The Chinese University of Hong Kong Ophthalmic Research Centre: from the past to the future

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Abstract

The Department of Ophthalmology and Visual Sciences of the Chinese University of Hong Kong was established in 1994 as the first academic ophthalmology department in Hong Kong. The department involves patient services, research, professional training, medical student teaching, post-graduate education, community services, international services and project development. For research, we conducted programs to advance ophthalmic surgery, clinical trials, epidemiology studies, investigative ophthalmology and basic sciences. A comprehensive ophthalmic research laboratory system is established for microscopies, genomics, molecular biology, cell biology, histopathology, analytical chemistry, experimental animals and bioinformatics. The Clinical Drug Trial Centre has been accredited by the China Food and Drug Administration since 2006. We work closely in clinical ophthalmology, research, training and education with the Shantou University / Chinese University of Hong Kong Joint Shantou International Eye Center. We have built a collaborative network in ophthalmic research with institutions in Hong Kong, mainland China and overseas. In 2015, we incorporated our Lim Por-yen Eye Genetics Research Centre, Pao So Kuk Macular Diseases Treatment and Research Centre and Lee Wing Kit Advanced Ophthalmic Training and Education Centre with the research laboratories to form the Chinese University of Hong Kong Ophthalmic Research Centre. While the existing Chinese University of Hong Kong Eye Centre mainly conducts clinical services and research, the 2 centers work together on patient orientated research to advance the investigation and treatment of common and rare eye diseases.

Key words: Cell biology; Genetics; Universities

Introduction

The Department of Ophthalmology and Visual Sciences of the Chinese University of Hong Kong (CUHK) is devoted to clinical services, research, training and education. The personnel and resources are shared by both clinical and laboratory research (**Figure**). Clinical research involves major subspecialties including corneal and external eye diseases, refractive surgery, cataracts, glaucoma, vitreoretinal diseases, orbital diseases, oculoplasties, neuro-ophthalmic diseases and pediatric eye diseases. Clinical research is conducted according to the good clinical practice and in collaboration with the Hong Kong Eye Hospital, Prince of Wales Hospital, Alice Ho Nethersole Hospital and other local, national and overseas hospitals. We study disease mechanisms including degeneration, inflammation, ocular nerve cells, cellular dysfunctions, oxidative stress, neoplasm, tumor, endocrine complications and genetics. We also conduct research using state-of-the-art equipment in ophthalmic imaging, electrophysiology and angiography. Some of our facilities in clinical research are depicted in **Table 1**.

For basic research, specialized equipment is installed such as

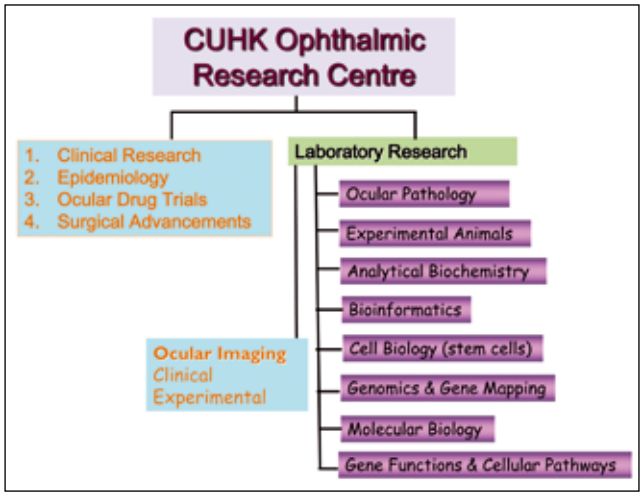

Figure. Clinical and laboratory research at the Chinese University of Hong Kong Ophthalmic Research Centre.
Clinical research includes refractive surgery, cataract surgery, corneal transplantation, ocular imaging, ocular epidemiology, visual electrophysiology, as well as different retinal and corneal diseases. Laboratory research includes etiology, molecular genetics, histopathology, biochemistry and therapeutic treatment of various eye diseases.

a multi-photon confocal microscopy, fluorescence-activated cell sorter and a high-throughput genotyping machine (**Table 2**). Research equipment designated for animal studies includes multi-focal electroretinogram and optical coherence tomography.

Our research has received much support from individual and institutional benefactors. In 2003, a generous donation by the late Mr Lim Por Yen enabled establishment of the Lim Por-yen Eye Genetics Research Centre to facilitate basic and translational research of genetic eye diseases. Another generous donation in 2005 by Mr Chang Bei Ming and Madam Chang Pao So Kok enabled establishment of the Pao So Kuk Macular Diseases Treatment and Research Centre to strengthen research and education in detection and treatment of macular diseases, including age-related macular degeneration (AMD), polypoidal choroidal vasculopathy (PCV), diabetic retinopathy, diabetic macular edema and retinitis pigmentosa (RP). In 2012, the establishment of the Lee Wing Kit Advanced Ophthalmic Training and Education Centre (AOTEC) raised the standard of our training, research and education to the next level. The AOTEC, named in memory of the late Mr. Lee Wing Kit, beloved father of the late Mr. Peter T.C. Lee, was built through a generous donation from Mr. Peter T.C. Lee and Mrs. Nancy Lee, with support from the University Grants Committee, the Hospital Authority, and the Food and Health Bureau of the HKSAR Government. Facilities in AOTEC include the Telemedicine Centre for international exchange of educational programs, grand rounds and clinical consultations, the Microsurgical Training Centre and the Virtual Reality Eye Surgery Training Laboratory. The latter 2 provide hands-on and virtual reality-enhanced training in ophthalmic microsurgery. The Hospital Authority Lions Eye Bank is also housed at AOTEC. Recently, we were awarded the Addition, Alteration and Improvement grant by the University Grants Committee to upgrade and enhance our teaching, education and training facilities in clinical ophthalmology and visual sciences.

Since 1999, we have contributed more than 1,200 scientific articles to science citation index peer-reviewed international journals, including *Ophthalmology*, *Archives of Ophthalmology*, *American Journal of Ophthalmology*, *Investigative Ophthalmology & Visual Science*, *Scientific Reports*, *Human Molecular Genetics*, *Proceedings of the National Academy of Sciences*, *American Journal of Human Genetics*, *Nature Communications*, *Nature Genetics* and *Science*. This has been achieved through the concerted efforts of staff and students, as well as collaboration with our local, national and international colleagues.

In this review, we describe the development of our laboratory-based research with emphasis on genetics, cell and molecular biology, and animal studies, with a brief discussion of future research direction.

Genetic studies

We have studied the genomics and molecular genetics of common complex eye diseases and rare Mendelian diseases. Our strategies include hypothesis-driven candidate gene screening and association studies, as well as the hypothesis-free familial linkage analysis and genome-wide association studies. Our goals are to identify the disease-causing and disease-associated genes for eye diseases so as to facilitate early-stage clinical diagnosis, provide guidance for clinical treatment, and offer genetic testing and counseling.[1]

Gene screening study

A genetic disease can be caused by disease-causing mutations in one or multiple genes, or by the interaction of multiple associated gene variants and environmental factors. A candidate gene study aims to determine the mutation spectrum and/or the association pattern of a specific gene for a disease in a population.[2] This strategy is comparatively low cost, and the candidate gene sequences and polymorphic changes can be resolved by Sanger's DNA sequencing and allelic discrimination (e.g. TaqMan genotyping) assays.[3]

Whole gene mutational screening aims to identify disease-causing mutations. Our first candidate gene screening study was the sequence analysis of the *PAX6* gene in a small number of Hong Kong Chinese patients with aniridia, in which 5 nonsense mutations and 1 frameshift mutation were discovered.[4] In addition, we identified a novel mutation (V53A) in the *myocilin* (*MYOC/TIGR*) gene in a Chinese patient with primary angle closure glaucoma and 3 other disease-causing *MYOC* mutations for primary open angle glaucoma (POAG).[5,6] In contrast, we confirmed that nonsense truncation mutations in *MYOC* are not disease-causing,[7] and we found no disease-causing mutation in

Table 1. Facilities at the Chinese University of Hong Kong Eye Centre.

Clinical equipment	Feature
Laser surgery / treatment	
1. Allegretto wave® Eye-Q Excimer Laser	Ophthalmic laser system for refractive surgery of the cornea
2. AMO Intralase IFS Femtosecond Laser Machine	Ophthalmic laser system for refractive surgery of the cornea
3. Alcon Yag Laser	Capsulotomy
4. Lumenis OMNI Laser	Fundal or glaucoma laser treatment
5. Topcon PASCAL Photocoagulator Laser	Fundal or glaucoma laser treatment
Anterior segment	
1. KONAN Specular Microscopy	Endothelial cell density and cell morphology measurement
2. Zeiss Anterior Segment Optical Coherence Tomography	Non-contact cross-sectional and 3D imaging for anterior segment of the eye
3. Bausch and Lomb's Orbscan II	Corneal shape measurement
4. NIDEK Pachymeter	Noninvasive ultrasonic technique for corneal thickness measurement
5. Zeiss IOL Master	Eyeball length, surface curvature and intraocular lens power measurement
6. Reichert Ocular Response Analyser	Intraocular pressure and corneal biomechanical property measurement
7. Lumenis Allergo Topolyzer	Corneal surface topographic measurement
8. NIDEK Non-contact Tonometer	Intraocular pressure measurement, the fluid pressure inside the eye and central corneal thickness
9. OCULUS Corvis® ST Tonometer	Intraocular pressure and corneal thickness measurement
Posterior segment	
1. Stratus Optical Coherence Tomography System	A noncontact, noninvasive diagnostic imaging technique for cross-sectional retinal structure analysis
2. Cirrus High-definition Optical Coherence Tomography System	A noncontact, noninvasive diagnostic imaging technique for cross-sectional retinal structure analysis
3. Heidelberg Spectralis Optical Coherence Tomography System	A noncontact, noninvasive diagnostic imaging technique for cross-sectional retinal structure analysis
4. Heidelberg Retinal Tomography III	3D imaging of human optic nerve head and retina.
5. Heidelberg Retinal Angiography II	For red free, fluorescein angiography, fundus auto-fluorescein and indocyanine green angiography imaging
6. Topcon Fundus Camera	Retinal image capture, including color, red free, fluorescein angiography, fundus auto fluorescein and indocyanine green angiography
7. Zeiss GDx VCC Scanning Laser Polarimetry	Retinal nerve fiber layer thickness measurement
Ocular function	
1. Espion Electroretinography System	Ocular electrophysiological test including visual evoked potential, electroretinography and electro-oculogram
2. Veris Multifocal-electroretinography System	Electrophysiological test of multifocal retinal function
3. Zeiss Humphrey Visual Field Analyzer	Non-intrusive evaluation of visual field
4. Zeiss Frequency Doubling Technology Perimeter	Visual field screening and full threshold testing
Other ophthalmic investigation	
1. NIDEK Autorefractor	Objective measurement of refractive error
2. TOMEY Ultrasound B-scan UD-1000	Producing a 2D image inside of the eye
3. TOMEY Ultrasound A-scan	Intraocular lens power calculations, axial length, anterior chamber depth, lens thickness and central corneal thickness measurements

the *oculomedin* (*OCLM/TISR*) gene in Chinese POAG.[8] Moreover, we identified differential mutation patterns of the *optineurin* (*OPTN*),[9] *WD repeat domain 36* (*WDR36*)[10] and *neurotrophin-4* (*NTF4*)[11] genes in Chinese POAG.

Our work on rare Mendelian eye diseases includes congenital cataract, corneal dystrophies, and inherited retinal degenerative diseases (such as RP, Bietti's crystalline dystrophy, and Best vitelliform macular dystrophy). For different forms of congenital cataract, we identified multiple novel mutations in 3 different crystallin genes (αA-crystallin gene, *CRYAA*, c.34C>T and c.292G>A;[12,13] βB-crystallin, *CRYBB2*, c.92C>G;[14] and γD-crystallin, *CRYGD*, c.43C>A and c.494delG[15,16]). For late-onset Fuchs endothelial corneal dystrophy, 4 mutations (E399K, G709E, T754M and c.99-100delTC) in the solute carrier family 4, sodium borate

Table 2. Facilities at the Chinese University of Hong Kong Ophthalmic Research Centre.

Laboratory equipment	Feature
Analytical chemistry	
1. Waters Solid phase extraction system	A high throughput platform to purify complex samples for liquid and gas chromatographic analysis
2. Waters High performance liquid chromatography - Fractionating system	Giving preparative separation and fractionating of a biological or herbal complex mixture
3. Agilent Gas chromatography mass spectrometry	Perform good sensitivity and reliability of routine analysis in a test sample
4. Waters Ultra-performance Liquid Chromatograph - FLR, Pda and ECD	Good resolution and accuracy for the identification and quantification of complex samples
5. Waters Liquid Chromatography tandem mass spectrometry	Highest quality nano to microscale 2-D separation for proteomics and metablomics investigation
6. Agilent Liquid Chromatography tandem mass spectrometry	Highest quality nano to microscale 2-D separation for proteomics and metablomics investigation
Animal laboratory	
1. Heidelberg Spectral-domain Optical Coherence Tomography System	A noncontact, noninvasive retinal imaging technique to take cross-section pictures of retina for live animals
2. Heidelberg Retinal Angiography II	For red free, fluorescein angiography, fundus auto-fluorescein and indocyanine green angiography imaging.
3. Espion Electroretinography System	Electrophysiological test of retinal function in animal eye diseases models
4. Zeiss surgical microscopes	Surgical microscopes for animal surgery
Cell biology	
1. Beckman Coulter MoFlo™ Astrios cell sorter	Cell sorting with specific marker
2. Beckman Coulter Cytomics FC500 flow cytometer	Cell marker analysis
3. Thermo Scientific steri-cycle carbon dioxide incubator	Cultured cell incubation
4. Forma Scientific class IIA/B3 biological safety cabinet	Cultured cell manipulation
5. NU-201-330E laminar fumehood	Tissue dissection
6. Millipore Milli-Q Alo	Water filtration
Genomics and molecular biology	
1. Fluidigm EP1 genetic analysis System	High throughput genotyping analysis
2. Roche LightCycler 480II	Real-time polymerase chain reaction
3. Applied Biosystems 3130xl automated sequencer	Direct DNA sequencing
4. BioRad ChemiDoc	Immunoblotting imaging
5. BioRad PCR machines	Polymerase chain reaction and molecular reactions
6. Beckman Coulter Allegra X-22R centrifuge	Sample unification
7. TaiTec Hybridization Incubator	Molecular reactions
8. Grant-bio orbital shaker-incubator ES-20	Bacterial cloning
Microscopy laboratory	
1. Nikon A1R MP multiphoton confocal microscopy	Precise fluorescence imaging
2. Nikon Eclipse Ni fluorescence microscopy	General fluorescence imaging
3. Nikon Eclipse Ti inverted fluorescence microscope	Live cell imaging
Pathology	
1. Thermo Shandon Excelsior automatic tissue processor	Preparation of tissue samples from chemical fixation to paraffin infiltration
2. Thermo Scientific CryoStar Nx50 cryostat	Preparing frozen tissue sections
3. Leica RM2135 microtome	Preparing paraffin sections
4. Leica EG1160 tissue embedding center	Paraffin block assembling
5. Leica DMLS light microscope	General slide
6. Leica M28 dissection microscope	Tissue dissection

transporter, member 11 (*SLC4A11*) gene,[17] and 2 mutations (D64D and N696S) in zinc finger E-box-binding homeobox 1 (*ZEB1*/*TCF8*) gene were identified.[18] In inherited retinal degenerative diseases, we found that disease-causing mutations in the rhodopsin (*RHO*) and retinitis pigmentosa 1 (*RP1*) genes account for approximately 2.0% and 2.2% of our Hong Kong Chinese RP patients respectively,[19-23] a lower percentage than in Caucasians. We identified the

first compound heterozygous truncation mutations in *RP1* causing autosomal recessive RP.[22] In Hong Kong Chinese RP patients, 2.9% carry the photoreceptor-specific nuclear receptor (*NR2E3*) mutations, compared with ~1% in Caucasian RP patients.[24] Furthermore, we identified novel mutations in the U5 small nuclear ribonucleoprotein 200 kDa helicase (*ASCC3L1*/*SNRNP200*) gene,[25,26] the eyes shut (*EYS*) gene,[27] the adenomatous polyposis coli (*APC*) gene in familial adenomatous polyposis with congenital hypertrophy of the retinal pigment epithelium,[28] the cytochrome P450, family 4, subfamily V, polypeptide 2 (*CYP4V2*) gene in Bietti's crystalline dystrophy,[29] the Bestrophin 1 (*BEST1*) gene in Best vitelliform macular dystrophy,[30] the tyrosinase (*TYR*) gene for type 1 oculocutaneous albinism,[31] and the sal-like 4 (*SALL4*) gene for Duane retraction syndrome.[32]

Familial linkage analysis

Family linkage analysis, based on the theory of linkage disequilibrium between known genetic markers with the disease-causing mutations, is a useful strategy to identify unknown disease-causing genes for inherited diseases that show a clear family history. The techniques have evolved from genome-wide linkage scan with microsatellite markers or single nucleotide polymorphisms (SNPs) to whole genome or exome sequencing.

Our first family linkage analysis of autosomal dominant high myopia on chromosome 18p revealed linkage at the D18S476 marker with a maximum LOD score of 2.4 in 5 Hong Kong Chinese families.[33] A novel locus for autosomal dominant high myopia (*MYP16*) on chromosome 5p15.33-p15.2 (17.45-cM interval) was mapped in 3 Hong Kong Chinese families with maximum LOD score of 4.81 at D5S2505, and the *IRX2*, *IRX1*, *POLS*, *CCT5* and *CTNND2* as disease-causing genes were excluded.[34] For glaucoma, in a 27-member Filipino family with autosomal dominant juvenile-onset POAG, we excluded mutations in the *MYOC*, *OPTN* and *WDR36* genes.[35] Our genome-wide scan identified the chromosomal region 5q22.1-q32 flanked by the D5S2051 and D5S2090 markers as a novel locus for POAG (*GLC1M*), with a maximum LOD score of 4.82.[36] We then excluded the neuregulin 2 (*NRG2*) and secreted protein acidic and rich in cysteine (*SPARC*) genes as the disease-causing gene in this locus.[37,38] In another study, chromosome 15q22-q24, a 16.6-Mb region flanked by D15S1036 and rs922693 with a maximum LOD score of 3.31, was mapped as a novel locus for autosomal dominant juvenile-onset POAG in a Hong Kong Chinese family.[39] The *NR2E3*, SMAD family member 6 (*SMAD6*) and ceroid-lipofuscinosis, neuronal 6 (*CLN6*) genes were excluded as disease-causing in this locus. In collaboration with University College London, we confirmed in 3 Hong Kong Chinese autosomal recessive RP families the *RP25* locus on chromosome 6 mapped by the 10K GeneChip Mapping Array.[40]

With the advent of next generation sequencing, including whole or targeted genome or exome sequencing, discovery of gene mutations is quick and cost-effective.[41] We have identified novel disease-causing genes and mutations for inherited retinal dystrophies,[42-44] including the U4/U6 small nuclear ribonucleoprotein Prp4 (*PRPF4*) and secreted phosphoprotein 2 (*SPP2*) genes for autosomal dominant RP,[45,46] Usher syndrome 2A (*USH2A*) mutations for Usher syndrome,[47] as well as mutations in Bardet-Biedl syndrome 2 (*BBS2*), McKusick-Kaufman syndrome (*MKKS*), ADP-ribosylation factor-like protein 6 (*ARL6*) and Meckel syndrome, type 1 (*MKS1*) genes for Bardet-Biedl syndrome.[48]

Genetic association study

Most common eye diseases such as AMD and POAG are late-onset, and their etiology is complex and a result of the interaction of multiple genetic and environmental risk factors, rather than a single mutation. It is difficult to have large pedigrees for linkage analysis. Association studies are conducted to identify the susceptibility gene variants that are associated with the disease phenotype, based on the proportional differences of a genetic polymorphism between patients and control subjects.[49] Association studies can be performed on a small scale by direct sequencing or allelic discrimination assay, or on a genome-wide scale by microarray or next generation sequencing.

Our first association study was on the apolipoprotein E (*APOE*) gene in AMD, where we found no significant association.[50] In contrast, we discovered a link between the *APOE* ε4 allele and normal tension glaucoma,[51] and its interaction with *MYOC* and *OPTN* polymorphisms in normal tension glaucoma,[52] while the *MYOC* promoter polymorphisms alone were not associated with POAG.[53] Moreover, we investigated other glaucoma-related loci, including lysyl oxidase-like protein 1 (*LOXL1*),[54,55] tumor necrosis factor (*TNF*),[56] tumor protein p53 (*TP53*),[56] atonal homolog 7 (*ATOH7*),[57] raftlin lipid raft linker 1 (*RFTN1*),[57] and chromosome 2p16.3,[58] in POAG. For AMD, we investigated formyl peptide receptor 1 (*FPR1*),[59] pigment epithelium-derived factor (*PEDF*),[60] and chromosome 8p21 and 4q12,[61] as well as the complement pathway and the high-density lipoprotein pathway. We have reported association of AMD with complement factor H (*CFH*; I62V), complement component 2 (*C2*; rs547154), and cholesteryl ester transfer protein (*CETP*; rs3764261).[62-66] We also compared the genetics of AMD with PCV and found that most AMD genes are associated with PCV, some with different effect sizes, e.g. SNPs at the *ARMS2-HTRA1* locus. We also found a PCV-specific association with the complement component 3 (*C3*) gene and the ATP-binding cassette, subfamily G, member 1 (*ABCG1*) gene.[66] Since uveitis and AMD share a similar pathogenesis of inflammation, we tested the association of complement pathway genes in uveitis and identified an association of anterior uveitis with complement factor B (*CFB*; rs1048709),[67] complement factor I (*CFI*; rs7356506),[68] and *CFH* (I62V),[69,70] but not *C2*.[67] The manganese superoxide dismutase (*SOD2*), chemokine (C-C motif) ligand 2 (*CCL2*) and *KIAA1109* genes are also associated with uveitis.[71,72] In addition, we confirmed the association of the paired box 6 (*PAX6*) promoter dinucleotide repeats with

high myopia,[73,74] the endothelin 1 (*EDN1*) K198N variant with diabetic retinopathy,[75] the transcription factor 4 (*TCF4*) and protein tyrosine phosphatase, receptor type G (*PTPRG*) genes with corneal dystrophies,[76,77] as well as the cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) and interleukin 13 (*IL13*) genes with Graves' ophthalmopathy in the Hong Kong Chinese population.[78,79]

Since the first report of genome-wide association study (GWAS) for AMD was published in 2005, it has become the most successful technology for mapping genes associated with complex diseases. We reported our first GWAS, which was also the first Chinese GWAS in eye genetics, in 2006 on AMD, where we discovered the high temperature requirement factor A1 (*HTRA1*) promoter polymorphism rs11200638 to be associated with exudative AMD in the Hong Kong Chinese population.[80] Further fine mapping analysis revealed functional variants related to the disease pathogenesis and confirmed *HTRA1* to be the strongest associated gene in Chinese.[81,82] Our second GWAS identified the association of caveolin genes (*CAV1/CAV2*) with POAG in a multi-institutional collaborative project.[83] Since then, we have collaborated with the Singapore Eye Research Institute and the Sichuan Provincial Key Laboratory for Human Disease Gene Study to discover 8 novel associated genes and variants for primary angle closure glaucoma (PACG; *PLEKHA7*, *COL11A1*, *PCMTD1/ST18* and *ABCC5*)[84,85] as well as POAG (*ABCA1*, *PMM2*, *CDKN2B-AS1* and *TGFBR3*).[86,87] For myopia, our collaboration identified 4 novel genes and loci associated with high myopia: chromosome 13q12.12,[88] vasoactive intestinal peptide receptor 2 (*VIPR2*),[89] syntrophin, beta 1 (*SNTB1*),[89] and zinc finger E-box binding homeobox 2 (*ZFHX1B*).[90] We also worked with the Consortium on Refractive Error and Myopia (CREAM), which comprised 37,382 individuals from 27 studies of European ancestry and 8,376 from 5 Asian cohorts. Genome-wide meta-analysis has deciphered 21 genes and loci associated with refractive errors (*CD55*, *PRSS56*, *CHRNG*, *CACNA1D*, *BMP3*, *LAMA2*, *CHD7*, *TOX*, *ZMAT4*, *RORB*, *CYP26A1*, *BICC1*, *GRIA4*, *RDH5*, *PCCA*, *ZIC2*, *GJD2*, *RASGRF1*, *MYO1D*, *KCNJ2* and *CNDP2*),[91] and 9 with axial length (*ALPPL2*, *CD55*, *C3orf26*, *GJD2*, *LAMA2*, *MIP*, *RSPO1*, *ZC3H11B* and *ZNRF3*).[92] In the GWAS on central corneal thickness and keratoconus, we identified 16 associated genes and loci (*USP37*, *GPR15*, *TIPARP*, *CWC27-ADAMTS6*, *RXRA-COL5A1*, *LCN12-PTGDS*, *FGF9-SGCG*, *FOXO1*, *TJP1*, *AKAP13*, *LRRK1*, *CHSY1*, *BANP-ZNF469*, *HS3ST3B1-PMP22*),[93] as well as on Vogt-Koyanagi-Harada syndrome, identifying 3 susceptibility loci (*IL23R-C1orf141* on 1p31.2, *HLA-DRB1/DQA1* on 6p21.3 and *ADO-ZNF365-EGR2* on 10q21.3).[94] With the development of the next generation sequencing technique, novel coding variants were identified for neovascular AMD, including the cholesteryl ester transfer protein (*CETP*) D442G variant,[95] and the ubiquitin protein ligase E3D (*UBE3D*) V379M variant.[96]

Although we have identified more than 70 loci/genes/variants associated or linked with different ocular diseases, the role of these genes in the disease pathogeneses has not been determined. Biological studies would be an important approach to decipher the functional roles of these genetically identified genes and variants.

Biological studies

Biological studies at CUHK Ophthalmic Research Centre comprise cell and molecular biology projects and animal studies, providing support for genetics analysis and supplementing clinical investigation. We aim to (1) characterize the function of the disease-causing/susceptible genes, (2) delineate the disease mechanisms, and (3) provide pre-clinical platforms for drug/treatment testing and disease modeling.

Characterization of disease-causing/susceptible genes

Genetic studies, such as candidate gene screening and association analysis, can identify disease-causing mutations and susceptible variants, respectively. To delineate the role of pathogenic mutations or to confirm the biological relevance of the associated variants to the diseases, *in vitro* experiments for gene function analysis are performed.

In the γD-crystallin gene (*CRYGD*; c.43C>A and c.494delG) for congenital cataract,[15,16] the c.494delG mutation deletes 1 nucleotide in the *CRYGD* open reading frame and is predicted to cause a frameshift and an early termination of translational product (G165fs).[16] Recombinant CRYGD mutant protein overexpressed in lens epithelial cell line (B3) leads to a reduced solubility of γD-crystallin protein and localization on the nuclear envelope (co-localized with lamin A/C), where the wildtype protein exists in both the nucleus and cytoplasm. The c.43C>A change is a missense mutation, causing the substitution of Arg at residue 15 to Ser (R15S). The recombinant mutant protein has similar solubility in detergent to the wildtype protein, but is predicted to raise the local hydrophobicity and create a hypothetical casein kinase II phosphorylation site.[15] Similarly, in the αA-crystallin gene (*CRYAA*, c.34C>T and c.292G>A),[12,13] the c.292G>A mutation causes the substitution of Gly at residue 98 to Arg (G98R). Compared to the cytoplasmic localization of wildtype CRYAA protein, the G98R mutant forms aggregates inside the endoplasmic reticulum (ER) and is predominantly detergent-insoluble.[13] Overexpression of CRYAA G98R mutant protein induces ER stress in B3 cells, and in turn leads to cell apoptosis. The c.34C>T change in *CRYAA* is a missense mutation, causing the substitution of Arg at residue 12 to Cys (R12C). The R12C mutant protein has similar detergent-solubility and cytoplasmic localization to the wildtype protein, but mutant-expressing cells exhibit a delayed expression of HSP70, compared with wildtype-expressing cells.[12]

We examined the effect of high myopia-associated dinucleotide repeat $(AC)_m(AG)_n$ polymorphism at the *PAX6* P1 promoter on its transcriptional activity.[73] Luciferase assays showed elevated transcriptional activity with

increasing length of $(AC)_m$ repeats ($(AC)_{Below20-22}$ vs $(AC)_{20-22}$: 1.27 folds, and $(AC)_{Below20-22}$ vs $(AC)_{Above20-22}$: 1.50 folds) although the transcriptional activity was not significantly elevated for increasing length of $(AG)_n$ repeat. At the same length of combined repeats, transcriptional activity of $(AC)_{23}(AG)_6$ was similar to that of $(AC)_{21}(AG)_8$, suggesting that both AC and AG repeats contributed to the transcriptional activity of the *PAX6* P1 promoter. This phenomenon may be due to influence of transcription factor binding sites within this region.[73] Gene function analysis of *NTF4*, in which we discovered mutations of POAG, showed that only the G157A variant protein, not the A182V variant, was detected by immunoblotting, while both wildtype and mutant *NTF4* mRNA were detected after transfection.[11] Compared to wildtype NTF4 protein, the G157A variant is less soluble in detergent but did not affect protein trafficking. Both NTF4 variants impaired NTF4 protein function, in which HeLa cells expressing NTF4 mutants were less stimulated to migrate than those expressing wildtype protein.[11]

Identification of disease mechanisms

Retinoblastoma (RB) is one of the most common causes of cancer in children.[97] Its etiology is the loss of function of retinoblastoma gene (*RB1*) alleles by loss of heterozygosity and gene mutations,[98] but this accounts for only 80 to 90% of RB patients. To search for novel RB-causing mechanisms, we identified promoter hypermethylation and impaired expression in O6-methylguanine-DNA Methyltransferase (*MGMT*),[99] MutL homolog 1 (*MLH1*),[100] and RAS-associated domain family 1A (*RASSF1A*) genes.[101] Loss of heterozygosity was detected in RB on chromosome 19, 20, 21, 22 and X,[102] whereas allelic loss at chromosome 13q31 with reduction in glypican 6 (*GPC6*) gene expression was found in 92% of RB patients.[103] In addition, B lymphoma Mo-MLV insertion region 1 (BMI1) is widely expressed in human RB, and it stimulates RB cell (Y79) proliferation and suppresses apoptosis with reduced expression of p14ARF and p16INK4 and upregulation of cyclin D1 and D2 as well as CHX10 and RAX.[104]

Ocular hypertension is the major risk factor for glaucoma. We established an experimental animal model to mimic ocular hypertension and retinal ganglion cell degeneration. We found that the JAK/STAT pathway, PI3K/AKT pathway and macrophages had a detrimental effect on RGC survival in rodents after acute ocular hypertension.[105-107] Acute intraocular pressure (IOP) elevation activates JAK/STAT pathway in RGCs, and blockage of JAK/STAT pathway by pathway inhibitor (AG490) further enhances RGC death when IOP is elevated.[105] Similarly, acute IOP elevation activates PI3K/AKT pathway in the inner nuclear layer and ganglion cell layer, where inhibition of PI3K/AKT pathway by inhibitors (LY294002 and KY12420) leads to RGC loss in rats after acute IOP elevation.[106] Interestingly, there are different responses of macrophages on RGC survival in rats with different autoimmune backgrounds. A significant increase in the number of macrophages and extensive RGC loss occurred in experimental autoimmune encephalomyelitis (EAE)-vulnerable Lewis rats after acute IOP elevation, but not in EAE-resistant Fischer 344 and Sprague Dawley (SD) rats. Removal of macrophages in Lewis rats but not in Fischer and SD rats reduced the extent of RGC loss.[107] Macrophage activation by zymosan had a detrimental effect on RGC survival in Fischer and SD rats after acute IOP elevation, confirming the negative effect of macrophages on RGC survival under ocular hypertension. Moreover, ocular hypertension can also be induced by the use of steroid, such as dexamethasone and triamcinolone that disrupts the trabecular meshwork (TM) cells.[108] To delineate this pathogenic mechanism, we performed global gene expression profiling on dexamethasone and triamcinolone acetonide-treated TM cells in culture by microarray.[109,110] We found 29 genes differentially expressed in dexamethasone-treated cells and 57 genes differentially expressed in triamcinolone-treated cells. *MYOC*, *GAS1*, *SENP1*, *ZNF343* and *SOX30* are the common genes regulated by both dexamethasone and triamcinolone.

The etiology of AMD is multi-factorial and influenced by environmental factors such as cigarette smoking and diabetes mellitus.[111,112] Vascular endothelial growth factor (VEGF), one of the initiators of choroidal neovascularization in exudative AMD, is highly expressed in aqueous humor of exudative AMD patients compared with controls, whereas pigment epithelial-derived factor (PEDF), acting antagonistically to VEGF,[113] also shows increased levels in exudative AMD patients.[114] Higher baseline aqueous VEGF level is associated with persistent angiographic leakage in exudative AMD patients.[115] In exudative AMD patients with 3 monthly intravitreal anti-VEGF agent (Avastin) injections, aqueous VEGF level reduced from 102.6 pg/mL at baseline to 18.3 pg/mL after 2 months, whereas PEDF level increased from 11.2 ng/mL at baseline to 38.7 ng/mL.[116] There was also significant central foveal thickness reduction at 6 months after Avastin injection.[115] Although the *HTRA1* gene shows a strong association with AMD,[117] the HTRA1 protein in vitreous humor is associated with VEGF ($r = 0.650$), especially in patients with retinal detachment ($r = 0.835$).[118] In cultured human fetal RPE cells, upon stress induction by tunicamycin and dithiothreitol, *HTRA1* and *VEGFA* expression is upregulated, but the 2 genes do not mutually regulate each other.[118]

MicroRNAs (miRNAs) are important in regulating cellular physiological functions, including stem cell differentiation and regenerative properties. We conducted global microRNA profiling for human limbal stem cells and central corneal epithelial cells and found that miRNA-145 promotes limbal stem cell differentiation by suppressing integrin β8 (ITGB8) expression.[119] In addition to miRNA-145, there are 36 microRNAs enriched in the limbal region, including miRNA-10b, 126, 143 and 155.[120] The target genes regulated by these miRNAs are involved in cell survival, cell apoptosis, cell movement, cell-matrix interaction, cell-cell adhesion as well as pathways of immune response and cellular protection. Apart from corneal cell differentiation, we also found miRNAs regulated retinal differentiation from

stem cells.[121] We have identified 71 differentially expressed human miRNAs in the retinal differentiation process of human periodontal ligament-derived stem cells (PDLSCs). The predicted miRNA target genes are closely related to neuronal differentiation processes, and 2 of them (VEGF and PTEN) are significantly upregulated during the retinal differentiation process. Furthermore, we have delineated the miRNA profile of nicotine-treated PDLSCs and identified 16 differentially expressed human miRNAs.[122] Among them, miRNA-1305 and miRNA-18b were significantly upregulated in PDLSCs obtained from cigarette smokers compared with those from non-smokers.[123]

Pre-clinical drug testing and experimental disease modeling

We have been working on herbal chemicals, small molecules and stem cells. We have selected herbal molecules with known chemical structure and reported benefits to vision. We have shown that green tea extract and its biological active components (catechins) can be easily taken up by the digestive system and distributed to different organs through circulation within 1 hour.[124,125] Although epigallocatechin gallate, the most abundant catechin in green tea extract, is a powerful antioxidant,[126] it can induce caudal retardation with abnormal axial flexion and delayed hind-limb formation in rat embryos.[127] A high dose (550 mg/kg) of green tea extract can increase oxidative stress in plasma, aqueous humor, vitreous humor, cornea and retina, but decrease it in the lens and choroid-sclera.[128] Oral administration of a high dose of green tea extract can alleviate ocular inflammation in a rat model of endotoxin-induced uveitis by reducing infiltrating leucocytes and macrophages, protein exudation in aqueous humor, the production of TNF-α, IL-6 and MCP-1 in aqueous humor, as well as downregulation of Toll-like receptor 4 (TLR-4), CD14 and nuclear factor-kappa B (NF-κB p65) in the iris and ciliary body.[129] In addition to anti-oxidation and anti-inflammation, epigallocatechin gallate, together with isoliquiritigenin from licorice and resveratrol from grapes, are anti-angiogenic. They can suppress VEGF-induced human umbilical vein endothelial cell proliferation and migration by activating focal adhesion kinase and upregulating PEDF.[130,131] Topical application of ginkgo biloba extract can result in steroid-induced changes in trabecular meshwork and lower the IOP in rabbits.[132]

Small molecules can reduce mutant protein misfolding and alleviate the ER stress response. We identified D384N mutation in *MYOC* gene from a POAG family with reduced protein solubility, aggregation-prone and nonsecreted.[133] Treatment with trimethylamine N-oxide (TMAO) improved the solubility of the D384N mutant, reduced its distribution in ER, alleviated ER stress, and rescued the cells from apoptosis. Meanwhile, TMAO treatment can induce moderate upregulation of heat shock protein 70 (HSP70). Coherently, TMAO suppressed corneal dystrophy-causing R124C transforming growth factor beta-induced (*TGFBI*) mutant-induced amyloid-beta (Aβ) fibrillar aggregation.[134] Another chemical chaperone, sodium 4-phenylbutyrate (4-PBA), can ameliorate cataract-causing G165fsX8 mutant of γD-crystallin protein insolubility and relieve its mislocalization from nuclear envelope. In addition, 4-PBA treatment can reduce cell apoptosis and upregulate HSP70 expression.[135] We investigated the therapeutic effect of neuropeptides in different ocular diseases and reported the extra-pituitary effect of growth hormone releasing-hormone receptor antagonist to alleviate endotoxin-induced ocular inflammation in an experimental animal model.[136]

Stem cells participate in disease pathogenesis and can be used in regenerative medicine.[137-139] Our center has a strong interest in tissue-specific stem cells (human adult stem cells), especially limbal progenitor cells, retinal progenitor cells and mesenchymal stem cells. We studied the *ex vivo* expansion of human limbal epithelial cells,[140] and the nuclear matrix changes in long-term culture of limbal epithelial cells.[141] Subsequently, we delineated the miRNA profile of human limbal progenitor cells and central corneal cells.[119,120] We also investigated the application of stem cells for retinal diseases.[142-144] We used a novel approach to study retinal cells by the trans-differentiation ability of human mesenchymal stem cells. In collaboration with the University of Miami, we directed human mesenchymal stem cells obtained from periodontal ligament to a retinal fate, expressing retinal lineage markers, including PAX6, RAX and rhodopsin.[145] We modified our initial induction protocol to direct human PDLSCs into retinal ganglion-like cells.[123] The differentiated PDLSCs expressed neuronal and retinal ganglion cell markers (ATOH7, POU4F2, β-III tubulin, MAP2, TAU, NEUROD1 and SIX3), formed synapses and gave glutamate-induced calcium responses with spontaneous electrical activity, indicating that the trans-differentiated stem cells exhibited the characteristics of functional neurons.

Future directions

The CUHK Ophthalmic Research Centre is equipped with different advanced research technologies. We are one of the pioneers in the field of *in vivo* imaging to monitor the progression of disease phenotypes and treatment responses in animals.[146] We have established animal models of glaucoma, optic nerve injury and AMD to serve as a platform for *in vivo* pre-clinical drug testing and disease pathogenesis studies.[147-150]

In eye genetics, although we have identified more than 70 loci/genes/variants associated or linked with different ocular diseases, the role of these genes/variants in the disease pathogenesis or protein function is yet to be characterized. *In vitro* cellular and molecular studies as well as animal experiments are important strategies to decipher the disease-causing mechanisms of these genetically identified genes and variants. Our research direction is disease-orientated and translational. We will focus on disease mechanism, drug discovery, molecular disease diagnosis, biomarker identification and advancing therapeutic treatments.

We find it a great privilege to have clinical ophthalmologists, visual scientists, and research students working together at

the CUHK Ophthalmic Research Centre. We look forward to more collaborative research projects with colleagues to advance ophthalmology and visual sciences in Hong Kong and regions beyond.

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Declarations

The authors declared no competing financial interests.

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