The Molecular Basis of Human Anophthalmia and Microphthalmia

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Abstract: Human eye development is coordinated through an extensive network of genetic signalling pathways. Disruption of key regulatory genes in the early stages of eye development can result in aborted eye formation, resulting in an absent eye (anophthalmia) or a small underdeveloped eye (microphthalmia) phenotype. Anophthalmia and microphthalmia (AM) are part of the same clinical spectrum and have high genetic heterogeneity, with >90 identified associated genes. By understanding the roles of these genes in development, including their temporal expression, the phenotypic variation associated with AM can be better understood, improving diagnosis and management. This review describes the genetic and structural basis of eye development, focusing on the function of key genes known to be associated with AM. In addition, we highlight some promising avenues of research involving multiomic approaches and disease modelling with induced pluripotent stem cell (iPSC) technology, which will aid in developing novel therapies.

Keywords: anophthalmia; microphthalmia; coloboma; eye; genetics; development; induced pluripotent stem cells; SOX2; OTX2; genes

1. Introduction

The development of the human eye is a tightly controlled morphogenetic process which requires precise spatial and temporal gene regulation [1–5]. Perturbation of early eye organogenesis, either due to genetic or environmental factors, can result in lack of eye induction or abortion of eye development [6]. Anophthalmia and microphthalmia (AM) are, respectively, the absence or reduced size of the ocular globe compared to the population age-adjusted mean (Figure 1) [7,8]. Both are part of the same clinical spectrum and, therefore, can overlap, as the phenotype can be difficult to delineate in severe cases [9,10]. True anophthalmia occurs when eye development is aborted at the stage of the developing optic vesicle around 3–4 weeks gestation, leading to absence of the eye, optic nerve and chiasm, which can be confirmed through MRI brain and orbit scans [9]. Frequently, a small cystic remnant is detectable termed clinical anophthalmia, which occurs when the optic vesicle has formed but subsequently degenerates, hence a hypoplastic optic nerve, chiasm or tract may be present [10]. In microphthalmia, the eye has a smaller volume, and can be associated with a reduced corneal diameter (microcornea defined as a horizontal diameter <9 mm in a newborn and <10 mm in children >2 years) [11]. An axial length of <19 mm at 1 year of age or <21 mm in an adult using ultrasound B-scan substantiates a diagnosis of microphthalmia, representing ≥2 standard deviations below normal [11]. The estimated prevalence of microphthalmia and anophthalmia is 1 in 7000 and 1 in 30,000 live births, respectively [12]. The effect on vision is dependent on the severity, size of the eye and associated ocular malformations [9–11]. AM are considered part of the phenotypic continuum with ocular coloboma, a structural malformation resulting from incomplete fusion of the
optic fissure, and are likely to have the same genetic basis [12–15]. Microphthalmia is reported in 3.2%–11.2% of blind children, but despite this there are no treatment options to improve visual function in microphthalmic patients, with current management focusing on maximising existing vision and enhancing cosmetic appearance [9,11,16].

Figure 1. Clinical images of anophthalmia and microphthalmia. (a) Bilateral anophthalmia. (b) Bilateral microphthalmia. (c) Unilateral anophthalmia with shell.

AM has high clinical heterogeneity, and is rarely an isolated condition, often associated with other ocular abnormalities within the microphthalmic or contralateral eye (complex), such as anterior segment dysgenesis, ocular coloboma, cataract or vitreoretinal dysplasia (Table 1) [11]. Furthermore, 33%–95% of AM occurs alongside additional non-ocular systemic malformations, with 20%–45% of individuals diagnosed with a recognised syndrome [6,7,10,17–19]. The phenotypic variation observed with AM may be due to the multitude of genetic variants, including genetic modifiers as significant intrafamilial variability is seen, and/or environmental factors, such as maternal vitamin A deficiency or alcohol consumption [7,20–23]. The genetic variation is attributable to chromosomal and monogenic causes, with over 90 identified associated genes (Table 1). Genetic diagnosis can be obtained through molecular testing, such as array comparative genomic hybridisation (aCGH) or whole exome/genome sequencing [11]. Identifying the underlying genetic cause can influence appropriate disease management with recruitment of the correct multidisciplinary team to screen for potential syndromic associations, as well as guide genetic counselling and family planning [6,11]. The molecular diagnosis can be established in approximately 70% of bilateral anophthalmia or severe microphthalmia cases, but less than 10% of unilateral cases, which consists of the majority of patients [6,9,11,12]. Through understanding the genetic regulation underlying human eye development, and clarifying the roles of signalling molecules, we can elucidate how aberrant gene expression results in eye developmental disorders. Additionally, it is possible to better understand genotype–phenotype relationships, prognosis and develop new treatments.
2. The Genetic Basis of Human Eye Development

2.1. Eye Field Specification

Human eye development is initiated at three weeks gestation in the anterior neural plate (Figure 2a) [2,24]. OTX2 is required for the specification of the forebrain, and allows the neural plate to become competent for eye development by coordinating expression of eye field initiators SIX3, RAX and PAX6 [2,3,24–27]. The role of Otx2 in initiating eye development has been shown through animal studies investigating eye development, which have shown Six3 activates Pax6 and Rx2 expression in medaka fish, and is sufficient to induce ectopic eyes in the presence of Otx2 in medaka fish and Xenopus [2,3,24–27]. OTX2 and SOX2 coregulation of RAX, along with SIX3-mediated repression of WNT allows upregulation of eye field transcription factors (EFTFs), including PAX6, RAX, LHX2, TBX3 and SIX6 (Figure 2a,b) [1,2,25,28,29]. Rax has been shown to be necessary for inducing eye development in mice, as Rax knockouts do not form optic structures [29,30]. In Xenopus, OtX2 and Sox2 have been shown to directly synergistically activate Rax through binding to a non-coding sequence 2 kb upstream of the Rax promoter, which is conserved across vertebrates [28]. Furthermore, Sox2 missense mutations identified in patients with ocular disorders were found to disrupt the interaction of Otx2 and Sox2, as well as Sox2 DNA-binding ability, perturbing the ability of Otx2 and Sox2 to regulate Rax expression [28]. The role of Six3 in early eye development has been shown in mice, where Six3 is required for repression of Wnt1 transcription, which is necessary for regionalisation of the forebrain, as Six3−/− mice show expansion of Wnt1 expression, truncated rostral forebrain and lack eyes [3,31]. EFTFs form a self-regulating network which are sufficient to coordinate the development of the eye through suppression of genes which antagonise eye development, such as WNT, and upregulation of genes required for eye development, such as BMP4, demonstrated in Xenopus by the ability of EFTF RNA cocktails to induce ectopic eye formation [1,2,4,25,32,33]. OTX2 is subsequently downregulated in the eye field [2,24].

Figure 2. The genetics of early optic vesicle development. (a) A single eye field is induced at the midline of the anterior neural plate, through OTX2 and SOX2 coregulation of RAX, along with SIX3-mediated repression of WNT, which allows upregulation of eye field transcription factors (EFTFs) including PAX6, RAX, LHX2, TBX3 and SIX6. (b) The eye field is formed expressing EFTFs, which form a self-regulating network sufficient to coordinate the development of the eye through suppression of genes that antagonise eye development and upregulation of genes required for eye development. (c) Secretion of factors from TGFβ, FGF and SHH families from the underlying axial mesoderm stimulate the anterior migration of prospective hypothalamic cells, causing the eye field to split in two to form bilateral optic primordia. (d) Cellular proliferation and bilateral migration of
the optic primordia is regulated by RAX. (e) The optic primordia evaginate from the forebrain through the cephalic mesenchyme forming bilateral optic pits/grooves. (f) Extended evagination of the optic pits through the cephalic mesenchyme results in the formation of bilateral optic vesicles.

2.2. Splitting of the Eye Field

At 22 days gestation, SIX3-regulated SHH establishes the midline and factors from TGFβ, FGF and SHH families secreted from the underlying axial mesoderm stimulate the anterior migration of prospective hypothalamic cells, causing the eye field to split in two (Figure 2c) [1–3,24]. The direct regulation of Shh by Six3 has been observed in mice, and loss of Shh signalling in mice leads to defective midline patterning, including cyclopia, also found in humans with mutations in SIX3 and SHH [1,3,34,35]. Experiments using medaka fish show that FGF signalling is required for neural patterning to establish a competence region for Shh signalling, and FGFs interact with Shh to activate downstream targets of Shh required for optic development [2,36]. Ndr2, a member of the TGFβ family, is required for midline signalling in zebrafish, and loss of Ndr2 expression leads to cyclopia [2,3,37]. Tracking the movement of neural cells in zebrafish has shown that the single eye field is split through the anterior migration of cells, and cells in Ndr2 mutant zebrafish fail to migrate, resulting in a single cyclopic eye [2,38]. RAX-positive cells of the eye field proliferate and migrate bilaterally, resulting in evagination of the optic primordia from the forebrain and the generation of bilateral optic pits/grooves, observed through fluorescent tagging of Rx3 in wild-type and mutant medaka fish (Figure 2d,e) [2,4,24,25,30,39,40]. From 22–27 days gestation, further evagination of the optic pits through the cephalic mesenchyme results in the formation of optic vesicles (Figure 2e,f) [2,3].

2.3. Lens Placode Formation

Within the overlying surface ectoderm, expression of SIX3 directly activates PAX6 and SOX2, stimulating the formation of a pre-placodal region from 22–27 days gestation (Figure 3a) [4,41–43]. Six3 regulation of the lens pre-placodal region has been demonstrated by conditional deletion of Six3 in mice, leading to downregulation of PAX6 and loss of Sox2 expression, as well as failure of the pre-placodal region to thicken [4,42]. Furthermore, ChIP, EMSA and luciferase reporter assays, show that Six3 activates Pax6 and Sox2 expression in mice [42]. LHX2-regulated BMP4 expression from the evaginating optic vesicle signals the thickening of the lens placode at day 27–28 of gestation (Figure 3b) [1,3,4,41,43–45]. The importance of Bmp4 in lens induction has been shown in mouse models, where homozygous mutant mice lack lenses, but the phenotype can be rescued by exogenous BMP4 protein [45]. Lhx2 has been shown to regulate Bmp4 expression in the optic vesicle of mice, as Lhx2−/− mice have downregulated Bmp4 in the optic vesicle, and failure of lens formation [43,46].
Figure 3. The genetics of optic cup and lens formation. (a) A preplacodal region develops within the surface ectoderm overlying the optic pit/groove, stimulated by expression of SIX3, which activates PAX6 and SOX2. (b) The lens placode is signalled to thicken through LHX2-regulated BMP4 expression from the evacuating optic vesicle. (c) The developing lens placode releases BMP and retinoic acid (RA), which bind to the optic vesicle, stimulating coordinated invagination of the optic vesicle and lens placode, which forms the lens pit. (d) The invagination of the optic vesicle results in the formation of a bilayered optic cup. (e) TGFβ signalling from the extraocular mesenchyme induces and maintains MITF and OTX2 expression in the outer layer of the optic cup, which forms the presumptive retinal pigmented epithelium (RPE). FGF signals from the surface ectoderm stimulate the inner layer of the optic cup to form a VSX2-expressing presumptive neural retina (NR). The continued invagination of the lens pit results in the formation of the lens vesicle. (f) From 47 days gestation, retinal differentiation occurs, with the inner layer of the optic cup forming the neural retina (NR) and the outer layer forming the retinal pigmented epithelium (RPE). The lens vesicle detaches from the surface ectoderm and forms a definitive lens by 58 days gestation.

2.4. Optic Cup Formation

The developing lens placode releases BMP and retinoic acid which bind to the optic vesicle, stimulating invagination of the optic vesicle from 31–33 days gestation (Figure 3c,d) [1,4,47–52]. Simultaneously, retinoic acid signalling from the surrounding pericocular mesenchyme aids invagination of the optic vesicle (Figure 3c) [48,51]. Retinoic acid requirement in optic cup formation has been studied in mouse models, where double knockouts of retinoic acid synthesising retinaldehyde dehydrogenases developed optic vesicles that failed to invaginate, which were rescued by maternal dietary retinoic acid administration [1,4,47–52]. Experiments in mice show that filopodia extending mostly from the lens ectoderm tether the lens to the presumptive retina, allowing coordinated invagination of the presumptive lens and optic vesicle, and loss of filopodia in mutant mouse models results in reduced lens pit invagination (Figure 3c–e) [4,53]. In mice and chicks, lens invagination has been shown to occur through apical constriction, but the mechanisms driving optic vesicle invagination are not entirely clear, although experiments disrupting the extracellular matrix in chick embryos, alongside computational investigation have shown the extracellular matrix is required for proper formation and invagination of the lens placode and optic vesicle, through spatial restriction of proliferating cells [54–57].

The invagination of the optic vesicle results in the formation of a bilayered optic cup (Figure 3d,e) [33]. TGFβ signalling from the extraocular mesenchyme induces and maintains MITF and OTX2 expression in the outer layer of the optic cup, which forms the presumptive retinal pigmented epithelium (RPE), demonstrated experimentally through the removal of the extraocular mesenchyme in developing chick eyes resulting in absent RPE, which was restored through addition of the TGFβ family member activin (Figure 3e,f) [4,24,44,58–61]. MITF and OTX2 in the distal optic vesicle activate RPE differentiation genes, such as QNR71, Tyr, Trp1 and Trp2 in mice [24,62]. Reduction of MITF signalling in a human embryonic stem cell (hESC) model of eye development disrupted RPE maturation [61]. Additionally, Otx1 and Otx2 mutant mouse models show loss of RPE-specific Mitf expression, and expansion of neural retina (NR)/optic stalk regions [63]. FGF signals from the surface ectoderm stimulate the inner layer of the optic cup to form VSX2-expressing presumptive NR, shown in chick development through Fgf8 localisation to the neural retina, and in vivo application of FGF8, inducing presumptive chick RPE to differentiate into ectopic NR through preventing expression of RPE marker Mitf, and inducing NR markers Rx and Srx1 (Figure 3e,f) [3,4,24,64]. VSX2 in the proximal optic vesicle represses RPE marker MITF, and is required for cell proliferation [24,65–68]. In Vsx2 mutant mice, Mitf is ectopically expressed in the NR, indicating Vsx2 represses Mitf expression in the NR [24,68]. Furthermore, mice with impaired Vsx2 production have a small eye phenotype due to decreased proliferation of retinal progenitor cells [24,69]. PAX6 expression in the presumptive NR maintains multipotency in retinal progenitor cells, allowing subsequent differentiation into the seven retinal types: retinal ganglion cells, horizontal cells, cone photoreceptors, rod photoreceptors, amacrine cells, bipolar cells and Müller glia [24,70]. Inactivation
of Pax6 in mice retinal progenitor cells using conditional gene targeting restricts the cell fate of retinal progenitor cells to amacrine cells [24,70].

The optic cup is patterned through antagonistic expression of PAX2 and PAX6, shown in mouse models as Pax6-/- mutants show expansion of the Pax2 expression domain into the optic stalk, and Pax2-/- mutants show expansion of the Pax6 expression domain into the optic cup, and analysis of the molecular interactions of Pax6 and Pax2 in mice show reciprocal transcriptional repression [24,32,71,72]. SHH-regulated VAX1/VAX2 expression induces PAX2 expression and represses PAX6 expression in the proximal optic vesicle, inducing optic stalk formation, demonstrated experimentally by injection of Shh mRNA into Xenopus embryos, resulting in expansion of the Vax1 and Pax2 domain, at the expense of the Pax6 domain, in addition to abrogation of Vax1/Vax2 in zebrafish, leading to the expansion of retinal tissue into the optic nerve territory [24,32,73-75]. The optic fissure forms on the ventral surface of the optic vesicle from four weeks gestation to enable the hyaloid vasculature to enter and supply the developing eye with nutrients [76]. This opening fuses by week seven of gestation through apposition of the epithelial margins around the vasculature, spatial specification along the proximal-distal axis of the fissure and basement membrane breakdown, resulting in the formation of a continuous epithelial layer [77-79].

3. The Genetic Basis of Anophthalmia and Microphthalmia

AM has a complex aetiology, with dominant, recessive, autosomal and X-linked inheritance, although most mutations associated with non-syndromic AM arise de novo sporadic [11,12]. Non-penetrance has been observed, where family members carrying the same variant as the AM proband exhibit no ocular phenotype, as well as variable expressivity, where relatives with the same variant exhibit a spectrum of AM and related ocular features [80,81]. Variable expressivity and non-penetrance may be due to genetic modifiers, and/or stochastic environmental factors which influence the levels of gene expression [80]. Some cases of AM have been shown to arise through parental germline mosaicism, where a parent expresses no ocular phenotype as the mutation is not present in the ocular-derived tissue, but is present in gonadal tissue due to postzygotic mutational events [10,80,82-84]. This complex pattern of inheritance has implications for providing meaningful genetic counselling [85]. Chromosomal abnormalities, including aneuploidy, triploidy, translocations, deletions and duplications account for 20%-30% of AM [9,10,85,86]. Over 90 individual genes are associated with AM (Table 1), most of which encode transcription factors involved in eye morphogenesis, or components of the retinoic acid signalling pathway [12,87,88]. Pathogenic monogenic mutations include missense, nonsense, deletions, insertions and splice-site variants [12,66].
Table 1. Genes associated with anophthalmia and microphthalmia. A—unilateral or bilateral anophthalmia. M—unilateral or bilateral microphthalmia. I—Isolated AM with no additional ocular features. C—complex AM (additional ocular features found in microphthalmic or contralateral eye of the patient listed). N—no syndromic features observed. S—systemic features observed.

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<th>Gene Name</th>
<th>OMIM#</th>
<th>Disease Name</th>
<th>Disease OMIM#</th>
<th>Anophthalmia/ Microphthalmia (A/M)</th>
<th>Isolated/Complex (I/C) (Associated Ocular Features)</th>
<th>Non-Syndromic/ Syndromic (N/S)</th>
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<td>M</td>
<td>C (coloboma, microcornea, nystagmus, esotropia, myopia, retinal detachment)</td>
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<td>604294</td>
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<td>614402</td>
<td>A, M</td>
<td>C (optic nerve hypoplasia, small optic nerve, cyst)</td>
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<td>SMOCI</td>
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<td>Microphthalmia with limb anomalies (MLA)</td>
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<td>Microphthalmia, isolated with coloboma 5 (MCOPCB5)</td>
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<td>Linear skin defects with multiple congenital anomalies 1 (LSDMCA1)</td>
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<td>I, C (corneal opacity/cloudy and vascular cornea, cyst, sclerocornea, glaucoma,)</td>
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<td>LRP5</td>
<td>603506</td>
<td>Osteoporosis-pseudoglioma syndrome (OPPG)</td>
<td>C (retinal detachment, persistent hyperplasia of the primary vitreous)</td>
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</tr>
<tr>
<td>91</td>
<td>PQBP1</td>
<td>300463</td>
<td>Renpenning syndrome (RENS1)</td>
<td>C (coloboma)</td>
<td></td>
<td></td>
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<tr>
<td>92</td>
<td>TUBB</td>
<td>191130</td>
<td>Symmetric circumferential skin creases, congenital, 1 (CSCSC1)</td>
<td>C (short palpebral fissure, retinal dysplasia, microcornea)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>93</td>
<td>MAPRE2</td>
<td>605789</td>
<td>Symmetric circumferential skin creases, congenital, 2 (CSCSC2)</td>
<td>C (short/slanting palpebral fissure, strabismus, ptosis)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>94</td>
<td>SALL1</td>
<td>602218</td>
<td>Townes-Brocks syndrome 1 (TBS1)</td>
<td>C (abnormal lens, aplastic optic nerve, small optic chiasm)</td>
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<tr>
<td>95</td>
<td>HDAC6</td>
<td>300272</td>
<td>Chondrodysplasia with platyspondyly, distinctive brachydactyly, hydrocephaly, and microphthalmia</td>
<td>I</td>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>ALX1</td>
<td>601527</td>
<td>Frontonasal dysplasia 3 (FND3)</td>
<td>C (coloboma)</td>
<td></td>
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<td>97</td>
<td>RERE</td>
<td>605226</td>
<td>Neurodevelopmental disorder with or without anomalies of the brain, eye, or heart (NEDBEH)</td>
<td>C (coloboma, optic nerve hypoplasia, anisometropia)</td>
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<tr>
<td>98</td>
<td>RAB18</td>
<td>602207</td>
<td>Warburg micro syndrome 3</td>
<td>C (microcornea, congenital cataract, small atonic pupil, progressive optic atrophy)</td>
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References: [201, 202, 203, 204, 205, 206, 207, 208, 209, 210]
There is significant inter- and intra-familial phenotypic variability amongst patients and this may be related to specific genotype–phenotype correlations, or yet unidentified genetic modifiers. In PAX6-related ocular disease, haploinsufficiency gives rise to a range of phenotypes; the majority of loss-of-function alleles result in classic aniridia (with full iris and foveal hypoplasia, nystagmus, cataract, glaucoma and corneal keratopathy [211]) and missense mutations in the DNA-binding paired domain result in a milder phenotype, such as dominant nystagmus with foveal hypoplasia and normal irides [212]. Interestingly, a recent report highlights recurrent missense variants, p.S54R and p.N124K, associated with severe bilateral microphthalmia, phenocopying SOX2-associated AM [213]. An electromobility gel-shift assay (EMSA) revealed that for p.S54R, an 85% reduction in binding to the PAX6 protein-responsive element termed the LE9 enhancer was seen compared to wild-type protein, but with no effect on binding to the SIMO element (an autoregulatory PAX6 binding site with enhancer activity, resulting in correct maintenance of PAX6 expression). The p.N124K variant showed a moderate reduction in binding to both DNA enhancer sequences. The precise location of variants can affect protein function, and the lack of established genotype–phenotype relationships highlight the need for more correlation studies in disorders with Mendelian inheritance and phenotypic heterogeneity, such as AM. This will guide clinicians on prognosis and identifying potential therapeutic targets [85]. Furthermore, exploring the genetic pathways frequently disrupted in AM will support the identification of novel causative genes, variants, and regulatory elements which may act as genetic modifiers [85]. The more common genes associated with AM, with evidence from animal and cellular modelling, will be outlined below. The main genes fall into two distinct categories (i) eye field initiating transcription factors, such as SOX2, OTX2, RAX, VSX2 and PAX6 or (ii) retinoic acid signalling pathway components, including STRA6, ALDH1A3 and RARβ [12,87,88].

3.1. Eye Field Initiating Transcription Factors

3.1.1. SOX2

Heterozygous variants of sex-determining region Y-box 2 (SOX2) are the most common known cause of bilateral anophthalmia and severe microphthalmia, accounting for 15%–40% of cases [8,12,19]. SOX2 mutations are associated with isolated unilateral or bilateral AM, complex AM, and Syndromic Microphthalmia 3 (MCOPS3), where extraocular features include brain anomalies, neurocognitive delays, seizures, sensorineural hearing loss, oesophageal atresia, short stature, microcephaly and genital anomalies (Table 1) [8,12,89]. Over 76 disease-associated variants of SOX2 have been identified, incorporating loss-of-function deletion, missense, frameshift and nonsense mutations [7,8,12,19]. The majority of AM pathogenic SOX2 mutations arise de novo (60%), but alleles can also be inherited in an autosomal dominant manner, causing AM due to SOX2 haploinsufficiency [7,8,12,89]. Disease-associated SOX2 variants have been inherited from asymptomatic parents as a result of mosaicism, such as a case of a mother with no ocular features who had a daughter with bilateral anophthalmia along with extraocular features of SOX2 syndrome as a result of germinal mosaicism [83,84]. Consequently, it is important to screen parents for accurate genetic counselling and future family planning [6,9–11].

SOX2 is a high-mobility group transcription factor [8,28]. In *Xenopus*, Sox2 and Otx2 have been shown to function together to coordinate Rax expression in the early eye field [28,29]. Furthermore, introduction of point mutations in *Xenopus* Sox2 protein (to replicate missense mutations found in human ocular abnormalities) resulted in disrupted Rax transactivation and reduced DNA and Otx2-binding ability of Sox2 [28]. These findings indicate a key role for Sox2 in early eye formation, through co-ordinated regulation of EFTF Rax in conjunction with Otx2, and consequently, disruption of Sox2 results in disrupted eye development through perturbation of the Rax signalling network [28,29]. The severity of ocular abnormalities observed in humans indicate the role of SOX2 may reflect that of *Xenopus*, with a conserved key function in transcriptional regulation of key genes in human early eye development.
A genotype–phenotype correlation has been suggested for SOX2 mutations in AM cohorts, as missense mutations, particularly in the DNA-binding or transactivation domains, often result in non-penetrance or a milder ocular phenotype, such as ocular coloboma, and fewer developmental and systemic abnormalities, including postnatal growth retardation [8,10,12]. However, a clear correlation has not been definitively proved [8]. Variable Sox2 ocular phenotypes have been observed in a gene-dosage allelic series of mouse models, which displayed a range of eye phenotypes where the severity correlated to the expression of Sox2, suggesting a relationship between SOX2 genotype and clinical phenotype in humans may exist, due to dosage sensitivity in eye development [10,214].

3.1.2. OTX2

Between 2%–8% of AM cases are estimated to be the result of a pathogenic mutation in OTX2 [8,9,18,90,91]. Combined, SOX2 and OTX2 variants account for at least 60% of bilateral severe cases of AM [12,19]. Variants of OTX2 include indel, missense, frameshift and nonsense mutations, and AM is usually the result of a heterozygous loss-of-function [8,12,19,91]. While 40% of OTX2 mutations arise de novo, 35% are inherited from an unaffected parent, which complicates inheritance patterns [8,12,19,91]. Frequency of non-penetrance is high, with carrier parents displaying no ocular phenotype, as well as variable expressivity, for example, a case where one patient displayed unilateral microphthalmia, and his brother had a right anophthalmic eye and left ocular coloboma [12,18,19,80,91]. This case was believed to be a result of gonosomal mosaicism, as neither parent was found to carry the mutation in blood or buccal cell DNA [18]. Mosaicism has been confirmed in other OTX2 cases, such as a patient with bilateral severe microphthalmia who was the daughter of a phenotypically normal mother shown to have low levels of the variant in blood and buccal DNA, indicating gonosomal mosaicism [12,18,19,80,91]. OTX2 patients encompass extremely variable phenotypes, including additional eye malformations such as anterior segment dysgenesis, retinal dystrophy and hypoplasia or aplasia of the optic nerve and optic chiasm, as well as syndromic features including pituitary abnormalities, hypopituitarism, brain anomalies, seizures and developmental delay (Table 1) [8,91,93,215]. Homozygous mouse knockouts of homeobox gene Otx2 lack eyes, while heterozygous knockout mouse models also show a highly variable phenotype, from acephaly, micrognathia, anophthalmia, microphthalmia to normal development, depending on the genetic background [8,25,63,80,215–219]. A relationship between genotype and clinical phenotype has been observed, as mutations in the second half of OTX2 seem to occur more frequently with abnormal pituitary function, although this correlation is not always seen [8,91,215].

OTX2 is required for neural development, and coordinates the expression of EFTFs SIX3, RAX and PAX6 to initiate eye field development, but is subsequently downregulated in the eye field by EFTFs including RAX [3,25,32,220–222]. Otx2 has been shown to be necessary for eye development in mouse and Xenopus, as early EFTFs Six3 and Pax6 only possesses the ability to induce ectopic eyes in the presence of Otx2 expression [25–27,63,223]. Furthermore, overexpression of Otx2 in Xenopus results in upregulation of Rax, indicating Otx2 is a positive regulator of Rax in early development [28].

OTX2 also plays a role later in eye development, during the regionalisation of the optic vesicle, where TGFβ signalling from the extraocular mesenchyme induces OTX2 expression in the distal optic vesicle [24]. WNT signalling then induces upregulation of OTX2 in the prospective RPE [24]. OTX2 is required for RPE maintenance and differentiation, including regulation of key RPE marker MITF [24,63,224]. Inactivation of Wnt signalling activator β-catenin in mice at the optic vesicle stage results in a lack of Otx2 and Mitf signalling in the optic cup, and expansion of the NR domain, leading to anophthalmia [24,59].

3.1.3. RAX

Biallelic mutations in the EFTF RAX are estimated to account for 2%–3% of AM [6,7,11,94]. Disease-associated loss-of-function variants of RAX include missense, nonsense, frameshift and splicing mutations, as well as whole gene deletions [6,12]. Heterozygous carriers of disease-associated variants display no ocular phenotype, indicating an autosomal recessive inheritance
pattern [6,7,12]. Homozygous or compound heterozygous RAX variants are often associated with bilateral severe AM, alongside neurological features such as intellectual deficiency and autism [7].

RAX has been shown to play a conserved role in establishment/proliferation of the eye field, as expression of ectopic Rax in Xenopus results in formation of ectopic retinal tissue [1,30], and zebrafish/mouse heterozygous knockouts of Rax have an eyeless phenotype [30,225–227]. The homeobox gene Rax is expressed early in eye development, and has been identified as necessary for the initiation of eye field development through regulation by Otx2 and EFTFs Six3 and Pax6 in targeted mouse knockout models [228]. Rax has been shown in zebrafish to coordinate the splitting of the eye field, by downregulating nlcam in the eye field, to decelerate the convergence of retinal progenitor cells to the midline of the neural plate [39,40]. Overexpression of nlcam results in abnormal migration of the eye field, converging further towards the midline, resulting in a small eye phenotype in zebrafish [39]. Furthermore, in chimeric mouse embryos of Rax−/− and wild-type cells, Rax-deficient cells were not incorporated into optic tissue, indicating a role for Rax in cellular segregation in the early developing eye [4,29].

3.1.4. VSX2

Biallelic mutations in VSX2 are thought to account for 2% of isolated microphthalmia, and were identified in 1.5% of all MAC (microphthalmia, anophthalmia and coloboma) cases in a screening of >880 samples [9,12]. Disease-associated variants include missense, nonsense, exon deletions and splice-site variants [66,95]. VSX2 has an autosomal recessive inheritance pattern, and homozygous or compound heterozygous VSX2 variants are usually associated with bilateral AM [12]. VSX2 variants can result in isolated AM, although complex cases often include cataracts, as well as iris abnormalities, retinal dystrophy and colobomas, and individual cases with systemic features have been reported, including developmental delay with behavioural problems, autism, cryptorchidism, ovarian defects, or hearing impairment, although no genotype–phenotype correlations have been identified [12,17].

VSX2 is a homeodomain-containing transcription factor [95]. Inactivation of the VSX2 orthologue Alx1 in zebrafish results in small eyes [95,229]. Vsx2 null mice are microphthalmic, and show reduced proliferation of retinal progenitor cells, as well as transdifferentiation of the neural retina into RPE [69,95,230,231]. Vsx2 has been shown to regulate retinal cell number via CycD1-mediated repression of the negative regulator of proliferation cyclin-dependent kinase inhibitor Kip1, in retinal progenitor cells [231]. Consequently, Vsx2 enables cell proliferation by preventing retinal progenitor cells from exiting the cell cycle [66,231]. This has been shown as in Vsx2 null mice, Kip1 is abnormally present in retinal progenitor cells, resulting in cell number decrease in the retina and a small eye phenotype [231].

In the optic vesicle, VSX2 expression marks the developing neural retina, while MITF is expressed in the developing RPE [32]. Fgf expression activates Vsx2 in mouse neural retina which represses Mitf transcription, and Vsx2 null mice show erroneous expression of Mitf in the neural retina [66,68]. Analysis of the function of VSX2 in human iPSC-derived optic vesicles showed WNT to be a downstream target [232]. Patient-derived iPSCs with a mutation in VSX2 found upregulation of WNT pathway components and RPE markers, demonstrating VSX2-mediated repression of WNT contributes to maintaining neural retina identity [232].

3.1.5. PAX6

PAX6 is a transcription factor most commonly associated with aniridia [92,233]. A study of >500 MAC cases found 1%–2% to be PAX6 related [12]. Heterozygous loss-of-function PAX6 variants have been found in patients with unilateral and bilateral AM, indicating an autosomal dominant inheritance pattern [7,12,92]. Patients with PAX6-associated microphthalmia often have a complex phenotype, with associated ocular abnormalities including aniridia, iris hypoplasia, coloboma and optic nerve hypoplasia (Table 1) [12]. Mosaicism has been reported in PAX6-associated microphthalmia, such as a case with two brothers with bilateral complex microphthalmia with a heterozygous missense mutation, and an unaffected mosaic mother [92].
Missense mutations in the paired-box domain of PAX6 have been more frequently been associated with MAC, which is the domain known to interact with SOX2 [12,213,234]. The complex formed by PAX6 and SOX2 is known to be key for initiation of lens development by activation of crystallin genes [3,234]. Correct formation of the lens is vital to optic cup development, indicating a potential pathway for PAX6 mutations in the paired-box domain to have an increased likelihood to cause AM.

The homeobox gene PAX6 has been shown to be a conserved regulator of eye development [42,235]. Pax6 mutant mouse models display small eyes alongside other ocular phenotypes [92,236,237]. Pax6−/− mice develop eyes, but they fail to be maintained, arresting in development prior to the optic cup stage [2,236–238]. PAX6 is one of the first EFTFs to be expressed, and ectopic expression of Pax6 in Xenopus can be sufficient to induce ectopic eye tissue [223,235]. Furthermore, misexpression of Pax6 in Xenopus results in erroneous upregulation of EFTFs including Six3, Rax and Lhx2 [25,223,235]. Pax6 is known to form functional complexes with Lhx2 in mice, which are required for the regulation of Six6 in the optic vesicle [235,238]. These results indicate PAX6 plays a conserved key role in initial eye formation by initiating a cascade of gene expression which regulates eye development [223].

Rax-deficient mice show loss of Pax6 expression in the region of the neural plate which would form the optic primordium, suggesting Rax is an upstream regulator of Pax6 in early eye field formation [32,228]. In Six3 mutant mice, Pax6 is downregulated in the preplacodal lens ectoderm, indicating Pax6 in the developing lens is also activated by Six3 [26,42]. Bmp7 has also been implicated in the initiation of Pax6 in lens induction, as in Bmp7 null mice, Pax6 expression is lost in the lens placode, resulting in lens defects [3,239].

Moreover, PAX6 is required for the dorso-ventral patterning of the optic cup, where PAX6 is highly expressed in the dorsal region and PAX2 is expressed in the ventral region [3,235]. In Pax6 mutant mice, there is dorsal expansion of Pax2 expression, while in Pax2 mutants there is ventral expansion of Pax6, demonstrating the antagonistic function of Pax6 and Pax2 in the optic cup [3,72]. In addition, this patterning is regulated in mice by Shh which promotes ventral expression of Vax1 and Pax2, while inhibiting expression of Pax6 and Rax [24,73,235,240]. In Vax1/Vax2 double-mutant mice, the optic nerve is transformed into retinal tissue, and Pax6 expression is increased in the ventral region of the optic cup, demonstrating the importance of Vax1/Vax2 in regulating Pax6 expression in the optic cup [75]. Lhx2 mutant mouse optic vesicles show lack of downstream target Vsx2 and display expansion of Pax6 ventrally, indicating Pax6 is partially regulated in the optic cup through Lhx2-mediated Vsx2 expression [32,46].

3.2. Retinoic Acid Signalling Pathway Components

3.2.1. STRA6

Biallelic loss-of-function mutations in STRA6, including missense, nonsense, frameshift and whole gene deletions, can lead to AM, usually manifesting bilaterally [8,12,97,241]. Commonly, pathogenic recessive STRA6 variants result in Microphthalmia, Syndrome 9 (MCOPS9), also known as Matthew Wood Syndrome, where syndromic features include pulmonary, diaphragmatic and cardiac defects, resulting in death within the first 2 years of life [12,17,96,242]. However, milder phenotypes have been observed, including a patient with bilateral anophthalmia and mild syndromic features, in addition to isolated AM in patients with homozygous missense STRA6 variants [19,20]. However, no genotype–phenotype correlation has yet been established [8,96,98].

STRA6 is a transmembrane receptor for retinol-binding protein, responsible for mediating vitamin A uptake into cells [8,20,98,243,244]. Cellular analysis showed that a missense mutation in STRA6 resulted in loss of vitamin A uptake [20]. Furthermore, the clinical phenotype is recapitulated in zebrafish models, with inhibition of retinoic acid resulting in microphthalmia, and developmental defects including heart morphogenesis [20]. Patients with STRA6 variants display variation in phenotype, such as one family where phenotype ranged from bilateral anophthalmia to unilateral
microphthalmia with contralateral ocular coloboma, indicating phenotypic variation may result from environmental factors such as variable vitamin A uptake, as well as genetic background [8,20,98].

The severity of phenotypes associated with STRA6 variants demonstrate the importance of retinoic acid signalling in development [98]. Retinoids can regulate development through enhancing or suppressing developmental gene expression [20,245,246]. In eye organogenesis, retinoic acid is required to signal the formation of the lens placode, in addition to the invagination of the optic vesicle [43,48,49]. Absence of Stra6 in zebrafish impairs retinoic acid receptor signalling and gene regulation, resulting in craniofacial and cardiac developmental defects, as well as microphthalmia [245]. The results demonstrated in animal and cellular modelling indicate a conserved function of STRA6 in mediating vitamin A uptake, and that disruption of STRA6 results in AM due to disruption of the retinoic acid signalling pathway, and consequently, disrupted expression of genes required for eye development, including lens placode development and optic cup invagination [245].

3.2.2. RARβ

AM is associated with recessive loss-of-function or dominant gain-of-function pathogenic variants of RARβ [8,21,87,88]. Variants include missense, nonsense and frameshift mutations [87]. Gain-of-function missense RARβ variants found in AM patients were shown to exhibit increased response to retinoic acid ligands compared to the wild-type, indicating an increase in retinoic acid signalling mediated by RARβ is sufficient to disrupt eye development [87,88]. The clinical manifestation of RARβ mutations resemble those of STRA6, including pulmonary and cardiac defects, often resulting in termination of pregnancy or neonatal death [8,12,87,88].

RARβ is one of three retinoic acid receptors, with RARα and RARγ, which transduce retinoic acid signalling to allow retinoic acid to regulate specific target genes in development [51,88]. The retinoic acid receptors form heterodimers which are key to oculogenesis [48,51]. A mouse model with all three retinoic acid receptors inactivated resulted in clinical anophthalmia, including lack of optic nerve [51]. In mouse models, RARβ-mediated retinoic acid signalling in the periocular mesenchyme has been shown to regulate apoptosis and the expression of Foxc1 and Pitx2, which coordinate the development of the anterior eye segment [51,247,248].

3.2.3. ALDH1A3

Mutations in the retinaldehyde dehydrogenase gene ALDH1A3 are associated with an estimated 10% of AM [21,99]. Disease-associated variants include missense, nonsense and exon skipping [99–101,249]. Biallelic ALDH1A3 mutations tend to be associated with bilateral AM, and can be isolated or complex (Table 1) [12]. Biallelic pathogenic AM ALDH1A3 alleles are commonly associated with a neurocognitive phenotype, but additional extracocular findings are rare [8,12,99]. Non-penetrance has been reported with ALDH1A3 disease-associated variants, such as a case of autosomal recessive ALDH1A3 variants where an asymptomatic sister carried the same homozygous mutation as two siblings affected with bilateral AM [101,249].

ALDH1A3 is part of the retinoic acid synthesis pathway, through oxidation of retinaldehyde to retinoic acid [8,48,99]. ALDH1A3 is one of three human retinaldehyde dehydrogenases, which have specific spatial and temporal expression patterns [48,50,250]. Pax6 mutant rat embryos are deficient in retinaldehyde dehydrogenase Raldh3 (Human ALDH1A3), indicating Raldh3 is genetically regulated by key oculogenesis transcription factor Pax6 [48,251].

The mouse retinaldehyde dehydrogenase gene Raldh2 (human ALDH1A2) is expressed in the periocular mesenchyme [50,250]. Loss-of-function mutations in Raldh2 inhibit optic cup invagination [49]. Consequently, ALDH1A2 in humans is thought to regulate retinoic acid synthesis signalling to the invaginating optic vesicle through signalling from the periocular mesenchyme [48]. However, in Raldh1−/−(Human ALDH1A1)/Raldh2−/− mouse embryos, retinoic acid synthesised by Raldh3 in the RPE acts on the neural retina to aid ventral invagination [48,50,250]. Raldh3−/− mouse mutant embryos initiate optic cup formation, but lack retinoic acid in the ventral optic cup, resulting in ventral retina shortening [250,252]. Consequently, there appears to be a conserved role for ALDH1A3 in synthesis
of retinoic acid in the developing ventral optic cup, with mutations resulting in defects in optic cup formation, including invagination [48,250].

4. Current Research and Future Directions

4.1. Modelling Eye Development Using iPSCs

Current understanding of eye development is heavily based on evidence from animal modelling, focusing on zebrafish, *Xenopus* and mouse [76,253,254]. While conserved molecular pathways indicate a fundamental similarity across vertebrate eye development, conflicting evidence from experiments performed on different model organisms indicates a degree of divergence in the morphogenesis of the eye [255,256]. For example, the development of the cornea is known to vary by species; stratification of the human corneal epithelium occurs early in development, resembling chick development, while the development of the mouse corneal epithelium does not fully stratify until 10 weeks after birth [255]. Furthermore, the migration of neural crest cells which form the corneal endothelium and stromal keratocytes occurs in two waves in reptiles, birds and primates, while in rodents, cats, rabbits and cattle, this occurs in a single wave of cell migration [256]. To comprehensively understand the molecular basis of human developmental eye defects and ultimately, develop functionally viable therapies, it is vital to obtain an accurate understanding of human eye development, and appreciate how it differs from that of model organisms.

One method for understanding human biology is the use of human induced pluripotent stem cells (hiPSCs) [257,258]. Long-term modelling of hiPSCs can enhance our understanding of normal human cellular behaviour, as well as better comprehending diseases by deriving patient iPSCs which can be used for investigating the basis of phenotypic heterogeneity, in addition to therapy screening and personalised medicine [257,259]. Current work in developing methodologies for organoid modelling of eye development show potential for gaining a working understanding of human eye morphogenesis, and developing therapies [259,260]. RNA-seq transcriptome analysis indicates that stem-cell derived optic cups recapitulate human eye development *in vivo*, through temporal expression of cell differentiation markers and retinal disease genes, as well as mRNA alternative splicing [261–263]. Early-stage optic vesicle-like structures generated from hiPSCs derived from a microphthalmic patient with a VSX2 null variant showed upregulation of WNT pathway components and RPE marker, *MITF*, which were rescued by pharmacological inhibition of WNT signalling [232]. These results showed a key role for VSX2 in WNT signalling and maintenance of the neural retina through WNT pathway suppression [232]. This study shows the potential utility of hiPSC modelling in improving understanding of human eye development, and developing novel therapies. However, a constraint of *in vitro* modelling is the lack of vasculature and surrounding tissues, which may result in loss of vital external cues for recapitulating *in vivo* development [264]. Additionally, whole organism modelling is necessary for understanding the effect of gene activity on organogenesis of multiple organs, which is particularly important when studying syndromic disorders [265]. Utilising knowledge gleaned from *in vivo* animal modelling in conjunction with human cellular modelling is key to obtaining a complete picture of the molecular basis of human eye development and the role of perturbed genetic pathways in congenital disorders, such as AM.

4.2. Genomics and Epigenetics

The functionality and availability of genetic tools has progressed rapidly in recent years. Next-generation sequencing is a powerful tool for understanding development and disease, allowing relatively cheap, high-throughput methods for analysing the genome, transcriptome and epigenome [266]. Whole exome/genome sequencing can identify novel disease-associated coding and non-coding variants, as well as be a useful tool in diagnosing patients with heterogeneous ocular disorders with numerous underlying genetic causes, such as AM (Table 1) [81,267]. Increasing the proportion of known variants associated with AM will improve the frequency of genetic diagnosis, which is important for directing disease management, including screening for potential syndromic features, as well as providing reliable genetic counselling and family planning [11,85]. RNA-seq has aided in
characterising gene expression in the developing retina, as well as elucidating genetic pathways affected in ocular disorders through differential transcriptomic analysis, such as the role of VSX2 in regulation of the WNT pathway in retinal organoid differentiation [232,268,269]. Improved understanding of temporal gene expression throughout eye development will aid in identifying potential disease-associated variants, as well as putative therapy targets.

A potential source of disruption to the molecular regulation of the developmental pathway is epigenetic disturbance. Epigenetic changes, such as chromatin remodelling and histone modification, result in altered gene regulation, and therefore, have a great potential for disrupting the ocular developmental pathway [270,271]. Heterogeneity in epigenetic patterning in stem cell cultures has been shown to impact the ability of cells to differentiate into retinai, providing reasonable evidence that epigenetic regulation is important to normal eye development, and consequently, may play a role in ocular malformations [272]. Epigenetic changes altering the secondary structure of DNA have been shown to induce neurodevelopmental disorders including autism [273]. Utilising epigenetic profiling techniques such as CHIP-seq to identify epigenetic variation associated with developmental ocular disorders will provide a better understanding of how these disorders arise, in addition to providing a new avenue of putative treatments to explore [266,274,275].

4.3. Proteomics and Metabolomics

Post-translational modifications can have a significant impact on protein activity, and consequently, cellular function, for example, post-translational modifications play a key role in the regulation of zebrafish retina regeneration [276–278]. Consequently, investigating the proteome alongside the transcriptome is key to understanding cellular behaviour in eye development. Additionally, the impact of downstream metabolites in temporal mediation of cellular behaviour has been revealed in recent years [279]. Advances in metabolomic investigation, such as metabolome profiling, will provide greater insight into different aspects of cellular processes and how metabolites impact development, in addition to providing new biomarkers for understanding the pathways disrupted in disease and targets for neonatal screening therapies [280,281].

4.4. Therapeutics

One of the fundamental reasons for investigating the molecular basis of human eye development and disease is to manipulate the knowledge gained into targeted therapies that will benefit patients. As AM is a structural eye defect arising from three weeks gestation, developing prenatal treatments has been difficult as the mother may not know they are pregnant and compounds may have an adverse effect on both the mother or the rest of the developing foetus. However, with the first in utero exploratory multicentre phase I/II clinical trial (NCT03706482) evaluating safety and efficacy of postnatal or prenatal intravenous administration of allogeneic expanded foetal mesenchymal stem cells for the treatment of severe osteogenesis imperfecta underway, there may be potential applicability for congenital ocular disorders in the future.

Small-molecule drugs, such as Ataluren, target in-frame nonsense mutations by interfering with ribosomal fidelity to enable near-cognate amino acids to be substituted rather than a release factor, thus enabling the production of full-length functional proteins. This drug had positive results as a prenatal treatment of aniridia in mouse models, demonstrating developmental plasticity of the eye. A phase 2 multicentre randomized, double-masked, placebo-controlled clinical trial (NCT02647359) is currently underway to assess the safety and efficacy of Ataluren for nonsense-mediated aniridia. If successful, this presents another avenue of post-natal treatment for certain genetic congenital diseases such as microphthalmia, where growth and visual maturation may be encouraged [282].

5. Conclusions

This review has highlighted the key molecular coordinators of early-eye development, and summarized current understanding of how disruption of these precise spatiotemporal regulators can lead to disruption of eye organogenesis, resulting in anophthalmia or microphthalmia (AM). Recent
advances in the field of genetics, including high-throughput next-generation sequencing technology has progressed knowledge of the genetic basis of AM, as well as improving diagnosis and genetic counselling [85]. Increasing genetic screening of AM patients using whole exome/genome sequencing will continue to improve the rate of diagnosis, through identifying novel disease-associated genes and isoforms [85]. Furthermore, increased genetic diagnosis will provide larger cohorts to analyse the AM genotype/phenotype relationship, including identifying environmental factors or genetic modifiers which may result in heterogeneity and variable expressivity observed in AM [85]. Additionally, utilising animal and cellular models of AM, in conjunction with genome-editing technology such as CRISPR, will further advance understanding of function and interactions of molecular regulators of eye development, and how pathogenic alleles result in AM, with the ultimate aim of creating therapies that may aid patients with AM [85,232].

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Conflicts of Interest: The authors declare no conflict of interest.

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