Final Report
Executive Summary

HSC Public Health Agency
Research and Development

HSC R&D Division Final Progress Report

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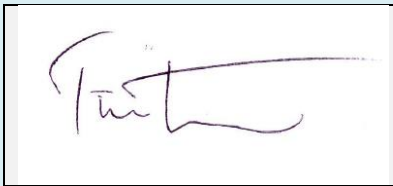
HSC Public Health Agency
Research and Development

HSC R&D Division Award Details

HSC R&D File Reference	STL/4748/13
HSC R&D Funding Scheme	*US Ireland R&D Partnership Programme*
Project Title	*COMP-Ang1: Vascular Normalization and Neuroprotection for Diabetic Retinopathy*
Award Holder Name (Employer)	Professor Tim Curtis (Queen's University Belfast)
Host Research Organisation	Queen's University Belfast
Award Duration	
Award Start Date	20.06.16
Award End Date	31.07.21
Name of Lead Supervisor: ***(only applicable to training awards)***	

Signature

Award Holder Signature:



Date: 22nd January 2022

HSC Public Health Agency
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Evidence Brief

(1 page: which may be used for dissemination by HSC R&D Division)

Why did we start? (The need for the research and/or Why the work was commissioned)	The current best end-stage treatments for diabetic retinopathy (DR) are either ineffective or become ineffective over time, motivating the search for alternative therapeutics and delivery methods to slow, stop or reverse the vision-threatening complications of this disease. Our aim was to develop a retinal gene therapy approach that would be effective in preventing the onset and development of diabetic retinopathy.
What did we do? (Methods)	We used in vitro and in vivo pre-clinical models of diabetic retinopathy to assess the effectiveness of modulating Angiopoietin1 (Ang1) signalling in experimental diabetes. We increased Ang1 signalling in the retina by expressing engineered COMP-Ang1 through intravitreal injection of gene delivery vectors. COMP-Ang1 peptide was also applied directly onto cells in culture. We investigated whether COMP-Ang1 can counteract the pathological changes associated with diabetic retinopathy in our experimental model systems.
What answer did we get? (Findings)	COMP-Ang1 was found to prevent the detrimental effects of high glucose in cell cultures, and to normalise retinal blood flow and visual performance in our in vivo pre-clinical models of diabetes. COMP-Ang1 expression was also found to prevent pathological remodelling of retinal blood vessels during diabetes but prevented blood vessel repair by vascular progenitor cells during later stages of the disease.
What should be done now? (Practice/Policy Implications and/or Recommendations)	The next step is to investigate in further preclinical studies the other arm of the angiopoietin signalling system, namely, Angiopoietin 2 (Ang2). Specifically, there is a need to determine whether counteracting the increase in Ang2 in the retina during diabetes, with or without COMP-Ang1 signal supplementation, provides a more efficacious strategy for the treatment of diabetic retinopathy.

Final Report

(no more than 20 pages)

Please structure the report using the headings below

Background

Diabetic retinopathy (DR) is the most common complication of diabetes and is a leading cause of new cases of legal blindness in the working age population of developed countries (Zheng et al., 2012). It is a progressive sight-threatening eye disease caused by hyperglycaemia, which damages the retinal vascular, glial and neuronal cells leading to vascular permeability, retinal ischemia, neovascularisation, retinal detachment, and blindness (Lechner et al., 2017, Stitt et al., 2016). Although good glycaemic control, treatment for hypertension and correction of dyslipidemia reduce the risk of DR, a substantial proportion of individuals still progress to the sight-threatening complications of the disease (Stitt et al., 2016). Visual impairment in DR can result from proliferative DR (PDR) or the development of diabetic macular oedema (DMO). Laser photocoagulation remains the mainstay treatment for proliferative DR (PDR) but is inherently destructive and risks permanent damage to peripheral vision (Stitt et al., 2016). While anti-VEGF therapy has substantially improved treatment for patients with DMO, it can be potentially harmful to neurons, does not reduce fibrosis, and does not improve vision in most patients (Stitt et al., 2016). Hence, there is an unmet medical need for new treatment strategies that better address the underlying pathogenesis of the disease.

Angiopoietin1 (Ang1) is a secreted ligand which binds to the receptor tyrosine kinase Tie-2 expressed on various cell types, including those of the vasculature and CNS (Teichert et al., 2017; Luck et al., 2021). Ang-1 binding to Tie-2 induces vascular survival, quiescence and maturation, as well as decreasing inflammation and leakiness in response to ischaemic and inflammatory conditions. Recent studies have also shown that Ang-1 has neuroprotective properties in both the brain and retina (Cahoon et al., 2015; Shin et al., 2010), although the underlying mechanisms have yet to be fully investigated. Ang1 also has the potential to activate a range of endothelial progenitor cell subtypes and patients with DR could benefit from the ability of these cells to repair damaged blood vessels in the retina (Balaji et al., 2015; Cahoon et al., 2015; Park et al., 2003). It is evident that enhancing Ang1 bioactivity in the retina could prevent the vascular and neurodegenerative changes that precipitate vision-loss in DR and testing this possibility constituted a major goal of the current US-Ireland Partnership Programme. Because Ang1 is a growth factor with a highly localised paracrine mode of action, intravitreal delivery of Ang1 is not clinically practical. In our preclinical proof-of-concept studies, we therefore aimed to increase retinal Ang1 expression through viral vector transfection (AAV) directing expression of recombinant COMP-Ang1 (a soluble and more potent variant of Ang1) (Cho et al., 2004).

Aims and objectives

The proposal brought together investigators from Queen's University of Belfast (Profs Curtis, Stitt and Medina), the Moran Eye Center (Prof Ambati, University of Utah, US) and Dublin City University (Assoc. Prof Phil Cummins) to address the following the specific aims:

1. To determine if broadening the tropism of AAV.COMP-Ang1 improves its retinal delivery.
2. To investigate the cellular signalling mechanisms underlying the protective effects of COMP-Ang1 in DR.
3. To determine whether COMP-Ang1 improves neurovascular structure and function in DR
4. To determine whether constitutive expression of COMP-Ang1 enhances endothelial progenitor cell (EPC) integration in diabetic retinal vasculature.

Methods

Across the three institutions involved in this tri-national project, a wide range of pre-clinical methods were utilised, including but not limited to: animal models of type 1 diabetes, ocular coherence tomography, histology, immunohistochemistry, visual behavioural testing, fundus imaging, cell culture models, barrier function measurements and gene and protein expression profiling by qPCR and Western blotting.

The Belfast group developed a fluorescent microbead-based method which makes use of a widely available, simple rodent retinal camera system (Micron IV from Phoenix Labs). This method allows for the mapping of regional capillary blood flow speed in a substantial area of the retina (1.6 mm x 1.6 mm in mice, 3.2 mm x 3.2 mm in rats) with practical limits of detection between 0.072-7.2 mm/s at 12Hz acquisition rate (in mice). An SOP and COSHH forms were created for this non-recovery procedure. The method requires the use of pigmented mice as in albino strains, out of focus microbead fluorescence from the choroidal circulation reduces the signal-to-noise. Required items are limited to the Micron IV imaging setup, mouse facilities, syringes and chemicals used (anaesthetics, eye dilating drops, microbeads and sterile saline). An important further improvement of the method involved writing Matlab code to visualise the raw data and to create “heat maps” of capillary flow speed. This new technique is currently being written up for publication as part of a larger paper detailing the effects of COMP-Ang1 gene therapy on blood flow changes in the diabetic retina.

Personal and Public Involvement (PPI)

The grant holder and staff engaged in several different outreach activities to publicise and explain the research as well as to draw the attention of the public to diabetic eye disease and retinal gene therapy. Prof Curtis gave radio interviews explaining the goals of the US-Ireland Partnership Programme Award. Dr Barabas hosted a stand in ‘Science Uncovered’ at the Ulster Museum in 2016 and 2017 as part of the European Researchers Night. A press release for the US-Ireland Partnership Programme Award written together with the Communications Office at Queen's and the HSC R&D Office (Belfast) was publicised by several media outlets including the Irish Times. This press release was also distributed by QUB via social media channels including LinkedIn and Facebook.

The Centre for Experimental Medicine, renamed to the Wellcome-Wolfson Institute for Experimental Medicine, hosted events within the framework of the Northern Ireland Science Festival every non-pandemic year of the project. In 2017 and 2018, the theme title was 'Know Your Enemy: Disease-Focused Research at Queen’s' where Prof Curtis and Dr Barabas helped to run stands on 'Cardiovascular' and 'Eye’ diseases', respectively. In 2018, Dr Barabas gave a soap-box presentation about modern eye research within the framework of the NI Science Festival and in 2019 new, “visual impairment glasses” were created and added to the interactive props used to allow visitors to experience some of the aspects of how different eye diseases, including diabetic retinopathy, affect vision. At the 2020 event, Dr Barabas organized the Eye Research component, showcasing live eye dissections, laser scanning confocal imaging of retinal samples and the use of in silico molecular modelling approaches.

Dr Barabas hosted a stand at QUB's "Global Thinking Locally" event, an interactive showcase at Belfast City Hall in May of 2017. In February 2019, Prof Curtis gave a presentation to donors and charity representatives about diabetic retinopathy research conducted in his lab while Dr Barabas delivered a lab demonstration in the use of cell cultures for diabetic retinopathy research. Prof Curtis gave a talk to a Diabetes PPI group in Newtownabbey in November 2019. In February 2020, Prof Curtis and Dr Barabas visited Methodist College Belfast and introduced pupils to vision research and potential new therapeutic approaches considered for tackling diabetic retinopathy.

After March 2020, the Covid-19 pandemic resulted in all public engagements and presentations being cancelled or postponed to safeguard public health.

Findings

To study the effects of COMP-Ang1 gene therapy on the development of early-stage diabetic retinopathy the Belfast group examined whether this therapeutic is capable of normalising capillary blood flow in the diabetic mouse retina. As described above, new methodology was developed to create retinal capillary blood flow maps in mice, and custom software written in MATLAB to facilitate the mapping protocol. This technique was initially validated in non-diabetic control mice. Mean capillary flow speeds determined in the mouse retina (1.41±0.09 mm/s) were found to be in close agreement with capillary velocity measurements conducted by other laboratories using more complex adaptive optics and confocal-based methods (Guevara-Torres et al., 2016; Paques et al., 2003). The mouse capillary flow speed data was also in line with a human study where the range of measured mean velocities in capillaries was 0.82-3 mm/s (de Castro et al., 2016; Warner et al., 2021). We divided the retinal flow maps into quadrants and found no statistically significant difference between mean capillary flow speed between the different quadrants. As expected, capillary flow speed decreased as the distance of the capillary from the optic nerve head increased with a rate of 1.2±0.2 s^{-1}. The slope of the decrease was not dependent on which quadrant it was determined in. Experiments were subsequently performed where streptozotocin (STZ)-induced diabetic mice of 4-6 weeks disease duration were intravitreally injected with PBS, control AAV2.GFP or AAV2.COMP-Ang1. Age-matched non-diabetic mice received PBS only. One month after vector injection, visual performance was assessed by OptoMotry and retinal capillary blood flow maps were created. Visual performance and nasal blood flow speeds were decreased in diabetic mice injected with PBS or AAV2.GFP compared with non-diabetic controls. Intravitreal injection of AAV2.COMPAng1 returned visual performance and nasal blood flow speeds back to control levels. These results confirmed that COMP-Ang1 gene therapy may be a useful therapeutic option for the early-stage treatment of diabetic retinopathy.

Later stage diabetic retinopathy studies in mice were performed by the US group with assistance from the Belfast and Dublin groups. The goal of these studies was to determine whether COMP-Ang1 gene therapy applied after the onset of vascular damage could rescue or repair damaged vascular beds and attenuate neuronal atrophy and dysfunction in the retinas of aged diabetic mice. These studies showed that AAV2.COMP-Ang1 exerts an anti-angiogenic effect in the retina when treatment is initiated well after the establishment of diabetic retinopathy. Whilst these results were encouraging, no significant improvement in retinal thinning, retinal ganglion cell death or diabetic retinopathy–associated visual loss was found in the aged diabetic mice treated with AAV2.COMP-Ang1. Furthermore, in the aged diabetic mice, it was discovered that AAV2 reporter and AAV2.COMP-Ang1 treatments led to the infiltration of IB4-positive inflammatory cells, which was not observed in mice treated during the early stages of diabetic retinopathy. The exact reason for this difference between younger and aged diabetic mice remains to be determined, but these results imply that in the future the AAV2.COMP-Ang1 viral dose may need to be optimised at different time points following the onset of diabetic retinopathy.

To investigate the cellular signalling mechanisms underlying the protective effects of COMP-Ang1 during the early stages of diabetic retinopathy the Dublin group, with assistance from the Belfast group, studied the effects of COMP-Ang1 on hyperglycaemia-induced changes in retinal barrier function *in vitro*. To this end, they first established a comprehensive and reproducible model of hyperglycaemia-induced human retinal microvascular endothelial cell (HRMEC) barrier disruption. Confluent HMECs were treated (0–72 hours) with D-glucose (5 or 30 mM) in the absence and presence of COMP-Ang1 (10–200 ng/mL), with L-glucose (30 mM) being used as osmotic control. Treatment with 30 mM d-glucose (but not l-glucose) triggered a time-dependent elevation in HRMEC monolayer permeability as determined using FITC-dextran transwell permeability assays. In parallel

significant reductions in the in mRNA/protein levels of the tight and adherence junction proteins, occludin, claudin-5, ZO-1, and VE-Cadherin were observed. These effects were all attenuated by COMP-Ang1 in a concentration-dependent fashion. Gene silencing studies were also undertaken using small interfering RNAs. These studies demonstrated a key role for the Tie2 signalling system in mediating the protective effects of COMP-Ang1 on hyperglycaemia-induced changes in HRMEC permeability and junctional expression levels.

The US and Belfast groups examined the potential of endothelial colony forming cells (ECFCs) to repair vascular beds damaged during diabetic retinopathy. Studies were performed to compare the outcomes of intravitreal versus intra-carotid artery delivery of ECFCs in retinas of adult diabetic mice. Moreover, the ability of AAV2.COMP-Ang1 to enhance the integration of ECFCs into the diabetic retina was evaluated. AAV2.COMP-Ang1 or AAV2 control virus was bilaterally injected intravitreally into four-month-old diabetic mice. 100 µl of PBS solution containing 100,000 ECFCs (derived from human cord blood) labelled with Q-dot 655 was systemically delivered via the right carotid artery and assayed after 24 or 72 hours. Alternately, 2 µl containing 2,000 ECFCs were injected directly into the vitreous. Intravitreal injection of ECFCs resulted in uncontrolled endothelial tube assembly primarily within the vitreous of AAV2.COMP-Ang1 treated eyes. In contrast, intra-carotid artery-injected ECFCs explicitly integrated within retinal vessels of diabetic mice, but not in those that had been pre-treated with AAV2.COMP-Ang1. These data are exciting as they suggest that intra-carotid delivery of ECFCs into the adult diabetic eye facilitates vascular repair. However, it appears that pre-treatment with AAV2.COMP-Ang1 may be detrimental to this process.

To study the tropism of AAV2.GFP and AAV2.COMP-Ang1 viruses in the diabetic retina, the Belfast and US groups undertook live fluorescence retinal imaging and immunochemistry-based studies on flat-mounted retinas. These studies confirmed retinal GFP expression in non-diabetic and diabetic mice intravitreally injected with AAV2-GFP using Micron IV imaging. *Ex vivo* confocal analysis of the retinal flatmounts, indicated that GFP and COMP-Ang1 transgene expression was mainly localised to retinal ganglion cells, amacrine, bipolar and to a lesser extent Müller cells. A key goal of our US-Ireland Programme was to evaluate the potential of gene therapy approaches for the treatment of diabetic retinopathy. While we found positive effects of AAV2-mediated COMP-Ang1 gene transfer during the early stages of diabetic retinopathy, we wanted to determine whether gene transfer was limited to the targeted retina. We therefore undertook PCR-based biodistribution studies. We found little evidence of transgene expression outside the injected eye. However, in eyes injected with AAV2-GFP, the GFP gene was detected in the right lens. Another observation with this gene delivery method was that in a few cases we observed highly localised expression of the transgene within the retina, suggesting that our intravitreal gene delivery approaches will require further optimisation in the future prior to testing this approach clinically. A panel of human retinal cell lines, including HRMECs, pericytes, astrocytes, and pigment epithelial cells were set up and optimised by the Dublin group for *in vitro* tropism studies. All cell cultures were characterised by qPCR for various markers (e.g. Tie1/2, Ang1/2, PECAM, GFAP, vWf) and AAV2.COMP-Ang1 delivery directly into HRMECs confirmed. Further progress on this aspect of the project, however, was severely disrupted by Covid-related operational issues at Dublin City University.

Thus far, and as detailed in our most recent Researchfish submission, this US-Ireland Partnership Programme Award has resulted in 12 publications in leading, peer-reviewed, journals in the Vision and Vascular Sciences area as well as numerous (>10) national and international conference presentations. A draft of our COMPAng1 blood flow study is almost completed and will be submitted for publication by April 2022.

Conclusion

Together with our collaborators in the RoI and US, we established that COMP-Ang1 efficiently counteracts many deleterious effects associated with the early-stage development of diabetic

retinopathy. However, its use during later stages of the disease process appears complicated by the fact that it causes inflammatory cell infiltration into the retina and prevents vascular repair by circulating endothelial progenitor cells.

Practice and Policy Implications/Recommendations

Since this project has been entirely pre-clinical, it has no immediate practice or policy implications. While AAV-mediated gene therapy has been approved clinically for the treatment of certain retinal dystrophies (e.g. Luxturna), a multi-species safety and efficacy study of AAV-mediated Comp-Ang1 gene therapy would be needed prior to translation into the clinical setting.

Pathway to Impact

Changes in the angiopoietin signalling system during diabetic retinopathy are complex and entail not only a decrease in Ang1 but also an increase in Ang2 signalling. The increase in Ang2 is detrimental as it reduces microvascular stability, increases capillary permeability, weakens blood-retinal barrier function, and increases vascular cell senescence and cell death. Thus, although our studies using COMP-Ang1 yielded promising results for the early-stage treatment of diabetic retinopathy, it is our view that this therapy could be significantly improved by simultaneously targeting Ang2. As a first step towards this goal, the US group have developed a gene therapy vector (AAV2.ScFv.Ang2), which is effective in blocking Ang2 signalling in cell culture experiments. Future studies will test the long-term therapeutic efficacy of this vector alone and in combination with AAV2.COMP-Ang1 to bring this work closer to clinical translation for patients with diabetic retinopathy.

References

Balaji S, Han N, Moles C, Shaaban AF, Bollyky PL, Crombleholme TM, Keswani SG. (2015) Angiopoietin-1 improves endothelial progenitor cell-dependent neovascularization in diabetic wounds. Surgery. 158:846-856.

Cahoon JM, Rai RR, Carroll LS, Uehara H, Zhang X, O'Neil CL, Medina RJ, Das SK, Muddana SK, Olson PR, Nielson S, Walker K, Flood MM, Messenger WB, Archer BJ, Barabas P, Krizaj D, Gibson CC, Li DY, Koh GY, Gao G, Stitt AW, Ambati BK. (2015) Intravitreal AAV2.COMP-Ang1 Prevents Neurovascular Degeneration in a Murine Model of Diabetic Retinopathy. Diabetes 64:4247-4259.

Cho CH, Kammerer RA, Lee HJ, Steinmetz MO, Ryu YS, Lee SH, Yasunaga K, Kim KT, Kim I, Choi HH, Kim W, Kim SH, Park SK, Lee GM, Koh GY. COMP-Ang1: a designed angiopoietin-1 variant with nonleaky angiogenic activity. (2004) Proc Natl Acad Sci U S A. 101:5547-5552.

de Castro A, Huang G, Sawides L, Luo T, Burns SA. (2016) Opt Lett. 41:1881–1884.

Guevara-Torres A, Joseph A, Schallek JB. (2016) Label free measurement of retinal blood cell flux, velocity, hematocrit and capillary width in the living mouse eye. Biomed Opt Express. 7:4228–4249.

Kuzmin DA, Shutova MV, Johnston NR, Smith OP, Fedorin VV, Kukushkin YS, van der Loo JCM, Johnstone EC. (2021) The clinical landscape for AAV gene therapies. Nature Reviews Drug Discovery 20:173-174.

Lechner J, O'Leary OE, Stitt AW. (2017) The pathology associated with diabetic retinopathy. Vision Res. 139:7-14.

Luck R, Karakatsani A, Shah B, Schermann G, Adler H, Kupke J, Tisch N, Jeong H-W, Back MB, Hetsch F, D'Errico A, De Palma M, Wiedtke E, Grimm D, Acker-Palmer A, von Engelhardt J, Adams RH, Augustin HG, de Almodóvar CR. (2021) The angiopoietin-Tie2 pathway regulates Purkinje cell dendritic morphogenesis in a cell-autonomous manner. Cell Rep. 36:109522.

Park YS, Kim NH, Jo I. (2003) Hypoxia and vascular endothelial growth factor acutely up-regulate angiopoietin-1 and Tie2 mRNA in bovine retinal pericytes. Microvasc Res. 65:125-131

Paques M, Tadayoni R; Sercombe R; Laurent P; Genevois O; Gaudric A; Vicaut E. (2003) Structural and Hemodynamic Analysis of the Mouse Retinal Microcirculation. Invest Ophthalmol Vis Sci. 44:4960-4967.

Shin HY, Lee YJ, Kim HJ, Park C-K, Kim JH, Wang KC, Kim DG, Koh GY, Paek SH. (2010) Protective role of COMP-Ang1 in ischemic rat brain. J Neurosci Res. 88:1052-1063.

Stitt AW, Curtis TM, Chen M, Medina RJ, McKay GJ, Jenkins A, Gardiner TA, Lyons TJ, Hammes HP, Simó R, Lois N. (2016) The progress in understanding and treatment of diabetic retinopathy. Prog Retin Eye Res. 51:156-186.

Teichert M, Milde L, Holm A, Stanicek L, Gengenbacher N, Savant S, Ruckdeschel T, Hasanov Z, Srivastava K, Hu J, Hertel S, Bartol A, Schlereth K, Augustin HG. (2017) Pericyte-expressed Tie2 controls angiogenesis and vessel maturation. Nat Com. 8:16106

Warner RL, Gast TJ, Sapoznik KA, Carmichael-Martins A; Burns SA. (2021) Measuring Temporal and Spatial Variability of Red Blood Cell Velocity in Human Retinal Vessels. Invest Ophthalmol Vis Sci. 62:1-7.

Zheng, Y., He, M., & Congdon, N. (2012). The worldwide epidemic of diabetic retinopathy. Indian J. Ophthalmol. 60:428–431.

Relevant Logos

Host institute logos:

Queen's University Belfast, Belfast, UK

Dublin City University, Dublin, Ireland

University of Utah, Salt Lake City, Utah, USA

Funding body logos:

US Ireland Partnership Programme

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