Human Genetics (2019) 138:1019–1026
https://doi.org/10.1007/s00439-018-01968-5

ORIGINAL INVESTIGATION

Prospects and modalities for the treatment of genetic ocular anomalies

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Received: 3 April 2018 / Accepted: 24 December 2018 / Published online: 2 January 2019

Abstract
Over the last three decades, genetic studies have made great strides toward the identification of genes and genetic mechanisms underlying congenital disorders of the eye. However, despite the vast knowledge available this has not translated into treatments to prevent or repair the damage in the clinical setting. Recently, new research in technologies, such as tissue regeneration, next generation designer drugs, and genome editing, have become available for some genetic disorders that might be applicable to congenital ocular diseases in the near future. Here, we provide an overview of the emerging therapeutic modalities and the future prospects they hold for debilitating ocular defects.

Introduction

During early development the human eye forms through a complex program of evolutionarily conserved spatiotemporal gene and protein interactions in tissues from different embryonic origins (Adler and Canto-Solera 2007). Defects in induction, proliferation, migration or differentiation during development can lead to a spectrum of congenital ocular disorders such as microphthalmia, anophthalmia and coloboma (collectively termed MAC), aniridia, cataract, anterior segment dysgenesis, retinal dystrophies, optic nerve defects and foveal hypoplasia. The severity of these defects depends on developmental timing and the extent to which growth and morphogenesis of the developing eye is disturbed (Graw 2010). Approximately, 50% of blindness in children has a genetic cause (Rahi and Cable 2003; Rhan et al. 2007), although not all genes have been elucidated. For example, already more than 80 genes are associated with MAC and still the genetic cause of many MAC cases is unresolved (Chassaing et al. 2014). For some genetic disorders such as aniridia it is straightforward to test for chromosome 11 deletions and *PAX6* sequence analysis, whereas for other diseases the underlying cause is not clear cut. This is a particular problem due to the high level of genetic, allelic and clinical heterogeneity that exists in many eye diseases. It is often frustrating for both parents and their physicians when a genetic test comes back negative or where it is difficult to clarify the significance of the DNA changes in terms of pathogenicity. Currently there are no treatments for congenital eye defects other than surgery where possible (e.g., congenital cataract) and detection *in utero* is not possible with routine antenatal screening.

Although much research has focused on Mendelian traits, this is further complicated by the effect of environmental factors, acting either independently or in combination with genes, as well as epigenetic factors. For example, dietary vitamin A deficiency results in a higher prevalence of ocular coloboma in some Asian countries (Hornby et al. 2002), whereas more recently a study has revealed a new mode of maternal transmission of mutations in *RBP4* where there is a restriction of placental vitamin A transport, associated with reduced penetrance of MAC (Chou et al. 2015). Other studies have found ocular associations with thalidomide toxicity (Miller and Stromland 1999) and fetal alcohol exposure in pregnancy (Brennan and Giles 2014). Furthermore, epigenetic mechanisms, including DNA methylation, histone modifications, chromatin remodeling, and non-coding RNAs, can result in the heritable silencing of gene expression without primary DNA sequence changes leading to ocular disease (Cvekl and Mitton 2010). For example, the ocular phenotype associated with spinocerebellar ataxia type seven is cone-rod retinal degeneration caused by a polyglutamine expansion in ataxin-7, that inhibits GCN5 histone acetyltransferase activity required for CRX (cone-rod homeobox)

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transactivation of photoreceptor genes (Palhan et al. 2005). These types of findings raise significant problems with regard to identifying new treatments for these common ocular birth defects. In addition, it suggests that a personalized medicine approach taking into consideration both genetic and biomarker profiles may be required (Porter and Black 2014).

Potential therapeutic options for congenital malformations of the eye

Prevention

Environmental factors are contributors to the occurrence of some ocular malformation defects, thus pre-conception preventative strategies can be considered, akin to folic acid supplementation to reduce the risk of spina bifida and other neural tube defects (NTDs) by about 80% (MRC 1991). A recent study examined pre-conception dietary supplementation in women and the risk of anophthalmia/microphthalmia in their offspring (Weber 2018). This study looked at 244 affected infants compared to 11,109 unaffected live-born controls. The odds of these phenotypes occurring were decreased in women who supplemented with folic acid, magnesium, and vitamin E compared to those who took no supplements. However, since these data were obtained by a post-birth dietary survey, there are a many limitations to this study. Actual measurements of nutrient intake and quantification of bioavailable levels needs to done before informing on pre-conception dietary recommendations.

Folic acid is an important nutrient that is involved in epigenetic modifications such as DNA methylation, histone modification and chromatin rearrangements through the one-carbon metabolic system (Ducker and Rabinowitz 2017) to ultimately produce S-adenosylmethionine. In addition, folate provides one-carbon units for the biosynthesis of thymidine, methionine and purines (Morscher et al. 2018), particularly important during proliferation in the developing embryo. One-carbon metabolism defects are implicated in neural tube defects (Leung et al. 2017). Both methionine and glycine cleavage cycles are required for normal neural tube closure and inhibitors of the methionine cycle cause NTDs in mice (Dunlevy et al. 2006). Furthermore, in mice that have defective glycine decarboxylase function (lack of one carbon units going into the folate cycle), neural tube closure was incomplete and about 30% of the mice had anophthalmia/ microphthalmia (Leung et al. 2017). This could be rescued by formate supplementation but not methyltetrahydrofolate. Thus, since folic acid enters the folate cycle as dihydrofolate and does not carry a one-carbon group, its efficacy in inhibiting neural tube defects is dependent on sufficient endogenous supply of one-carbon groups from mitochondria. Thus, perhaps formate supplementation may be more effective at inhibiting developmental defects rather than folic acid.

With respect to folate's role in DNA methylation most of the knowledge comes from studies on NTDS. Mutation in any of the three methyltransferase genes results in NTDs in mice (Wilde et al. 2014). The majority of folate-associated methylated CpG dinucleotides overlap with a specific enhancer (H3K4me1) in human stem cell-derived neural cultures suggesting that folate controls gene expression via regulation of enhancers (Valensisi et al. 2017). In a number of studies suggest that abnormal DNA methylation plays a major role in human NTDs. For example, a common SNP (rs1801133) in the folate pathway gene *MTHFR* is associated with a higher risk of NTDs (Friso et al. 2002). NTDs in progeny are also associated with a mutation in the methionine synthase reductase gene in the maternal grandparents (Padmanabhan et al. 2015). In the eye, a very recent report suggests that abnormal methylation of the *Pax6* promoter at transcription factor binding sites is associated with cyclopia and anophthalmia (Martin-del-Campo et al. 2018). Furthermore, defects in a methytransferase gene (*dnmt3bb.1*) in zebrafish results in retinal hyperplasia and increased expression of *crx*, and *opn1lw1*, whereas excessive Dnmt3bb.1-dependent methylation globally represses eye genes in cavefish resulting in loss of eyes. (Gore et al. 2018). These studies suggest that therapies aimed at targeting epigenetic mechanisms might reduce the occurrence of ocular malformations. One recent study showed that the histone methylation inhibitor DZNep significantly delayed photoreceptor loss in a mouse model of retinitis pigmentosa (Zheng et al. 2018).

Tissue regeneration and transplantation approaches

It has been broadly accepted that when a child is born with an ocular development abnormality that the only treatment option is surgery where that is possible (e.g., cataract, eyelid coloboma repair, corneal transplant, prosthetic implant, etc). This is based on the embryological precedent that tissues form during timed stages of development, and once those have passed then the regulatory genes that control their formation are usually switched off. Stimulating structural and functional repair of complex tissue after birth, or after injury, would be an attractive proposition. However, unlike metazoans the regenerative capacity in mammalian species is limited or absent due to constraints from immune responses, physiological adaptation and angiogenic capacity (Iismaa et al. 2018). Despite these constraints, much progress has been made in elucidating key molecular mechanisms that may provide therapeutic targets for the development of future regenerative therapies, as well as previously

unidentified developmental paradigms and windows-of-opportunity for improved regenerative repair.

In some ocular malformation disorders it may be possible to use tissue regeneration or transplantation to treat specific aspects of the phenotypes. Regeneration of tissue refers to both regular renewal throughout life (i.e., tissue homeostasis) or in response to injury (reparative regeneration). The drivers for tissue homeostasis to maintain physiological function are different to the injury signaling for tissue repair. In some organs, such as the brain and visual system, there is a little regenerative capacity, as the birth of new neurons is restricted to the embryonic or early postnatal period (Lillien 1994). However, this has been challenged by carbon dating of neuron birth in the brain which has demonstrated that ~35% of hippocampal neurons turn over at a rate of ~1.75% per year, mediated through proliferating neural stem cells (Monje et al. 2003; Spalding et al. 2013). In addition there is increasing evidence that endogenous stem/progenitor cells may play regenerative and reparative roles in response to traumatic brain injury (Sun 2014). Examples of physiological regeneration in the eye include replacement of corneal epithelium and eyelashes. Thus, it might be possible to use the intrinsic cues to stimulate corneal repair postnatally in some malformation defects.

The corneal epithelium is renewed by limbal epithelial stem cells (LESCs) that reside in the palisades of Vogt between the cornea and conjunctiva. Birth defects affecting the cornea such as aniridia, occur due to deficiency of LESCs ultimately leading to corneal opacification. Corneal transplantation alone is not a feasible option for these patients because the corneal grafts only replace the central cornea and not the limbus so they ultimately fail due to the lack of LESCs in the patient (Ahmad et al. 2010). Alternatives include transplantation of allogeneic limbal tissue from donors (Dua and Azuara-Blanco 2000) or cultivated allogeneic LESC transplantation (Rama et al. 2010). The overall success rate of these surgeries is high (75%), however, the lack of healthy limbal donor tissue, immune responses and stromal scarring are problematic (Joe and Yeung 2014). Pioneering research has revealed that human embryonic stem cells (ESCs) or human induced pluripotent stem cells (iPSCs) have self-renewal capabilities and have the potential to be differentiated into any cell type (Takahashi et al. 2007; Ahmad et al. 2007). Both ESCs and iPSCs have been differentiated into corneal epithelial and limbal lineages that could be suitable for human transplantation (Brzeszczynska et al. 2014; Sareen et al. 2014). Most recently, three-dimensional corneal organoids have been differentiated from iPSCs (Foster et al. 2017) containing mucin-secreting goblet cells, stromal keratocytes, an endothelial layer and a Descemet's-like basement membrane, which could replace the use of donor tissue (Susaimanickam et al. 2017). There are several ongoing clinical trials investigating either autologous or HLA matched donor limbal stem cells in patients with limbal stem cell deficiency (ClinicalTrials.gov).

A recent landmark study demonstrated a strategy for treating congenital cataract using naturally regenerated lenses (Lin et al. 2016). Although congenital cataract can be treated by capsulorhexis and implantation of an artificial intraocular lens, the procedure is not without risk of complications such as poor surgical outcomes, secondary glaucoma and issues with refractive correction in the developing eyes. These young infants have yet to develop normal lens refractive power, and clinical sequelae such as strabismus and amblyopia can occur. This study showed that removal of the native lens with a modified minimal capsulorhexis in rabbit, macaques, and in human infants under the age of 2 years, allowed the endogenous lens epithelial stem/progenitor cells to regenerate a transparent, biconvex lens. Eight months after surgery visual acuity and accommodation power were significantly improved over pre-operative baseline in human infants, and there were no surgery-related complications. The authors suggest that the use of endogenous stems cells to replace a cataractous lens could provide a new approach in ocular regenerative therapy. However, there are several issues that were not addressed with this study: (1) they did not take into account that the majority of bilateral infantile cataract is caused by genetic defects in the lens epithelial stem cells. Therefore, the underlying defect is still present and patients may then require further surgery; (2) the mean visual acuity achieved by children in this trial was at the threshold for legal blindness, whereas current recommended cataract surgery between 6 and 8 weeks of life is twofold better than that reported in this trial. As reported by Solebo and co-workers (2018) further work needs to be done to support the conclusion that this technique is superior to current therapeutic modalities.

In some vertebrates, such as teleost fish and amphibians, the development of the retina continues throughout life, due to the existence of a population of cells found the ciliary marginal zone (CMZ) (Wetts 1989). Although retinal histogenesis is thought to occur only during the early stages of embryonic development, there is mounting evidence suggesting a CMZ-like region in the adult mammalian eye (Martinez-Navarrete 2008). This is based upon the expression of the protein nestin (a marker of stem cells) in the peripheral Müller glia cells (Bhatia et al. 2009). The Müller glia-derived progenitors in post-embryonic fish retina generate cells that follow the rod photoreceptor lineage (Raymond et al. 2006; Lust and Wittbrodt 2018). A sub-population of Müller glia with stem/progenitor characteristics has also been identified *in situ* within the adult human retina (Lawrence et al. 2007). Although Müller glia cells appear to be quiescent, epidermal growth factor exposure in cultured explants leads to significant cell proliferation (Bhatia et al. 2009). A recent proteomic study in zebrafish showed that

after damaging the retina with intravitreal injection of ouabain, there were key differences in protein expression levels between degenerating (↑Galectin 1, ↑HSP90-α2, ↑ApoA1b, ↑Vitellogenin 6) and regenerating retina (↑Tubulin beta 2A, ↑vimentin) (Eastlake et al. 2017). This suggests that regeneration likely involves complex interactions that link proliferation with cellular remodelling and metabolic changes.

Injection of insulin and FGf2 into the postnatal chick retina results in Müller glial cell expression of Pax6 and Chx10 transcription factors (Das et al. 2006). In a neonate rat retina study, treatment with the neurotoxin *N*-methyl-D-aspartic acid (NMDA) stimulated Müller cell proliferation and the production of bipolar cells and rod photoreceptors, whereas retinoic acid treatment stimulated bipolar cell genesis (Ooto et al. 2004). In an alternative strategy, mis-expression in the neonate retina of Math3/NeuroD resulted in amacrine cell genesis from Müller glia, whereas Math3/Pax6 induced horizontal cells and Crx/NeuroD induced photoreceptor cells (Ooto et al. 2004). These observations suggest that the adult mammalian retina has the potential for regeneration. Thus, it may be possible to tap into the regenerative capacity of endogenous progenitors using intrinsic and extrinsic cues to stimulate the differentiation of these Müller glia cells into retinal neurons. Müller glia may become a target for drug delivery and regenerative therapies for disorders such as Leber congenital amaurosis.

Nonsense suppression

Meta-analysis has revealed that approximately 11% of all inherited diseases are caused by nonsense mutations based on reported mutations in the Human Genome Database 2012 (Lee and Dougherty 2012), suggesting that targeting patients with this type of mutation could be relevant to many patients with a broad range of genetic diseases (Mort et al. 2008). Nonsense suppression is a therapeutic approach aimed at treating genetic diseases caused by premature in-frame nonsense mutations (PTCs). This approach utilizes compounds that inhibit translation termination at PTCs, allowing translation to continue, so that partial levels of deficient protein function can be restored (between 5 and 20%). Proof-of-principle for this approach was demonstrated for a nonsense mutation in the *CFTR* gene that causes cystic fibrosis (Howard et al. 1996). In this study, the aminoglycoside G418 was able to suppress the nonsense mutation and restore significant levels of functional protein in cultured cells. In recessive diseases even small amounts of functional protein can be of therapeutic benefit in improving function or halting disease progression (Wagner et al. 2001). Since then more than 100 studies have tested this approach in different animal models and with a variety of different drugs (Wang and Gregory-Evans 2015). In the eye, nonsense suppression has been tested in retinitis pigmentosa (Guerin et al. 2008; Schwarz et al. 2014), choroideremia (Moosajee et al. 2016), ocular coloboma (Moosajee et al. 2008), usher syndrome type 1 (Goldman et al. 2012), usher syndrome type 2A (Neuhaus et al. 2017) and in aniridia syndrome (Wang et al. 2017).

The landmark research in nonsense suppression therapy for *PAX6* mutations that cause aniridia has resulted in a paradigm shift in the way we think about ocular tissue plasticity (Wang and Gregory-Evans 2015; Wang et al. 2017). Our lab analyzed the efficacy of Ataluren in a *Pax6*-deficient mouse model of aniridia (*Pax6-Sey*) carrying a naturally occurring nonsense mutation. We developed a novel eye drop formulation of Ataluren which we called 'START' therapy, that was given twice daily after birth. The therapy reversed corneal, lens, and retinal malformation, and restored electrophysiological function of the retina. In addition, the therapy and prevented the disease from progressing, and on removal of the drug the malformation did not return, demonstrating the stable efficacy. A further addition to nonsense suppression therapy is the idea of combining this therapy with a nonsense-mediated decay inhibitor (Linde et al. 2007; Gonzalez-Hilarion et al. 2012). This would mean that potentially more native mutant mRNA transcript would be available for nonsense suppression. This would be particularly valuable where low read-through does not produce enough functional protein, especially in recessive diseases.

The preclinical studies of Ataluren efficacy for *Pax6* nonsense mutations has led to the first clinical trial for aniridia. The STAR study is a phase 2, multicenter, randomized, double masked, placebo controlled study of the safety and efficacy of Ataluren (PTC124) for the treatment of nonsense mutation aniridia (NCT02647359). Enrolled patients take an oral dose of Ataluren or placebo three times per day for 48 weeks, followed by open-label Ataluren for another 48 weeks. An eye drop formulation is not yet available, however, since aniridia has some systemic pathologies including metabolic, endocrine and neurological deficits (Netland et al. 2011) an oral formulation may be beneficial. With the development of new nonsense suppression drugs with more efficient PTC read-through capability and improved safety profiles, it is likely that patients with other ocular diseases caused by nonsense mutations will be enrolled in future clinical trials.

These studies have suggested that the eye retains significant developmental plasticity into the post-natal period. This could, therefore, provide a window of opportunity to treat congenital blindness. In the case of aniridia, if treatment was started at birth then we may be able to promote foveal development as this continues until about 4 years of age (Hendrickson et al. 2012). Furthermore, the progressive corneal keratopathy in aniridia could be delayed or halted completely. We have also shown that nonsense suppression can close the optic fissure in a *pax2*-deficient zebrafish model

of ocular coloboma. However, since optic fissure closure occurs at 5–7 weeks post-conception in humans, nonsense suppression drugs would need to be taken prior to conception similar to folic acid supplementation to prevent neural tube defects. Although we did not observe any toxic effects on organ development in zebrafish there are likely to be considerable ethical and toxicological hurdles to be overcome before use in humans is possible, and probably only then in families with inherited mutations.

Gene editing

New tools in molecular biology such as clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated (Cas) gene system are revolutionizing the modification of the genome in microorganisms (Horvath and Barrangou 2010; Wiedenheft et al. 2012), eukaryotic cells (Hu 2016) and in a number of experimental ophthalmology paradigms (Burnight et al. 2018). Using CRISPR-Cas9 reagents DNA double strand breaks (DSB) are repaired through two different conserved repair pathways: NHEJ (non-homologous end joining) and HDR (homology directed repair). Using the correct DNA templates, these repair pathways can be used to repair mutations or knock-out defective mutations, allowing the repaired genes to remain under the control of their endogenous regulatory elements, to avoiding overexpression toxicity. Examples of gene editing in ocular disease include co-injection of Cas9 mRNA and a single guide RNA into embryos targeting the dominant mutation in the mouse *Crygc* gene which effectively rescued the cataract phenotype, and was subsequently transmitted to the next generation (Wu et al. 2013). CRISPR-Cas9 has also been used in patient-specific induced pluripotent stem cells (iPSCs) to repair an RPGR point mutation responsible for X-linked retinitis pigmentosa (Bassuk et al. 2016). Furthermore, a gene editing approach has been used in Leber congenital amaurosis type 10 (LCA10). This is caused by a common intronic splicing mutation in *CEP290* (IVS26 c.2991 + 1655 A > G), a centrosomal protein localized to the connecting cilium of photoreceptors (Drivas et al. 2013). CRISPR-Cas9 was used to excise this disease-causing *CEP290* mutation in primary fibroblasts derived from LCA10 patients (Maeder et al. 2015). Critically, this gene editing correction led to increased amounts of wildtype CEP290 protein and correction of the ciliogenesis. Interestingly, two pharmaceutical companies are hoping to bring LCA10 gene editing to clinical trial in the near future (Allergan and Editas Medicine). The most recent article in ocular disease targets gain-of-function mutations in retinitis pigmentosa. Here, the authors use gene editing to ablate endogenous rhodopsin from both alleles using a mutation-independent CRISPR/Cas9 strategy combined with replacement of wildtype rhodopsin to compensate for loss of endogenous rhodopsin protein (Tsai et al. 2018). In this approach, any rhodopsin mutation could be targeted and would be a cost-effective approach. However, off target effects (e.g., Cas9-gRNA recognizing other similar genome sites) was not evaluated in this study.

In 2017 the National Academy of Sciences, Engineering, and Medicine suggested that modifying human embryos should be allowed, but only to correct mutations that cause serious disease or conditions, and when no reasonable alternatives exist (The National Academies Press). The cardiomyopathy associated with a mutation in the *MYBPC3* gene is a common cause of sudden cardiac deaths in young people. One way to correct the genetic defect could be by gene editing. Recently, CRISPR-Cas9 technology was used to correct a pathogenic heterozygous *MYBPC3* mutation in human preimplantation embryos through homology-directed repair mutation in human embryos (Ma et al. 2017). In this study about 72% of the injected embryos, which were fertilized with sperm carrying the heterozygous *MYBPC3* mutation, carried two copies of the non-mutated gene copy after CRISPR/Cas9-treatment. There has been criticism of this research because the lack of robust verification of gene correction, since alternative outcomes (e.g., rearrangements, long deletions and loss of heterozygosity) could be used to explain the absence of mutation detect in the embryos (Egli et al. 2018). Implanting embryos with undetected mutations could have severe consequences after development.

The application of CRISPR-Cas9 to congenital ocular malformations may in the future be possible, for now though only somatic defects of a malformation could potentially be targeted postnatally. Examples might include correcting the mutation in *PAX6* in limbal stem cells to prevent corneal opacification in aniridia; knocking out PITX2 dominant-negative mutations causing glaucoma or targeting an undeveloped fovea in ocular albinism. Clinical implications of gene editing in human embryos are clearly substantial with further optimization in terms of mosaicism, off-target editing and detection of unexpected abnormalities in edited embryos before implantation.

Conclusions

We are now entering an era of rational drug design, tissue engineering and gene editing interventions that could reach many patients with ocular disorders. Because the treatment modalities are in general mutation or patient-specific, the horizon for personalized medicine is quickly approaching. A step-change is required in the availability of genetic testing for ocular diseases so that patients can be directed to relevant clinical trials. Furthermore, national patient registries and natural history data for each phenotype need to be developed. Overall, the future is promising for many of these untreatable ocular malformation defects.

Compliance with ethical standards

Conflict of interest The authors declare there is no conflict of interest.

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