Background- This is a re-submission of a *CIHR* grant renewal initially submitted in October 2016, which received a one-year bridging award of $100,000 from July 1 2017-June 30 2018. We are grateful to the application's four reviewers for their efforts and kind assessment of the Oct. 2016 application. In this re-submission we have addressed the reviewer comments as outlined below:

Additional Preliminary data- Since the original application was submitted in October 2016, we have made important progress on this research program:

a. We have characterized the molecular phenotype of all corneal-dystrophy causing point mutants of SLC4A11.[1] These data indicate that the vast majority of Fuchs endothelial corneal dystrophy (FECD) mutants of SLC4A11 traffic normally to the cell surface. Thus, in most FECD, disease arises from a functional defect, rather than retention of the protein inside the cell. Some of these retain water flux function, meaning that SLC4A11 has a critical role other than membrane transport, which is consistent with the central hypothesis of this application, that SLC4A11 has a critical role in endothelial cell attachment to the underlying basement membrane Descemet's membrane (DM). (See Submitted manuscript in Appendix.)

b. We have proceeded with work to engineer SLC4A11 as a cell surface adhesion protein (CSAP) therapeutic. As described briefly in Aim 2B, we modified the pre-protein form of glycophorin A (GPA) to receive the Descemet's membrane (DM) binding region of SLC4A11, extracellular loop 3 (EL3). We mutated the normal site of signal sequence cleavage so that glycophorin (normally a single transmembrane segment (TM) protein) would be expressed with SLC4A11 EL3 between the two TMs (the uncleaved signal and the usual TM). The protein (called GPA-SLC4A11-EL3) expresses to high levels, does not aggregate, and associates as a dimer, as we had hoped (Appendix Fig. 1). We were excited by very recent data revealing that GPA-SLC4A11-EL3 promotes adhesion to Descemet's membrane as effectively as full length SLC4A11 (Appendix Fig. 2). This small (143 amino acids, 15.7 kDa) construct holds great hope as a virally-delivered therapeutic.

c. We raised an antibody against a portion of SLC4A11 EL3 (see Fig. 5 of proposal). Using the 96 well cell adhesion assay, we found that cells expressing SLC4A11 adhered to a much greater extent to wells coated with DM components than did vector-expressing control cells. In the presence of anti-EL3 antibody, SLC4A11 expressing cells adhered no more strongly to wells than did control cells. This strongly supports the identification of EL3 as the DM binding region of SLC4A11.

d. Three FECD-causing FECD mutations map to SLC4A11 EL3 (Y526C, T561M and S565L) (see Fig. 5 of proposal). Since the last grant submission, we have expressed and tested these mutants. Each traffics normally to the cell surface, meaning disease arises from loss of normal function, rather than from failure to reach the cell surface. These mutants have normal water flux activity, meaning some function other than membrane transport is compromised. Consistent with a central role of SLC4A11-mediated DM adhesion in FECD pathology, each of the three FECD mutants failed to support WT levels of cell adhesion in the 96 well assay.

Response to Reviewer 1

Use a CRISPR strategy- Thank you. This is a great idea, which we have added to Aim 2B.

Viability of CEC transduction strategy- We have added citations (see Aim 3) to the manuscript indicating the ability to transduce corneal endothelial cells (CECs) with adeno-associated virus, including one study in which transduction was successful using eye drops in patients. Successful CEC transduction using AAV (human *ex vivo*, up to 94%)[2] or LV (rat, human, sheep CEC-ex vivo; >80%)[3,4] has been reported. In vivo adenoviral transduction of CEC in the rat model has also been reported.[5] We do appreciate that ideally we would have our own data illustrating the ability to transduce CECs. We do have experience with viral transduction of primary cultured cardiomyocytes as seen in two of our publications.[6,7] Now that we have one year of bridge funding in place, we (Brunette group) are working to establish CEC adenoviral transduction, but time lines were too short to have this in place for this resubmission.

Cohort sizes- We appreciate the reviewer's concern about cohort sizes required to achieve significance in studies. In this re-submission we have increased the proposed cohort sizes and included a note that we will adjust the N-value depending on the magnitude of difference observed between groups.

Alternate strategies in Aim 1- As described in *preliminary data b* (above), we have already used an alternate strategy (the glycophorin engineered construct) to develop an optimized therapeutic CSAP. The reviewer may have been concerned that the alternative strategies section of Aim 1 is admittedly quite short. This reflects our confidence that the three approaches proposed to identify CSAPs in corneal endothelial cells would be successful. This set of approaches seemed comprehensive to the applicants.

The reviewer was concerned that success of the proposal rests on success of Aim1, a point with which we do not disagree. That said, we believe that the key minimal outcome of Aim 1, identification of a corneal endothelial cell CSAP required to mediate adhesion to Descemet's membrane, has already been achieved. As outlined in the proposal and in new preliminary data above, there is compelling evidence that SLC4A11 is a corneal endothelial cell CSAP. Aim 1 proposes to identify additional CSAPs in order to: 1. Obtain a complete view of the mechanism of CEC/DM interaction, and 2. To mitigate possible unforeseen difficulties in the use of engineered CSAP as a therapeutic. We also note that Aim 1b has also achieved a minimum objective required to move to Aim 2, expression of an engineered therapeutic CSAP: GPA-SLC4A11-EL3 (Appendix Fig. 1). Our very recent data indicate that GPA-SLC4A11-EL3 is an effective CSAP (Appendix Fig. 2).

Response to Reviewer 2

Viability of CEC viral transduction strategy- Please see response to *Reviewer 1*, who had the same comment.

Response to Reviewer 3

We thank Reviewer 3 for the positive assessment of the application.

Response to Reviewer 4

Potential difficulties in engineered CSAPs- We also were concerned about properties of the engineered CSAPs. Indeed, we thought that the proteins might misfold and fail to accumulate to a significant extent, or aggregate. We were relieved when glycophorin A (GPA)-based engineered CSAP expressed to a high level and associated as a dimer, just as does native GPA, as described above and in Aim 2B. Data on successful expression of this engineered potential therapeutic CSAP is seen in Appendix Fig. 1.

Evidence that SLC4A11 is a CSAP- Additional preliminary data is described above. We have also done a more thorough job of outlining evidence in grant section "Observations supporting hypotheses."

References

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