Animal and cellular models of microphthalmia

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Abstract

Microphthalmia is a rare developmental eye disorder affecting 1 in 7000 births. It is defined as a small (axial length ≥2 standard deviations below the age-adjusted mean) underdeveloped eye, caused by disruption of ocular development through genetic or environmental factors in the first trimester of pregnancy. Clinical phenotypic heterogeneity exists amongst patients with varying levels of severity, and associated ocular and systemic features. Up to 11% of blind children are reported to have microphthalmia, yet currently no treatments are available. By identifying the aetiology of microphthalmia and understanding how the mechanisms of eye development are disrupted, we can gain a better understanding of the pathogenesis. Animal models, mainly mouse, zebrafish and Xenopus, have provided extensive information on the genetic regulation of oculogenesis, and how perturbation of these pathways leads to microphthalmia. However, differences exist between species, hence cellular models, such as patient-derived induced pluripotent stem cell (iPSC) optic vesicles, are now being used to provide greater insights into the human disease process. Progress in 3D cellular modelling techniques has enhanced the ability of researchers to study interactions of different cell types during eye development. Through improved molecular knowledge of microphthalmia, preventative or postnatal therapies may be developed, together with establishing genotype–phenotype correlations in order to provide patients with the appropriate prognosis, multidisciplinary care and informed genetic counselling. This review summarises some key discoveries from animal and cellular models of microphthalmia and discusses how innovative new models can be used to further our understanding in the future.

Plain language summary

Animal and Cellular Models of the Eye Disorder, Microphthalmia (Small Eye)

Microphthalmia, meaning a small, underdeveloped eye, is a rare disorder that children are born with. Genetic changes or variations in the environment during the first 3 months of pregnancy can disrupt early development of the eye, resulting in microphthalmia. Up to 11% of blind children have microphthalmia, yet currently no treatments are available. By understanding the genes necessary for eye development, we can determine how disruption by genetic changes or environmental factors can cause this condition. This helps us understand why microphthalmia occurs, and ensure patients are provided with the appropriate clinical care and genetic counselling advice. Additionally, by understanding the causes of microphthalmia, researchers can develop treatments to prevent or reduce the severity of this condition. Animal models, particularly mice, zebrafish and frogs, which can also develop small eyes due to the same genetic/environmental changes, have helped us understand the genes which are important for eye development and can cause birth eye defects when disrupted. Studying a patient’s own cells grown in the laboratory can further help researchers understand how changes
in genes affect their function. Both animal and cellular models can be used to develop and test new drugs, which could provide treatment options for patients living with microphthalmia. This review summarises the key discoveries from animal and cellular models of microphthalmia and discusses how innovative new models can be used to further our understanding in the future.

**Keywords:** cells, development, eye, human, iPSC, microphthalmia, mouse, optic vesicles, organoids, *Xenopus*, zebrafish

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**Introduction**

Microphthalmia describes a small underdeveloped eye and is defined as having a total axial length of <19 mm at 1 year of age or <21 mm in an adult measured on B-scan ultrasound, determined as being ≧2 standard deviations below the age-adjusted mean. It is a rare condition, with an estimated prevalence of 1 in 7000 live births, resulting from disrupted eye development between 4–8 weeks gestation either due to genetic or environmental factors. Currently no preventative or restorative treatments exist to improve vision.

Prospective UK incidence studies have indicated that environmental causes, such as maternal vitamin A deficiency or alcohol consumption, contribute to approximately 2% of microphthalmia cases. There are over 90 identified monogenic causes, as well as large chromosomal aberrations. The most common mutations associated with microphthalmia are in transcription factors that control correct gene expression during early eye development, such as SOX2 and OTX2 which account for 60% of severe bilateral microphthalmia, along with RAX, VSX2 and PAX6. These transcription factors regulate signalling pathways (e.g. WNT, BMP, TGFβ and SHH) which stimulate morphogenic movements and specialisation of cells within the developing eye. Retinoic acid signalling is vital for early eye morphogenesis, and functional variants in this pathway frequently cause microphthalmia, including STRA6, ALDH1A3, RARβ and RBP4. Inheritance patterns comprise autosomal dominant, recessive and X-linked, although germline mosaicism has been reported for multiple microphthalmia-associated variants, making deciphering inheritance patterns and providing appropriate genetic counselling challenging.

Most pathogenic mutations associated with non-syndromic cases arise de novo sporadically, and include missense, nonsense, frameshift and splice-site variants. A molecular diagnosis can be obtained in approximately 70% of severe bilateral microphthalmia cases, but less than 10% of unilateral cases, which consists of the majority of patients. This discrepancy may be the result of de novo mutations, mosaicism and haploinsufficiency in unilateral patients, or due to genetic/epigenetic modifiers, but has not been thoroughly investigated.

Heterogeneity in clinical phenotype is observed amongst patients. Microphthalmia can manifest as an isolated condition with a continuum of severity and laterality, often in association with anophthalmia (in the contralateral eye) or ocular coloboma (in the same or contralateral eye), which are considered part of the same spectrum of ocular disorders (collectively known as MAC). An affected eye can display other ocular features (complex) such as cataract, anterior segment dysgenesis or retinal dystrophy, and 33–95% of patients exhibit systemic features (syndromic) (detailed by gene in Harding and Moosajee 2019). Variable expressivity and non-penetance have also been observed in microphthalmia probands. The severity of microphthalmia on visual function depends on the stage in which eye development was disrupted, and so the degree to which ocular structure and cellular function is perturbed, as well as associated ocular malformations present.

Identification of causal microphthalmic genes and the pathways disrupted in eye development
will provide insight into pathogenesis as well as potential therapeutic targets to treat infants born with microphthalmia, through ocular delivery of drug compounds to stimulate postnatal growth and development. Furthermore, uncovering environmental factors and genetic modifiers is key to begin understanding variable penetrance. By recognising the effects of specific variants on clinical phenotype, we can establish genotype–phenotype correlations, provide important prognostic indicators and allow assembly of the correct multidisciplinary team and for families to receive informed genetic counselling and access to family planning advice.

As microphthalmia arises within the first few weeks of gestation, it is difficult to study eye development in humans, both morphologically and molecularly. Consequently, much of our understanding of microphthalmia derives from animal and cellular models. This review highlights the key disease models used to study microphthalmia, as well as the innovative technologies which will further our understanding and aid the generation of pioneering treatments.

Eye development and microphthalmia

Eye formation begins relatively early in vertebrate embryogenesis, from 3 weeks gestation in humans, embryonic day 8 (E8.0) in mice, 12 h post fertilisation (hpf) in zebrafish and embryonic stage 12.5 in Xenopus (Table 1). The molecular mechanisms of early eye development and the pathways relating to microphthalmia are reviewed in detail by Harding and Moosajee. Briefly, the eye is initially specified in the anterior neural plate through the upregulation of eye field transcription factors (EFTFs), including RAX, PAX6 and SIX3, which form a self-regulating network of genes coordinating eye development. The eye field then splits in two as the cells migrate anteriorly away from the midline of the neural plate, evaginating towards the surface ectoderm. Concurrently, the lens develops from a pre-placodal region within the surface ectoderm, and signalling from the evaginating optic vesicle stimulates the thickening of the lens placode, which then reciprocally induces invagination of the optic vesicle to form a bilayered optic cup. The outer layer of the optic cup becomes the retinal pigmented epithelium (RPE), while the inner layer forms the presumptive neural retina (NR), which later differentiates into the specialised cell types of the retina. The opening along the inferior surface of the optic cup (the optic fissure), which allows the hyaloid vasculature to support eye development, closes by week 7 in humans (Table 1).

Animal models of microphthalmia

Mature eye structure is similar across vertebrate species (Figure 1), with light entering via the pupil through the transparent cornea and lens, which refracts the light through ciliary muscle movement, before reaching the stratified, light-sensing NR at the back of the eye (whose function is supported by the RPE) where specialised retinal cells detect light (photoreceptors) and convert it into electrical signals, which are transmitted to the brain via the optic nerve. Investigating disease progression and molecular pathways in models with known genetic causes can help understand disease mechanisms. Due to their conserved ocular development and physiology, numerous mouse, zebrafish and Xenopus lines with a microphthalmia phenotype have been generated, many of which have overlapping causal molecular changes to patients (Table 2, Figure 2, Table S1). Microphthalmia has been studied in other animal models, including chick and drosophila; however, this review will only explore findings from mouse, zebrafish and Xenopus, as these are the most common models utilised to explore the molecular basis of microphthalmia due to their ease of experimental manipulation together with conserved genetics (Table 3).

Mouse

Advantages of mouse models of ocular development. Mice are the most common animal model for studying development and disease. They are easy to manage in a laboratory environment, being small with a short generation time, and are relatively cost effective. The mouse is the best characterised mammalian model system for hereditary disease, and 99% of its genome is conserved compared with humans. Eye development between humans and mice is similar (Table 1), and the mature ocular structure resembles the human, albeit lacking the cone-rich macula, instead with few cone photoreceptor cells distributed evenly throughout the retina (Figure 1). The size of the mouse eye permits morphological analysis without the need for advanced technical equipment.
Table 1. Stages of early eye development in humans and animal models.

<table>
<thead>
<tr>
<th>Species</th>
<th>EFTF expression</th>
<th>Splitting of eye field</th>
<th>Optic vesicle evagination</th>
<th>Optic cup invagination</th>
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<td>Xenopus</td>
<td>6–10 somite stage</td>
<td>10–12 somite stage</td>
<td>12 somite stage</td>
<td>12–15 somite stage</td>
<td>Hatching period (long pec stage)</td>
<td>Zuber; Zuber et al.; Ledford et al.; Holt; Feldman; Henry et al.</td>
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</tbody>
</table>

Days, days gestation; E, embryonic day; EFTF, eye field transcription factor; hpf, hours post fertilisation; stage, *Xenopus* Nieuwkoop and Faber developmental stage.

Figure 1. Diagrams of mature eye structure in human, mouse, zebrafish and *Xenopus*, and images of microphthalmic eyes in human and zebrafish. (a) Human eye with a cone-rich macula responsible for central vision, and a small lens which refracts light, along with the cornea. (b) Mouse eye with an enlarged lens compared with humans and lacking a cone-rich macula, with cones instead dispersed throughout the retina. (c) Zebrafish eye with thick neural retina layer and spherical lens which alone is responsible for focusing light. (d) *Xenopus* eye with a large, spherical lens encompassing most of the vitreous. (e) Clinical image of patient with unilateral left microphthalmia with and without prosthetic shell. (f) Wildtype and microphthalmic zebrafish at 76 h post fertilisation (hpf).
**Generation of microphthalmic mice.** Historically, forward genetics was used to create phenotypes randomly in animals, and those of interest were screened to identify genetic mutations.\textsuperscript{25,156} However, recent advances in genome editing technologies mean it is now common to directly modify specific genes to create mouse models using targeted or conditional mutagenesis, thereby using reverse genetics to generate specific mutants.\textsuperscript{25,156} Mouse phenotyping centres, such as the International Mouse Phenotyping Consortium (IMPC, https://www.mousephenotype.org/) are used to screen targeted mutants for ocular phenotypes.\textsuperscript{25,155} Many mutant mouse lines have been generated (Table 2), and 269 genes or loci have been linked to microphthalmia, from which key discoveries have been made and are described in Graw\textsuperscript{25} (Figure 2, Table S1).

**Drawbacks of mouse ocular models.** Despite the shared genetics of mice and humans, discrepancy in ocular phenotypes implies divergence in molecular regulatory mechanisms between the species. For example, common microphthalmia-associated genes involved in retinoic acid signalling, such as ALDH1A3 and RARβ, do not produce a microphthalmic phenotype in mouse models, which instead suffer from ocular disorders including lens and retinal anomalies.\textsuperscript{3,25,160,161} On the other hand, mutations in some genes produce a more severe ocular phenotype in mice than typically observed in humans, such as Pax6 which was first studied as a classical anophthalmia model as many mutants display no eyes, while microphthalmic models often develop many additional eye defects (Table 2).\textsuperscript{25} A further problem with mouse models is that inbred strains can be associated with background ocular disorders; for example, 5–10% of C57BL/6 mice and related strains develop sporadic microphthalmia/anophthalmia, depending on age, environment and additional induced mutations, most likely as the result of a polygenic disease basis.\textsuperscript{156} Therefore, choice of strain is important for translating results in mice to understanding human eye development and disease, as well as when testing novel therapies.

**Understanding molecular pathways in microphthalmia.** Conservation of genetics makes mice a practical model for investigation of genetic pathways in microphthalmia development. Through transcriptional and proteome analysis of knockout mouse models, downstream targets of disease-causing genes can be identified to resolve complex molecular networks. Transcription factor Pitx3 is known to regulate a large number of molecular elements which are important for eye development.\textsuperscript{25} Homozygous deletion of the promoter region of Pitx3 or homozygous nonsense mutations resulting in overexpression of truncated protein lead to severe microphthalmia and aphakia due to halted lens development.\textsuperscript{123,162–164} Investigation of molecular targets through EMSA and ChIP assays demonstrated Pitx3 binds to an evolutionarily conserved region of Foxe3, resulting in increased transcriptional activity.\textsuperscript{165} Foxe3 mutants display a similar phenotype to Pitx3 including microphthalmia and aphakia (Table 2), reflecting the phenotypic similarity of patients with pathogenic mutations in PITX3 causing anterior segment dysgenesis 2 (OMIM #610256) and FOXE3 producing Cataract 11 (OMIM #610623), both of which result in microphthalmia, cataract, anterior segment disorders and sclerocornea, indicating conservation of molecular regulatory mechanisms.\textsuperscript{16,166} Understanding the interaction and shared pathways of genes in eye development helps to clarify genotype-phenotype correlations in patients, as well as identify potentially effective therapeutic targets.

**Modelling variable ocular and syndromic phenotypes.** One of the earliest mouse models of microphthalmia was an ‘eyeless’ mouse (Rax\textsuperscript{ey} – MGI: 3809647) in which 10% of mice were reported to develop ‘small’ or ‘medium’ sized eyes, later determined to be the result of a point mutation in transcriptional regulator Rax, creating a hypomorphic mutant protein with a partial loss-of-function.\textsuperscript{25,56} Variation in eye size observed in patient cohorts and animal models [like Rax and Pax6 mutant mice (Table 2)] is the result of microphthalmia and anophthalmia (no eye) sharing the same clinical spectrum and genetic basis, which may be the result of dose-dependent gene function.\textsuperscript{3} The availability of allelic series of mouse mutants with a wide range of ocular disorders makes them an ideal model to study the effect of gene dosage on eye development (Table 2). For example, Sox2 mutants with a range of pathogenic variants display a spectrum of disease severity which correlates to the expression level of Sox2 in the progenitor cells of the NR, validating a role of dosage sensitivity in microphthalmia (Table 2).\textsuperscript{51} Mitf variants have differing effects on gene function: (i) semi-dominant mutations affecting the DNA-binding or transcriptional activation domains yield proteins which do not bind to DNA but still dimerise to other proteins, thereby impairing their functional DNA-binding ability; (ii) recessive variants...
<table>
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<tr>
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<th>Genotype/allele ID</th>
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| BMP4       | Mouse  | MGI:3711773        | Bmp4tm1Bhp/Bmp4 + | Mi, An, ONH, RD, Cat, CO, AC, ASD, IH, | Dunn et al. 
| BMP7       | Mouse  | MGI:3629218        | Bmp7tm2Rob/Bmp7tm4(Bmp4I Rob) | Mi | Zouvelou et al. 
| GDF6       | Zebrafish | ZDB-ALT-900203-555 | gdf6as327/s327 (dark half) | Mi | French et al. 
| SMOC1      | Mouse  | MGI:4941783        | Smoc1Tnlcb-lacZ,GFP/Smoc1Tnlblcb-lacZ,GFP | Mi, ONH, RD | Okada et al. 
| SHH        | Mouse  | MGI:3759227        | ShhTnlam1Amc/ShhTnlAmc | Mi, ONH, RD | Wang et al. 
|           |        | MGI:3812210        | ShhTnl1Ch9/Shh + | Mi, An | Ratzka et al. 
|           |        | MGI:3589447        | ShhTnl1Ch9/Shh + | Mi, Ak | Grobe et al. 
|           |        | MGI:3042780        | ShhTnl1Ch9/ShhTnl1Ch9 | Mi | Bulgakov et al. 
|           |        | MGI:3851497        | ShhTnl1.1Rseg/ShhTnl1.1Rseg | Mi | Chan et al. 
|           |        | MGI:3851498        | ShhTnlAmc/ShhTnl1.1Rseg | Mi | Chan et al. 
| Zebrafish  |        | ZDB-ALT-900413-636 | shha252/sshha252 (syu) | Mi, RD | Brand et al. 
| MAB21L2    | Zebrafish | ZDB-ALT-140130-18  | mab2112 au10/mab2112 au10 | Mi, Col, LA, ASD | Gath and Gross 
|           |        | ZDB-ALT-150611-1   | mab2112G45Sls5*/mab2112G45Sls5* | Mi, Col, CO | Deml et al. 
|           |        | ZDB-ALT-150611-2   | mab2112RS1,F52del/mab2112RS1,F52del | Mi, An, Col, RD, ASD | Deml et al. 
| PORCN      | Mouse  | MGI:6368187        | PorcnTnl1.1Lcm/Porcn + | Mi, Col, RD | Bankhead et al. 
| FRAS1      | Mouse  | MGI:2657302        | Fras1b/Fras1bl | Mi | Phillips 
| FREM1      | Mouse  | MGI:3026630        | Frem1crf11/Frem1crf11 | Mi | Kile et al. 
| TCTN2      | Mouse  | MGI:5292219        | Tctn2Tnl1.1Reit/Tctn2Tnl1.1Reit | Mi | Sang et al. 
| COL4A1     | Mouse  | MGI:4822250        | Col4a1D456/Col4a1 + | Mi, LA, Cat | Favor et al. 
|           |        | MGI:5308056        | Col4a1Detaex4d/Col4a1 + | Mi, ONH, RD | Labelle-Dumais et al. 
|           |        | MGI:4822242        | Col4a1ENPU91/Col4a1 + | Mi, LA, Cat, CO | Favor et al. 
| PTCH2      | Zebrafish | –                  | ptch2uta4/ptch2uta4 | Mi, LA, Cat, ASD | Lee et al. 
|           |        | –                  | ptch2uta5/ptch2uta5 | Mi, RD | Lee et al. 

(Continued)
Table 2. (Continued)

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<td>West et al.</td>
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<td>Timmer et al.</td>
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<td>Shaheen et al.</td>
<td>146</td>
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<td>six3a&lt;sup&gt;v129/six3a&lt;sup&gt;v129&lt;/sup&gt;, six3b&lt;sup&gt;v87/six3b&lt;sup&gt;v87&lt;/sup&gt;</td>
<td>Mi, RD</td>
<td>Samuel et al.</td>
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<td>Snx3&lt;sup&gt;1.1(KOMP)Vicg&lt;/sup&gt;/Snx3&lt;sup&gt;1.1(KOMP)Vicg&lt;/sup&gt;</td>
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<td>Mouse Genome Informatics and the International Mouse Phenotyping Consortium</td>
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<td>DAG1 Mouse</td>
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<td>Dag1&lt;sup&gt;tm2Kcam&lt;/sup&gt;/Dag1&lt;sup&gt;tm2Kcam&lt;/sup&gt;</td>
<td>Mi, Bu, CO</td>
<td>Satz et al.</td>
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<td>Plaster et al.</td>
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<td>Mi, ONH</td>
<td>Cheng et al.</td>
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Mouse genotype ID and phenotypic data was taken from the Mouse Genome Informatics database [http://www.informatics.jax.org/]. Zebrafish allele ID was taken from The Zebrafish Information Network (ZFIN) database [https://zfin.org/]. Xenopus data was taken from Xenbase [http://www.xenbase.org]. Data from December 2020.

A, aniridia; AC, absent cornea; Ak, aphakia; An, anophthalmia; ASD, anterior segment dysgenesis; Bu, buphthalmos; Cat, cataract; CO, corneal opacity; Col, coloboma; IH, iris hypoplasia; LA, lens abnormalities; LS, small lens; Mi, microphthalmia; ONH, optic nerve hypoplasia; RD, retina dysplasia.
affect Mitf transcription or produce mutant proteins which do not dimerise, and hence do not interfere with DNA binding of other proteins. This results in a variable ocular phenotype between heterozygotes, homozygotes and compound heterozygotes (Table 2). Similarly, patients with biallelic MITF pathogenic mutations exhibit COMMAD (coloboma, osteopetrosis, microphthalmia, macrocephaly, albinism, and deafness) syndrome (OMIM #617306), but haploinsufficient heterozygotes display more mild symptoms of Waardenburg syndrome (OMIM #193510), and patients with semi-dominant heterozygous mutations have the more severe overlapping disorder Tietz albinism-deafness syndrome (OMIM #103500), neither of which include microphthalmia. Consequently multiple models are required to understand the full spectrum of ocular and systemic features which can be caused by disruption of an individual gene.

Beyond the ocular phenotype, mutant mice with comprehensive phenotype annotation can be used to study any systemic involvement associated with candidate genes. For example, Otx2+/− mice display microphthalmia and otocephaly, alongside reduced fertility in males, reflective of the abnormal development of the hypothalamic–pituitary–gonadal axis seen in humans with OTX2 mutations causing syndromic microphthalmia 5 (MCOPS5 – OMIM #610125). These features coincide as, in addition to controlling oculogenesis, Otx2 regulates the expression of genes involved in pituitary development, such as Hesx1.168,173,174 Investigation of extraocular phenotypes in mice can provide information on the effect of different variants and genetic/environmental factors on systemic involvement, thus unlocking genotype–phenotype relationships.

Identification of novel variants through mouse studies. Forward genetic approaches and phenotypic screening to generate and catalogue eye phenotypes lead to successful discovery of many disease-causing microphthalmia genes, including Mitf, first identified in the early mi mouse line, and subsequently in a multitude of different lines with a range of ocular defects including a small eye (Table 2).25,167,175–178 Targeted mutagenesis is
### Table 3. Advantages and disadvantages of mouse, zebrafish, Xenopus and 2D/3D cellular models of microphthalmia.

<table>
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<th>Availability of material</th>
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<th>Genetic conservation with humans</th>
<th>Morphological similarity to humans</th>
<th>Availability of genetic/phenotypic data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Large (3 mm)</td>
<td>Breed in medium numbers (5–10 pups per litter)</td>
<td>High</td>
<td>1–2 months</td>
<td>High</td>
<td>High</td>
<td>Very good</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>Medium (1–2 mm)</td>
<td>Breed in large numbers (&gt;100 fertilised eggs/clutch)</td>
<td>Medium</td>
<td>3–5 days</td>
<td>Low</td>
<td>High</td>
<td>Good</td>
</tr>
<tr>
<td>Xenopus</td>
<td>Large (3–6 mm)</td>
<td>Breed in large numbers (&gt;100 fertilised eggs/clutch)</td>
<td>Medium</td>
<td>3–5 days</td>
<td>Medium</td>
<td>High</td>
<td>Poor</td>
</tr>
<tr>
<td>Cellular – 2D</td>
<td>N/A</td>
<td>Easy to expand (although can be difficult to obtain primary patient/embryonic tissue)</td>
<td>Medium</td>
<td>N/A</td>
<td>Human</td>
<td>Human</td>
<td>Very good</td>
</tr>
<tr>
<td>Cellular – 3D</td>
<td>Small (100–500 µm)</td>
<td>Protocols to obtain mature structures can be inefficient (and difficulty obtaining primary patient/embryonic tissue)</td>
<td>High</td>
<td>2–6 months (plus 2–3 months to reprogramme primary cells to iPSCs if required)</td>
<td>Human</td>
<td>Medium [mature structure does not contain vasculature etc]</td>
<td>Very good</td>
</tr>
</tbody>
</table>

now performed more frequently, allowing validation and further exploration into novel candidate genes, although it is time-consuming and costly to test the effect of genes of uncertain significance, unless performed by large consortiums such as IMPC.25,155

**Genetic modifiers in microphthalmia aetiology.** The vast number of mouse lines with a microphthalmic phenotype means the effect of the genetic background can be investigated by inducing multiple mutations in the same mouse model, or the same mutation in multiple strains. Cx50<sup>−/−</sup> mice have microphthalmia with nuclear cataracts.179,180 When studying Cx50 knockouts in both 129S6 and C57BL/6J strains, genetic modifiers were found to influence cataract severity due to differentially altered solubility of crystallin proteins, while eye growth was unaffected by genetic background.181 Understanding oligogenic effects on ocular development using these techniques could aid in understanding the variation of MAC spectrum and additional ocular/extraocular features observed within and between families with the same molecular diagnosis.

**Modelling environmental causes of microphthalmia in mice.** Only 2% of microphthalmia cases were attributed to environmental factors in a UK prospective incidence study; however, this varies in different regions of the world.2,3,6–8 These include maternal vitamin A deficiency, in utero exposure to toxic/teratogenic substances such as alcohol, and certain infections, for example rubella.8,182–186 For decades, mice have been used to study the effect of the maternal environment on eye development on embryos, due to their in utero gestation, for example, induction of microphthalmia through maternal exposure to toxic trypan blue at 7 days’ gestation.187,188 More recently, a study of maternal diabetes showed embryonic glucose exposure mimicking hyperglycaemia and diminished expression of Wnt-PCP pathway genes, resulting in altered cytoskeletal organisation, cell shape and
cell polarity in the optic vesicle and ultimately ocular defects including anophthalmia and microphthalmia. Likewise, the effect of maternal diet, including folic acid deficiency, alcohol consumption, pharmaceuticals and infection has also been explored in relation to mouse eye development. Studies on the environmental influences on ocular development are vital to understanding pathogenesis and providing appropriate clinical guidance and care during pregnancy.

### Zebrafish

**Advantages of zebrafish models of ocular development.** Zebrafish (*Danio rerio*) are a popular organism for studying vertebrate eye development and related disorders. Zebrafish are easily maintained and breed in large numbers at low cost, with a generation time of 2–4 months. They have many advantages over other organisms such as mice, including external fertilisation, transparency of embryos permitting direct visualisation of organogenesis, rapid eye development leading to adult-like patterning by 72 hpf and a highly organised heterotypical photoreceptor mosaic, which is cone-rich similar to humans, unlike mice. Overall, zebrafish eye development closely resembles that of humans, although there are some distinctions. Hollow optic vesicles extend from the forebrain at 27 days’ gestation in humans, while in contrast zebrafish optic vesicles begin as a solid mass of cells, which undergo cavitation by 14 hpf (Table 1). The thickening of the highly proliferating NR and thinning of the RPE through cell flattening occurs earlier in zebrafish development, and ultimately a wider NR layer is present in the adult zebrafish with squamous epithelial cells in the RPE, where cells in the mature human RPE maintain a cuboidal shape. Beyond this, the mature eye is remarkably similar between humans and zebrafish, except that in the fish, like many aquatic vertebrates, the larger, spherical lens is solely responsible for focusing light, without contribution from the cornea.

**Generation of microphthalmic zebrafish.** There is significant genetic conservation between humans and zebrafish, with 70% of human genes corresponding to at least one zebrafish orthologue, and 84% of known human disease-causing genes having a zebrafish counterpart, providing potential to model a wide scope of human conditions. Moreover, zebrafish are highly amenable to genetic manipulation, meaning mutations can be induced easily. Injection of genome modification tools at the single-cell stage of the fertilised egg allows induction of genetic changes which display a phenotype in the F0 generation. Knockdown morphants can be generated by injection of an antisense oligonucleotide morpholino, which is complementary to the mRNA of interest, and leads to transient gene knockdown in the embryo for up to 5 days post fertilisation (dpf). Injection of TALENs or CRISPR/Cas9 gene editing tools can be used to generate specific mutations in models, and establish stable mutant lines, which prevent mosaicism and are important to carry out a more complete investigation of phenotypes.

**Drawbacks of zebrafish ocular models.** Due to a whole-genome duplication which occurred in zebrafish ancestry, many orthologues of mammalian genes have two copies. Consequently, careful experimental planning is required when undertaking any genetic manipulation to avoid genetic compensation/redundancy. Role-sharing between multiple orthologous genes can lead to variations in phenotypic severity, e.g. missense mutations in the *sunrise (sri)* pax6b homozygous line replicate the milder microphthalmia phenotype observed in patients with some missense *PAX6* mutations, while morpholino-induced knockdown of *pax6a* shows more extreme phenotypes, including reduced body size and abnormal brain development, in addition to microphthalmia. Moreover, morpholinos can produce variable phenotypes, particularly regarding eye morphology (often spanning the MAC spectrum), and concerns have been raised with regards to their reliability, given disparities between morpholino and mutant phenotypes. However, off-target effects can be controlled for by co-injecting with *p53* morpholino to mitigate non-specific phenotypes.

**Understanding molecular pathways in microphthalmia.** The shared molecular basis of human and zebrafish eye development means complex genetic networks underpinning microphthalmia can be resolved using transgenic/mutant zebrafish lines to establish the function of genes during eye development (Table 2). The functional role of the *shh* signalling pathway in retinal cell proliferation and survival was established using *syu* mutants, which have homoygous *shha* deletions causing reduced eye size due to decreased mitosis and
increased apoptosis in the retina.\textsuperscript{103,104} \(rx3^{-/-}\) mutants display an eyeless phenotype and expanded forebrain, similar to isolated microphthalmia 3 (OMIM \#611038) in patients with biallelic \textit{RAX} mutations.\textsuperscript{57} Transcriptome analysis of these mutants showed downregulation of transcription factors regulating eye development (such as \textit{mab21l2}) and retinoic acid signalling pathway components (including \textit{aldh1a3}), with upregulation of Wnt signalling pathway components which function in brain development and are associated with microphthalmia and multiple neural disorders. Investigation of \textit{mab21l2} morpholino-induced knockdown showed a similar phenotype to \(rx3^{-/-}\) mutants, validating the downstream role of \textit{mab21l2} in the \(rx3\) ocular regulatory network, and its role in microphthalmia development.\textsuperscript{208} Other genetic knockdowns inducing microphthalmia include: transcription factors \textit{otx2},\textsuperscript{39} \textit{rax},\textsuperscript{209} \textit{six6}\textsuperscript{210,211} and \textit{alx1}\textsuperscript{212} retinoic acid signalling components \textit{rarr}\textsuperscript{213} and \textit{aldh1a3}\textsuperscript{160} TGF\(\beta\) signalling component \textit{gdf6}\textsuperscript{214} and SHH signalling component \textit{ptch1}.\textsuperscript{215} There are few established microphthalmic mutant zebrafish lines (Table 2), and most exist from ENU mutagenesis screens.

\textit{Modelling variable ocular and syndromic phenotypes}. Heterogenous ocular and systemic features observed in patient cohorts are mirrored in genetically modified zebrafish, allowing for further analysis of the sources of phenotype variation, whether genetic, epigenetic or environmental. Functional knockdown using \textit{vsx2} morpholinos shows concentration-dependent reduction in eye size. This dosage effect of \textit{vsx2} may explain the variable MAC phenotype observed in \textit{VSX2} patients with isolated microphthalmia 2 (OMIM \#610093) or colobomatous microphthalmia 3 (MCOPCB3 – OMIM \#610092).\textsuperscript{216} Loss-of-function biallelic variants in human \textit{STRA6} leads to syndromic microphthalmia 9 (MCOPS9/Matthew-Wood Syndrome – OMIM \#601186), where severe systemic features include pulmonary, diaphragmatic and cardiac defects, resulting in death usually within the first 2 years of life.\textsuperscript{2,217-219} This phenotype is recapitulated by morpholino-induced knockdown, which exhibits microphthalmia, curved body axis, cardiac oedema and craniofacial defects due to disrupted retinoic acid signalling in the developing eye.\textsuperscript{220} A less severe phenotype was observed when an alternative morpholino was used where a small concentration of RNA was still detectable, indicating a dose-dependent mechanism which may explain the milder or isolated microphthalmia/coloboma phenotype (MCOPCB8) observed in some patients with homozygous \textit{STRA6} mutations, including certain missense variants.\textsuperscript{9,217} Ocular and cardiac malformations in \textit{stra6}-knockdowns were partially rescued by reduction of retinoic acid binding protein 4 (\textit{rbp4}) using morpholino knockdown or 1-phenyl-2-thio-urea (PTU)-mediated inhibition (which downregulates \textit{rbp4} mRNA expression at larval stages), demonstrating potential avenues for treatment \textit{via} targeting of retinoic acid signalling pathways.\textsuperscript{220-222}

\textit{Identification of novel variants through zebrafish studies}. When a new microphthalmia candidate gene or variant of unknown pathogenic significance is identified in a family through genetic investigation, zebrafish can be used to provide evidence of pathogenicity through expression studies in the early developing eye and through rapid gene knockdown in F0 fish and with validation of resulting phenotype. A novel association of \textit{TMX3} with microphthalmia was validated with morpholinos targeting the \textit{tmx3} zebrafish orthologue \textit{zgc:110023}, resulting in significantly smaller eye size at 2 dpf.\textsuperscript{17} This phenotype was rescued by injection of human wildtype \textit{TMX3} mRNA, but not by injection of the patient mutant mRNA (p.Arg39Gln), confirming a functional effect of the \textit{TMX3} variant on eye growth.

Equally, phenotypic annotation of zebrafish knockdowns can be used to identify putative novel genes to screen unsolved patient cohorts. For example, \textit{BCO1} encodes a key enzyme for vitamin A formation and causes microphthalmia when knocked-down at the larval stage.\textsuperscript{223} Similarly, \textit{rbm24a} has been found to positively control the mRNA stability of \textit{sox2} transcripts, with gene knockdowns resulting in a small-eye phenotype.\textsuperscript{224-227} So far, no pathogenic mutations have been successfully detected in human orthologues \textit{BCO1} or \textit{RBM24} in microphthalmic patients, although disease-causing variants of \textit{RBM24} are known to cause cardiomyopathy. Nevertheless, examining these genes for functional mutations in patients without a known genetic cause through next-generation sequencing could improve molecular diagnosis rates by broadening the mutational spectrum and inclusion in future panel-based diagnostic testing.\textsuperscript{228}

\textit{Genetic modifiers in microphthalmia aetiology}. Rapid generation of phenotypes in F0 fish provides
an efficient method examine gene combinations to decipher epistatic interactions and oligogenic inheritance, aiding the investigation of multigenic factors in microphthalmia pathogenesis. Patients with pathogenic mutations in transcription factor TFP2A display a variable ocular phenotype including microphthalmia, coloboma and cataract as part of branchio-ocular-facial syndrome (BOFS – OMIM #113620), but homozygous loss-of-function tfap2a zebrafish mutants and morpholino-induced knockdown display no ocular phenotype. Heterozygous mutations in BMP4 cause syndromic microphthalmia 4 (OMIM #607932), but bmp4−/− zebrafish have normal eye morphology. Transcription factor tf7l1a plays a role in the Wnt signalling, but zebrafish tf7l1a−/+ and tf7l1a−/− mutants do not have a disrupted ocular phenotype. However, injection of tfap2a morpholinos tf7l1a−/+ and tf7l1a−/− mutants results in coloboma/anophthalmia, respectively, while injection into bmp4−/− mutants causes microphthalmia and/or coloboma, tf7l1a and bmp4 variants sensitize the developing eye to the effects of additional deleterious mutations, implying human hypomorphic TFP2A variants may contribute to developmental eye disorders when on a background with additional mutations, potentially explaining phenotypic variability. More severe microphthalmia has also been noted in tfap2a−/− zebrafish combined with mutations which when in isolation show no ocular phenotype (e.g. in hexx1) or a mild reduction in eye size (e.g. in cct5 or gdf6a).

Zebrafish studies show genetic interactions also influence syndromic heterogeneity, as otx2 morpholino knockdowns display mild microphthalmia and shortening of the pharyngeal skeleton, but the combination of otx2 and other otocephaly gene knockdowns (including pgap1, prx1 and msx1) result in more severe mandibular malformations, similar to craniofacial anomalies in patients with OTX2-associated otocephaly-dysgnathia complex. This work demonstrates that otx2 interacts with other genetic loci to regulate development throughout the body, which may explain the high systemic variation observed in patients with OTX2-associated microphthalmia. Further investigation of multigenic factors in syndromic microphthalmia using zebrafish could clarify variability observed within families.

Modelling environmental causes of microphthalmia in zebrafish. Relatively few studies of environmental factors influencing eye growth have been performed; however, phenotypic variability observed within families indicates environmental factors could account for certain cases of variable penetrance and expressivity. Fertilisation and development of zebrafish ex vivo allows for easy modification of the embryonic environment. Vitamin A deprivation through pharmacological inhibition of enzyme retinaldehyde dehydrogenase in early wildtype zebrafish embryos results in a dose-dependent reduction in eye size, with high doses causing systemic features reminiscent of the MCOPS9 phenotype including cardiac oedema and mortality within the first days after treatment. Variable severity of MAC observed between siblings with retinoic signalling component STRA6, the molecular cause underlying MCOPS9, indicates environmental factors such as maternal retinoic acid intake may be the cause of clinical heterogeneity. Modelling these factors in zebrafish, where external conditions can be manipulated, can help explain the role of environmental factors in variable ocular and systemic phenotypes.

Xenopus

Advantages and drawbacks of Xenopus models of ocular development. Xenopus have similar advantages as disease models to zebrafish, including external fertilisation and development and low cost. Unlike zebrafish, Xenopus are tetrapods, hence are evolutionarily more similar to humans, with Xenopus tropicalis sharing 79% of their genes with humans. Xenopus embryos are also larger in size, and able to tolerate extensive surgical manipulation, with transplantation of single cells to other parts of embryos in order to understand the role of interacting tissues and environments in development. However, like zebrafish, Xenopus genomes can contain duplicated genes, therefore clear understanding of compensation and subfunctionalisation is important when evaluating data. For example, genetic manipulation of six6 in Xenopus shows diverged functionality of the duplicated genes, where knockdown of six6.L results in a more severe microphthalmia phenotype than knockdown of six6.S.

Much of the early understanding of eye field specification, cell fate determination and the key regulators of oculogenesis were obtained from Xenopus studies. The development and mature eye structure is extremely similar between humans
and *Xenopus*; nevertheless, the main difference is that *Xenopus*, like many amphibians and fish, can regenerate certain eye structures beyond embryogenesis. *Xenopus* have especially high capacity for ocular regeneration, and can produce new retinal cells through functional stem cell populations, and restore lost/damaged lens through transdifferentiation of the corneal epithelium. Overall, conservation of cellular and developmental processes, as well as genomic synteny with mammals, makes *Xenopus* a valuable resource for studying eye development and microphthalmia.

**Generation of microphthalmic Xenopus.** Microphthalmic phenotypes can be generated in the F0 generation using morpholino-induced knockdowns or injection of genome editing tools at the single-cell stage, without the need for time-consuming crosses. For example, over 85% of TALENS-injected embryos to induce targeted gene disruption of *pax6a* and *pax6b* reveal perturbed eye formation and a spectrum of anophthalmia/microphthalmia phenotypes. Gain-of-function experiments have often been performed in *Xenopus* to understand molecular networks, as embryos tolerate injection with mRNA.

**Understanding molecular pathways in microphthalmia.** Size, external development and regenerative properties of *Xenopus* embryos allows surgical manipulations to be performed which can provide new insights into the molecular pathways at the initiation of eye development. Early transplantation experiments were invaluable to establishing the timing of eye induction. Following this work, ectopic expression of EFTFs showed eye field initiation can only occur in the presence of *Otx2*, demonstrating a permissive role in regulating early eye development. Fluorescent tissue induced to express EFTFs and *Otx2* transplanted to different regions of host embryos form functional, organised eye-like structures, demonstrating the need for these factors alone to stimulate and coordinate ocugenesis. This understanding of the early regulators of eye development gleaned from *Xenopus* has been essential for extricating the molecular pathways underlying microphthalmia.

**Modelling variable ocular and syndromic phenotypes.** Developmental and genetic conservation with humans means *Xenopus* can be used to study both ocular and systemic phenotypes caused by microphthalmia-associated gene disruption. Overexpression of the epigenetic regulator *SMCHD1* through injection of wildtype or mutant mRNA results in craniofacial anomalies including microphthalmia, recapitulating the Bosma arhinia microphthalmia syndrome (BAMS – OMIM #603457) phenotype observed in patients with heterozygous missense mutations, confirming a gain-of-function mechanism. This phenotype is not recapitulated in mouse models, due to apparent redundancy of *Smchd1* function in rodents.

Morpholino-induced knockdown of co-repressor gene *bcor* in *Xenopus* produces a microphthalmia phenotype, along with systemic features including skeletal and central nervous system abnormalities. These knockdowns phenotypically reflect *BCOR*-associated syndromic microphthalmia 2 (OMIM #300166), also known as oculofaciocardiodentodysplasia syndrome as hallmarks include cataracts, microphthalmia, facial, cardiac and dental anomalies. Downregulation of transcription factor *Pitx2* in this model highlighted an upstream regulator role for *bcor*, demonstrating a shared pathway in *Xenopus* and humans, as heterozygous *PITX2* variants can cause anterior segment dysgenesis 4 (OMIM #137600) or Axenfeld–Rieger syndrome (OMIM #180500), where patients also exhibit dental hypoplasia and skeletal anomalies. Knockdown of *bcor* in zebrafish does not produce a small eye, instead displaying a less severe ocular coloboma phenotype, and no ocular phenotype has been observed in mouse models of *Bcor*.

**Identification of novel variants through Xenopus studies.** Rapid ocular development, along with tolerance for genetic manipulation and injection of additional genetic material, means genes suspected to be involved in microphthalmia pathogenesis can be easily assessed in *Xenopus* using morpholino-induced knockdowns or overexpression to evaluate hypermorphic variants. MicroRNAs (miRNAs) are post-transcriptional regulators of gene expression. While not currently associated with microphthalmia, their role in eye development and disease is being revealed. Targeted knockdown or overexpression of *miR-199* in *Xenopus* results in small eyes and reduced cell proliferation in the eye field due to disruption of EFTF expression including *rax1*. This phenotype is rescued by blocking the *miR-199* binding site, demonstrating a distinct role of miRNAs in eye development and ocular maldevelopment.
and a novel set of targets for drug treatments. Additional candidates for patient screening originating from *Xenopus* overexpression modelling include siah-2,252,253 E-NTPDase,254 PNAS-4,255 and ppary256 and knockdowns of sdr16c5,257 frs3258 and psf2.259 Although none of the candidates listed have yet been identified in microphthalmic cohorts, as frequency of next-generation sequencing escalates and large databases such as from the 100,000 genomes project can be analyzed in more depth, there is increased capability to identify novel genes in patients through screening performed in animal models.260

**Modelling environmental causes of microphthalmia in Xenopus.** External development of *Xenopus* embryos allows for evaluation of adverse effects of environmental changes on ocular development. Alcohol consumption during pregnancy can cause Foetal Alcohol Spectrum Disorder (FASD), leading to microphthalmia, short stature, microcephaly and facial anomalies. Exposure of *Xenopus* embryos to ethanol between the late blastula and early/mid gastrula stages (stage 8.5–18) recapitulates phenotypic aspects of FASD, including shortened rostro-caudal axis, microcephaly and microphthalmia, due to antagonism of vital retinoic acid signalling pathways through competitive inhibition.261 This knowledge could be beneficial for understanding how genetic and environmental interaction impact eye development and help explain clinical heterogeneity in microphthalmic cohorts.

**Human cellular models of microphthalmia**

As discussed, differences exist in genetic regulation and disease manifestation between humans and animals. Hence, *in vitro* human cellular disease models can overcome species-dependent variation for studying molecular mechanisms and therapeutic compound testing, while also reducing the use of animal experimentation.

**Generation and advantages/drawbacks of different types of cellular models**

**Primary cell lines.** Cells derived directly from patients with molecularly confirmed cause allow researchers to study how specific variants disrupt human cell function, and thereby investigate genotype-phenotype correlations from a molecular and cellular perspective. Furthermore, developing and testing the effects of drugs on patient-derived cells increases capacity to determine drug efficacy, creating more reliable data for which treatments might be successful in clinical trials as well as potential for more personalized medicine options.262–265 However, a drawback of primary cell lines is as they senesce, they display changes in function and morphology, and eventually stop replicating; for example, primary RPE cells cannot be passaged more than 4–6 times.266 Additionally, developmental cell types relevant to the onset of microphthalmia such as retinal progenitor cells cannot be derived from adult tissue, and consequently must be isolated from embryonic tissues, which are in short supply and have ethical implications surrounding their usage.267,268

**Immortalised cell lines.** Immortalised cell lines provide an unlimited supply of cells at a relatively low cost and are easy to maintain.269 They are useful for studying various molecular functions in cellular processes, as they are generally tolerant of transfection with exogenous genetic material, and so can be induced to express genes of interest, enabling investigation into their mechanism of action in health and disease. However, misidentification and contamination remain widespread problems in producing reliable data from cell lines.270,271 Moreover, due to genetic manipulation required to produce the immortalised line, cells may no longer represent their cell type of origin, such as the epithelial phenotype of ARPE-19 cells which diminishes within 3–4 passages, partially due to loss of key claudin tight junctions resulting in reduced functionality.272,273

**Embryonic stem cells (ESCs)/Human induced pluripotent stem cells (hiPSCs).** Embryonic stem cells (ESCs) and human induced pluripotent stem cells (hiPSCs) have the capacity to differentiate into any lineage, and therefore can model cellular functions and molecular regulation in any cell type, at different stages of development.262,274–276 By providing an unlimited source of cells for disease modelling, ESCs/hiPSCs are an excellent resource for research and developing therapies, although can be expensive and more difficult to culture than other cells.277 HiPSCs are also a promising source of cells to treat disease by transplanting into patients, either as differentiated cells, or in a pluripotent/multipotent state.278,279 Cell-based therapies are being developed for multiple ocular diseases, such as age-related macular degeneration and retinitis pigmentosa, and show initial success with many ongoing clinical trials.278–280
3D cellular models. Traditionally, cells are grown as a monolayer of a specific cell type on a flat surface. However, 2D cell culture has been shown to alter cell morphology, gene expression and function.\(^{283-285}\) Furthermore, monoculture of a single cell type lacks the cross-cell-type signalling necessary to recapitulate the \textit{in vivo} complexity.\(^{286,287}\) Recreating the natural environment experienced in the developing eye using 3D culture techniques with multiple interacting cell types facilitates collection of more clinically relevant data.\(^{284,288}\) Organoids mimic development through restricted division of progenitor cells and expression of distinct cellular adhesion molecules which allow temporal and spatial organisation of multiple cell types, in a manner similar to that of organs.\(^{288}\) As such, organoids allow study of human organogenesis at developmental stages which would be otherwise inaccessible, such as within the first weeks of pregnancy.

Optic cup-like organoids were first generated by the Sasai group, using mESCs in 2011, then human ESCs in 2012.\(^{289-292}\) Their work showed self-organisation of cells into distinct layers reflecting the NR and RPE of the developing optic cup, although with inconsistent efficiency in forming stratified retina, which may have been the result of missing surface ectodermal signalling forming stratified retina, which may have been the result of missing surface ectodermal signalling.

Attempts to combat background genetic/epigenetic variability include creation of isogenic lines through CRISPR/Cas9 gene editing to induce/correct patient mutations, to reduce noise and generate more reliable data.\(^{318,319}\) It should also be noted that lack of additional signals such as the embryonic axis means organoid structures are often highly heterogeneous, with random relative positioning of tissue regions, such as RPE and NR in retinal organoids.\(^{288}\) Additionally, current constraints of \textit{in vitro} organoid modelling include lack of vasculature thus poor nutrient diffusion, and absent surrounding tissues, which may result in loss of vital external developmental cues.\(^{35,288,294}\)

However, advances in co-culturing techniques and organ-on-a-chip technologies may provide a solution for more complex cellular modelling, by facilitating signalling between different cell types, and a more vasculature-like perfusion of nutrients across organoids.\(^{286,320,321}\) The potential of these more advanced culturing systems for drug toxicity screening has been demonstrated through chloroquine and gentamicin treatment to induce retinopathies, although successful use of retinal organoids in drug discovery screens has yet to be reported.\(^{320,322}\)

Understanding molecular pathways in microphthalmia. Studying the genetic basis of microphthalmia directly in human cells has a clear advantage over animal models, as genetic pathways and potential therapies can be studied without possibility for divergence or functional redundancy. By modelling gene function at a cellular level in human tissue, a more translational understanding of microphthalmia pathogenesis can be established. Homozygous frameshift mutations in \textit{FAT1} have been associated with colobomatous microphthalmia, ptosis, syndactyly and facial dysmorphism in patients.\(^{323}\) Study of RPE cells showed FAT1 localised to cell–cell junctions required for optic fissure fusion in eye development, and knockdown of \textit{FAT1} using short hairpin RNA (shRNA) resulted in disruption of \(\beta\)-Catenin, ZO-1 and F-actin fibres at junction sites, and a failure of RPE cells to form an organised epithelial monolayer.\(^{323}\) These disruptions were not observed in differentiating RPE tissue from \textit{in vivo} \(\text{Fat}^{−/−}\) mouse models, although mice did display a microphthalmia and coloboma phenotype. The ability to study molecular pathways in human cells allows for clarity in where molecular mechanisms are conserved and where they deviate from animal models.

In 2014, Phillips \textit{et al.} generated optic vesicle-like models of early eye development with iPSCs derived from a microphthalmic patient with a homozygous \(\text{VSX2R}^{R208Q}\) mutation.\(^{324}\) Molecular techniques including RNAseq and ChIPseq identified that WNT pathway components were direct targets for...
VSX2 DNA binding and transcriptional downregulation in retina development. Upregulation of the WNT pathway in the VSX2-disrupted models resulted in erroneous RPE differentiation, partially rescued by pharmacological inhibition of the WNT pathway.\textsuperscript{329} Furthermore, supplementation with growth factors including FGF9 partially rescued the phenotype in mutant organoids, although suppression of FGF9 alone in wildtype organoids did not produce a phenotype, indicating redundancy of pathways in retinal development.\textsuperscript{325} The valuable insights gained from this study demonstrate the ability of these 3D cellular models to advance our understanding of how individual genes function in human eye development and which pathways are disrupted in microphthalmic patients with the corresponding variant.

Modelling variable ocular and syndromic phenotypes. Studying the impact of disease-causing proteins on cell function can elucidate the effect of different alleles and genetic/epigenetic background on phenotypic variability. FZD5 is a transmembrane receptor which regulates WNT signalling in the early optic vesicle.\textsuperscript{326} Investigation of FZD5 in HEK (human embryonic kidney) cells revealed that transfection of microphthalmic patient-originated cDNA produced truncated protein which did not localise to the outer cell membrane or mediate WNT signalling like wildtype protein, instead inhibiting the pathway due to antagonistic competition, resulting in a dominant-negative effect.\textsuperscript{326} Heterozygous pathogenic mutations in FZD5 have predominantly been identified in coloboma cohorts, but in one large family with the frameshift variant c.656del CinsAG, p.Ala219Glufs*49, two members were non-penetrant.\textsuperscript{326,327} Animal models also display variable MAC phenotypes, such as zebrafish with Fzd5 knockdown or overexpression of mutant protein,\textsuperscript{326} and Fzd5\textsuperscript{−/−} mice which exhibit 50% penetrance of mild microphthalmia/coloboma.\textsuperscript{328} This may be the result of overlapping microphthalmia/coloboma and Fzd8, as triallelic Fzd5\textsuperscript{−/−};Fzd8\textsuperscript{−/−} mutants develop severe retinal coloboma and microphthalmia with full penetrance. No FZD8 protective alleles were identified in non-penetrant individuals from whole exome sequencing; however, further analysis of human cellular models could help identify other effects of genetic background or compensatory gene mechanisms on FZD5 function.

Cell models are not representative of the whole organism and hence it is more difficult to explore systemic manifestations. However, they can be used to extrapolate tissue involvement through investigating the transcriptome and molecular pathways, as gene ontology tools can link developmental pathways in other parts of the body to shed light on syndromic features. For example, transcriptomic analysis of zebrafish optic fissure tissue identified differentially expressed genes between optic fissure and dorsal retina which are known to be involved in heart development (tbx2a/3a), providing new pathways to explore through cellular research.\textsuperscript{329} In addition, patient-derived fibroblasts with a heterozygous splice-site NAA10 variant show reduced cell proliferation and disrupted retinoic acid signalling, which may explain both microphthalmia and extraocular growth defects observed in patients with syndromic microphthalmia 1 (Lenz microphthalmia syndrome – OMIM #309800).\textsuperscript{330}

Identification of novel variants with cellular models. Converting genomic annotations from animal models to humans can be misleading, due to divergence in genetic regulation of eye development. Evidence from human cellular studies can therefore be more practical for identifying and validating novel candidates. Generation of transcriptomic and epigenomic data from human-derived 3D microphthalmic models could provide datasets from which pathway components and disease mechanisms can be identified, providing both validation for putative genetic causes found in patients as well as resources to discover new genes to screen in microphthalmic cohorts by next-generation sequencing. To date, few 3D cellular disease models have been generated, but as protocols grow more efficient, and multi-omic technologies become more affordable, cellular modelling could become an effective strategy for detecting molecular causes of microphthalmia.

Modelling environmental causes of microphthalmia in cells. The effect of exogenous chemicals on cellular function can be quickly investigated in 2D cell culture, due to efficient diffusion of compounds. Retinoic acid treatment of ARPE-19 cells induced dose-dependent increase in RARβ mRNA and protein within 24h, which was inhibited by treatment with antagonist LE135.\textsuperscript{331} Utilising more complex 3D models, toxins and potential treatments can be applied directly to mature human ocular tissues without bioavailability and drug metabolism issues, allowing greater understanding of effect on ocular development and its regulation. Importantly, using
patient-derived cells can shed light on the effects of environmental factors on different genetic backgrounds and particular modifiers, leading to more precise clinical advice and care.

Conclusion

Through work studying patients, animals and cellular models, considerable progress has been made in understanding the genetic basis of eye development, and how dysregulation of molecular pathways can result in microphthalmia. Over 90 monogenic causes of microphthalmia have been identified, and yet molecular diagnosis can only be made in less than 10% of unilateral patients and few genotype–phenotype correlations have been established. Numerous animal models for microphthalmia have been generated; however, many still have not been genetically characterised (including 25% of mouse lines) and several causative microphthalmia genes have not been disrupted in animals. Many known human genetic variants have not been studied in detail due to a lack of a corresponding model. Screening animal lines for novel candidate genes/genetic modifiers and functionally validating variants identified in patients could increase understanding of the roles of disease-causing genes, improve molecular diagnostic rates and provide patients with appropriate multidisciplinary care and genetic counselling by clarifying genotype–phenotype relationships.

Cutting-edge developments in 3D cellular modelling techniques show potential as an animal-free approach for deepening understanding of human eye development and molecular disease mechanisms at early stages of oculogenesis, which would otherwise be inaccessible to study, as well as providing promising results in understanding patient-specific mutations and developing novel therapeutics. Nevertheless, whole-organism modelling in animals is necessary for understanding the systemic effect of gene disruption and screening drugs, particularly when studying syndromic microphthalmia. Research on a combination of animal and cellular models is essential to gaining a clear picture of the molecular basis of microphthalmia and developing life-changing treatments.

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The authors declare that there is no conflict of interest.

Ethics statement

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Supplemental material

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