have suggested that the optic cup develops autonomously and is self-shaping (Eiraku et al., 2011). Though the reality is more complicated (see below), the observation that the optic cups forms in the absence of lens has suggested that the invaginating lens pit does not push on the presumptive retina as the optic cup forms. To extend this line of questioning further, we can ask whether there are any changes in the shape of the optic cup when the invaginating lens placode and presumptive retina are uncoupled. Arguably, an uncoupling occurs in *Cdc42* conditional mutant mice in which the presumptive lens does not generate any connecting filopodia. When we analyze the shape of the optic cup in this mutant, we find that it is changed compared to a control (Fig. 3). Obviously, this suggests that the invaginating lens placode does influence the shape of the optic cup. As in the reciprocal interaction, the changes in the shape are subtle and so again it might be argued that the lens finetunes the shape of the cup. It is also worth noting that when optic cups are generated in culture, very few of them are perfect in shape and a proportion never undergo the evagination of the presumptive retina that generates the bilayered optic cup (Eiraku et al., 2011; Nakano et al., 2012). This observation contrasts with the *in vivo* development of the eye where the structure is perfect nearly all of the time. This contrast suggests that while the optic cup can form its basic shape autonomously some of the time, if it is to do so all the time, the influence of surrounding tissues is required. Below, we review the evidence that the surface ectoderm of the eye produces signals that are critical for morphogenesis of the optic cup.

4.3. The “bimetallic strip” mechanism of optic cup morphogenesis

In the mouse, when the lens placode and adjacent ectoderm are prevented from producing Wnt ligands, there are profound consequences for eye development. One consequence is that the optic cup does not form its normal, near-spherical shape but is flat and saucer shaped (Carpenter et al., 2014). This contrasts with the hypothesis that the optic cup is self-shaping and shows very directly that signaling ligands produced by the ectoderm influence optic cup morphogenesis. With the available data, the most compelling hypothesis to explain this function of Wnt ligands is that they are produced by the surface ectoderm and stimulate a Wnt/ β -catenin response in the presumptive RPE adjacent to the retinal rim (Carpenter et al., 2014). It is also understood that the Wnt/ β -catenin response upregulates the retinoic acid

response pathway, known to be critical for optic cup morphogenesis in the RPE. In turn, the Wnt/ β -catenin and retinoic acid responses stimulate the production of cells within the RPE that helps to shape the optic cup.

It has been suggested that the mechanism by which RPE cells shape the optic cup is, in principle, similar to the mechanism by which a bimetallic strip bends when it is heated. A bimetallic strip is a temperature sensing device that consists of two different metals bonded along their length. If these metals have different expansion coefficients, then the bimetallic strip will bend when it is heated (<https://www.youtube.com/watch?v=sP5NwEkd3ds>). In the same way, as more RPE cells are produced in the bilayered optic cup, they will induce curvature (Fig. 2).

The bimetallic strip model of optic cup morphogenesis is consistent with a number of published observations. First, it has been shown that the presumptive RPE is Wnt/ β -catenin responsive according to the expression of Wnt/ β -catenin pathway components and reporters (Cho & Cepko, 2006; Fuhrmann, Levine, & Reh, 2000; Liu, Mohamed, Dufort, & Wallace, 2003; Liu, Thurig, Mohamed, Dufort, & Wallace, 2006; Liu et al., 2007; Trimarchi, Cho, & Cepko, 2009) and the expression of Wnt/ β -catenin target genes (Eiraku et al., 2011; Fuhrmann, 2008; Westenskow, Piccolo, & Fuhrmann, 2009). Second, assessment of the stiffness of the ocular epithelia using the atomic force microscope has indicated that while the RPE is stiff, the presumptive retina is not (Eiraku et al., 2011). This suggests, as does the bimetallic strip model that the RPE acts as a scaffold and is perhaps the dominant structural determinant of optic cup curvature (Fig. 2).

How do we reconcile the apparently conflicting observations that *in vivo*, optic morphogenesis is dependent on Wnt ligands from the surface ectoderm while in culture, they can form in the absence of the surface ectoderm? The simple reconciliation is that in the ES-derived optic cups, these critical natural signaling ligands are provided as media supplements. Retinoic acid is a standard component of “optic cup medium” and supplementary Wnt ligands have been shown to enhance the developmental process (Eiraku & Sasai, 2012).

5. CONCLUSIONS

A defining message of the current studies in eye morphogenesis is that many small influences can lead to dramatic shape changes when each of those subtle influences are focused on a single structure. The eye has a structure that is more complex than most organs, and so it will be interesting to discover if this principle holds elsewhere.

What does the future hold for studies of morphogenesis, including those focused on the eye? As the field progresses, it will be important to quantify morphogenesis numerically and, perhaps using force sensors (Grashoff et al., 2010), define the forces, in real time, that drive morphogenesis. This, coupled to a more sophisticated understanding of the signaling pathways that drive the morphogenesis machinery, will steadily build a comprehensive model of the process.

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Figure 1.

Drawing describing the basic features of eye morphogenesis. (A) At E9.5 in the mouse, the optic stalk (os) and optic vesicle (ov) have evaginated from the diencephalic neural tube (nt) and approached the overlying surface ectoderm (se). (B) By E9.75, the thickened surface ectoderm of the lens placode (lp) has formed. (C) By E10.5, the lens pit (pt) has undergone a coordinated invagination with the bilayered optic cup (oc) that incorporates the forming retinal pigmented epithelium. (D) At E11.5, the lens vesicle (lv) has separated from the surface ectoderm and presumptive corneal epithelium (cor).

Figure 2.

Summary schematic of eye morphogenesis pathways. This schematic describes some of the pathways involved in morphogenesis of the mouse eye from E9.5, when the lens placode overlies the optic vesicle, to E10.5 when the lens pit has invaginated and the optic cup has become bilayered. The surface ectoderm, presumptive retina, and presumptive retina pigmented epithelium are color-coded in red, green, and gray, respectively. Within the red box, we summarize the action of three GTPases that have distinct actions on lens placode invagination. Cdc42 and IRSp53 are required for the generation of filopodia within the presumptive lens that connect to the presumptive retina and permit force transmission between the two epithelia. They are required for a full invagination of the lens pit and for subtle shaping of the optic cup. This is an example where lens pit invagination depends on extrinsic forces. The GTPases RhoA and Rac1 are required for the generation of intrinsic forces for lens pit invagination because they control cell length (Rac1 through an Arpc2/cortactin response) and apical constriction (RhoA through a Rho kinase (ROCK) phosphomyosin pathway). RhoA activity is likely controlled by the TRIO guanine nucleotide exchange factor and in turn, this is required for the apical localization of Shroom3, an actin and ROCK-binding protein required for apical constriction. At the transcriptional level, Shroom3 is positively regulated by Pax6. During lens pit invagination, the coordinated morphogenesis of the optic cup is in part stimulated by Wnt/Catnb pathway ligands that are produced by nonlens surface ectoderm. These ligands stimulate the production of cells within the developing retinal pigmented epithelium (RPE). The RPE thus expands against the relatively static retina and, with the principle of a bimetallic strip, induces curvature in the optic cup. The red arrows indicate the movements of morphogenesis for the each epithelial layer.

Figure 3.

Inductive morphogenesis in the epithelia of the developing eye. (A) The average shape of the basal surfaces of the lens pit and presumptive retina in a wild-type, E10.5 mouse eye. The black line represents that average shape and the green (gray in the print version) shaded area, two standard deviations from the average shape. (B) The average shape of the basal surfaces of the lens pit and presumptive retina in an *Le-crc; Cdc42^{fl/fl}*, E10.5 mouse eye. In this mutant (*Cdc42 M*), there are no filopodia connecting the epithelia. As a consequence, the interepithelial distance has increased. The lens pit (C) has lost about one-third of the invagination distance and the retinal cup (D) is also flatter. Since genetic changes in this mouse line are restricted to the presumptive lens, this provides some evidence for force transmission between the epithelia. These data were generated as described previously (Chauhan et al., 2009).