A SENSITIVE ASSAY FOR MONITORING WILD-TYPE AND MUTANT MYOCILIN SECRETION

Serena Zadoo[1], Annie Nguyen[1], Gulab Zode[2], John D. Hulleman[1,3]

1 Department of Ophthalmology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390-9057

2 Department of Cell Biology & Immunology and the North Texas Eye Research Institute, University of North Texas Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, TX 76107

3 Department of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390

INTRODUCTION

GLAUCOMA

- Chronic blinding disease characterized by gradual, irreversible loss of vision from retinal ganglion cell death
- 2nd leading cause of bilateral blindness worldwide, projected to affect ~80 million by 2020[1]
- Majority of glaucoma cases are primary open angle glaucoma (POAG)
  - Aqueous humor outflow clogged through ciliary muscles
  - Associated with ocular hypertension[1, 2]
  - Inherited nature of POAG established with identification of genes linked to monogenic POAG[3]
- Myocilin (MYOC), also known as the trabecular meshwork (TM)-inducible glucocorticoid response (TIGR) gene was the first to be linked to inherited POAG[4]

MYOCILIN (MYOC)

- MYOC gene encodes a 57 kDa, secreted glycoprotein[5] of unknown function
- Sites of expression include brain, skeletal muscle, heart, and eye with highest levels within the human TM[6-9]
- Over 100 glaucoma-causing MYOC mutations lead to an autosomal-dominant, gain-of-toxic-function, inherited form of POAG[10]
- Heterozygous missense mutations in MYOC primarily located within the olfactomedin (OLF) domain in the C-terminal portion of the protein (Fig. 1)

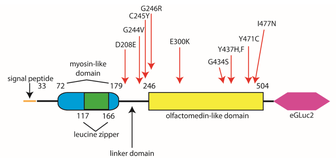


Fig. 1: Schematic of MYOC eGLuc2 reporter fusion construct. eGLuc2 (pink hexagon) was appended to the C-terminus of MYOC to enable luminescence-based detection. Predicted molecular weight of construct is 76.5 kDa. MYOC mutants used in this study are indicated and their position is designated with a red arrow.

- Single point mutations in MYOC compromise folding of MYOC and case substantial defects in protein's secretion efficiency – producing insoluble intracellular protein aggregates[7,12-15] and potentially amyloid[16]
- Proposed mechanism of POAG pathogenesis: formation of MYOC aggregates in the endoplasmic reticulum (ER) of TM cells[12,17,18] → ER stress → unfolded protein response (UPR) → TM cell death[19] → stress may ultimately cause dysfunctional aqueous humor outflow → increased intraocular pressure (IOP), RGC death, and optic nerve damage[20]

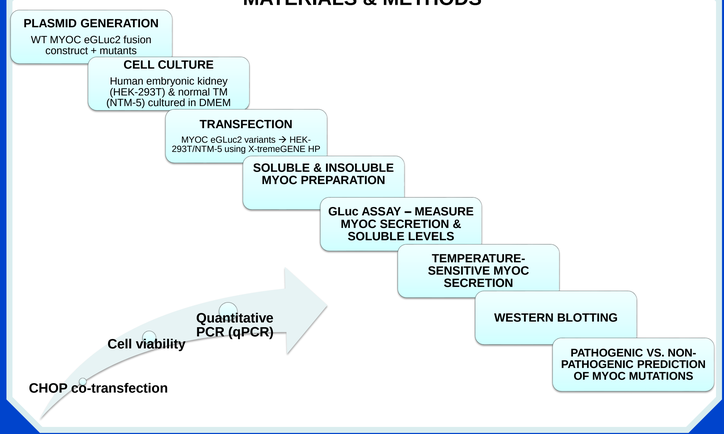
QUANTIFYING MUTANT MYOC SECRETION: GLuc ASSAY

- Gold standard for detection of mutant MYOC secretion: SDS-PAGE/western blotting[7,15,19]
  - Time-consuming, costly, and usually does not detect small amounts of protein
- Small (19.8 kDa), highly sensitive, naturally secreted *Gaussia* luciferase (GLuc) has been used successfully to quantitatively monitor a number of biological processes[21-23]
- Advantages of using GLuc as a reporter protein:
  - Yields an extremely bright signal – easy to measure small amounts of the protein
  - Compared to conventional non-enzymatic fluorescent reporters such as green fluorescent protein (GFP), red fluorescent protein (RFP), or even enzymatic reporters such as secreted alkaline phosphatase (SEAP):
    - GLuc is a secreted protein and thus is processed normally through ER/Golgi pathway → theoretically, if appended to a secreted protein, GLuc is less likely to disrupt folding and secretion of that protein
    - Luminescence assays have substantially reduced background signals in comparison to fluorescence assays, significantly increased sensitivity, and the ability to easily monitor GLuc in conventional phenol-red containing media
    - GLuc is far more sensitive (5,000 - 20,000 fold[22]) than conventional secreted protein reporters
- Focus of this study: develop, characterize and use the GLuc assay as a rapid, sensitive, and inexpensive method to quantify secreted levels of wild-type (WT) and mutant MYOC based on GLuc luminescence readouts in both human embryonic kidney (HEK-293T) cells and normal TM (NTM-5) cells
- Development could serve as an effective platform for identifying new drugs to potentially treat MYOC-associated POAG

RESCUING MUTANT MYOC SECRETION

- Defects in mutant MYOC secretion have been partially rescued by either growth temperature reduction[7,15,19] or administration of chemical chaperones such as phenyl butyric acid (PBA)[24,25]
- These treatments correlate with a reduction in MYOC-mediated TM cell death[19] and a reduction in glaucoma-associated phenotypes in mice[24, 26] respectively
- Enhanced mutant MYOC secretion, due either to growth temperature reduction or chemical chaperone addition, is likely the result of an environment conducive to proper folding of mutant proteins[27] and/or a reduction in ER stress
- MYOC secretion inversely correlated to disease severity and follows a strong genotype-phenotype correlation[14]
- Identifying cellular conditions or small molecule compounds that can rescue mutant MYOC misfolding and restore secretion, albeit partially, could serve as much-needed treatments for MYOC-associated glaucoma

MATERIALS & METHODS



RESULTS

Fusion of a naturally secreted luciferase to myocilin (MYOC) does not alter its secretion profile or soluble intracellular levels

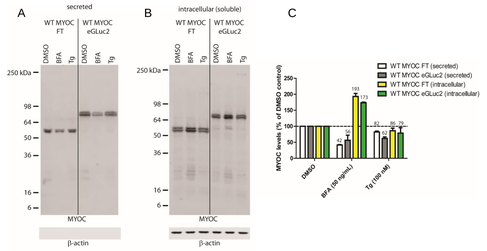


Fig. 2. Secreted and soluble intracellular FLAG-tagged (FT) and eGLuc2-tagged WT MYOC behave similarly. (A, B) HEK-293T cells were transfected with the indicated constructs followed by treatment with vehicle (DMSO), brefeldin A (BFA, 50 ng/mL), or thapsigargin (Tg, 100 nM). Conditioned media (A) or the soluble intracellular portion (B) was run on a reducing SDS-PAGE followed by western blotting. (C) LI-COR quantification of MYOC FT and MYOC eGLuc2. (n = 3 ind. experiments, ± S.D.).

Y437H MYOC eGLuc2 behaves similarly to Y437H MYOC FT

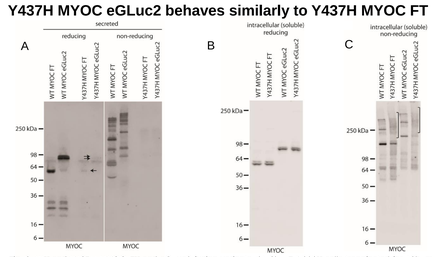


Fig. 3. WT and Y437H MYOC FT and eGLuc2 fusion variants. (A-C) HEK-293T cells transfected for 48h, media changed (2% FBS media) and incubated for 24h prior to western blotting conditioned media (A) or soluble cell lysates (B, C). Single arrow – secreted Y437H MYOC FT, double arrow – Y437H MYOC eGLuc2. Reducing and non-reducing conditions used to compare disulfide bonding ability. Mixed disulfide intermediates denoted by bracket. (n = 3 ind. experiments).

Utilization of GLuc assay to measure secreted & soluble intracellular MYOC levels

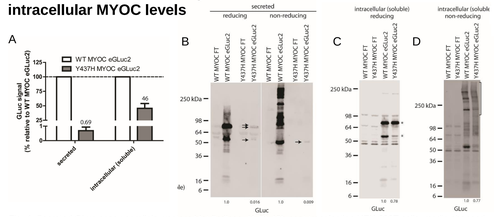


Fig. 4. Secreted GLuc assay parallels western blot findings. (A-D) 48h post-transfection HEK-293T cells, media changed, cells incubated 24 h in 2% FBS media. (A) Conditioned media or soluble intracellular lysates analyzed by GLuc assay. (n = 3 ind. experiments, ± S.D.). (B-D) Blots reprobed with GLuc antibody. (B) Conditioned media analyzed under reducing or non-reducing conditions. Single arrow-cleaved Y437H MYOC eGLuc2, double arrow-full length Y437H MYOC eGLuc2. Numbers indicate relative MYOC protein levels (LI-COR quantification). (C,D) Soluble intracellular cell lysates under reducing (C) and non-reducing conditions (D). Mixed disulfide intermediates denoted by bracket. (n = 3 ind. experiments).

GLuc assay can sensitively monitor Y437H MYOC secretion within 30 min of media change

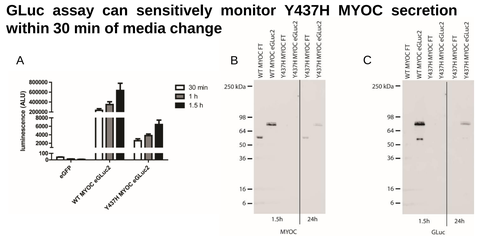


Fig. 5. GLuc assay can easily measure Y437H MYOC eGLuc2 levels before they are detectible by western blotting. (A) 48h post-transfection HEK-293T cells had media change to 2% FBS media. 30 min – 1.5 h after media change, conditioned media aliquots (20 µL) taken and analyzed by GLuc assay. (n = 3 ind. experiments, ± SEM). (B, C) 1.5h post-media change conditioned media analyzed by western blotting. 24h conditioned media sample from Y437H MYOC expressing cells included as a positive control. Representative images of 3 independent experiments.

GLuc assay extended to measure additional POAG-associated MYOC mutants

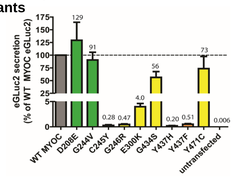


Fig. 6. Pathogenic & non-pathogenic MYOC eGLuc2 mutants. GLuc luminescence assay of secreted WT MYOC (gray bar), predicted polymorphic (nonpathogenic) mutants (green bars – D208E, G244V), predicted pathogenic mutants (yellow bars – C245Y, G246R, E300K, G434S, Y437H, Y471C) and engineered mutant (orange bar – Y437F), from 48h post-transfected HEK-293T cells. Untransfected cell media used as control. (n ≥ 3 independent experiments, ± SD).

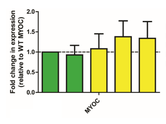


Fig. 7. Secretion differences between WT and mutant MYOC not due to differences in transfection efficiency, or transcriptional changes. RNA extracted from transfected HEK-293T cells, MYOC gene expression analyzed via qPCR. Data presented as fold change in expression levels relative to WT MYOC. (n ≥ 3 ind. experiments, ± SD)

Secretion of MYOC eGLuc2 variants from NTM-5 cells parallels secretion from HEK-293T cells

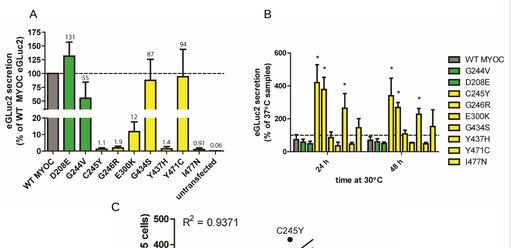
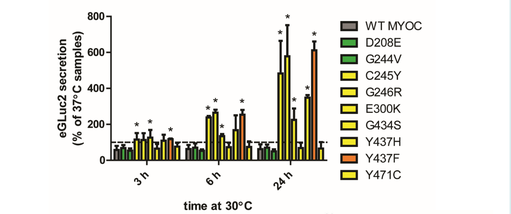


Fig. 8: MYOC secretion from NTM-5 cells parallels secretion from HEK-293T cells. (A) GLuc luminescence assay of secreted MYOC eGLuc2 variants (colors described in Fig. 6). Conditioned media aliquots from NTM-5 cells 48h post-transfection assayed for MYOC eGLuc2 fusion protein. (n = 3 ind. experiments, ± SD). (B) Temperature-sensitive secretion of MYOC variants in NTM-5 cells. NTM-5 cells transfected for 48h, media changed, and grown at 37°C or 30°C for up to 48h. 24h and 48h post-temperature shift media aliquots assayed for eGLuc2. Results presented as % of 37°C samples' secretion. (n = 3 ind. experiments, ± SD, * = p<0.05 vs. WT MYOC by a t-test). (C) Temperature-sensitive secretion of MYOC between HEK-293T and NTM-5 cells 24h post-temperature shift. Average of 3 ind. temperature-shift experiments in HEK-293T and NTM-5 cells plotted against each other.

Compromised secretion of MYOC mutants is improved by growth at permissive temperature



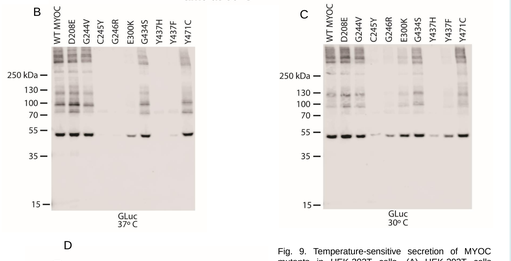


Fig. 9. Temperature-sensitive secretion of MYOC mutants in HEK-293T cells. (A) HEK-293T cells transfected for 48h at 37°C. Media changed and cells incubated at 37°C or 30°C for up to 24h. 3h, 6h, and 24h post-temperature shift aliquots assayed for eGLuc2 secretion. Results presented as % of 37°C sample secretion. (n ≥ 3 ind. experiments, ± SD, * = p<0.05 vs. WT MYOC by a t-test). (B) Western blot analysis of eGLuc2-tagged MYOC variants in HEK-293T cell culture media under non-reducing conditions at 37°C. (C) Western blot analysis of cells grown at permissive (30°C) temperature. Representative blots of 3 ind. experiments. (D) Western blotting findings parallel GLuc assay results. MYOC (from the 37°C expt) quantified by luminescence assay (y axis, avg. of 2 ind. experiments) or western blotting (signal from the entire lane, x axis, avg. of 2 ind. experiments) plotted against each other.

Mutant MYOC protein secreted during permissive temperature originates from intracellular stores – is not newly synthesized

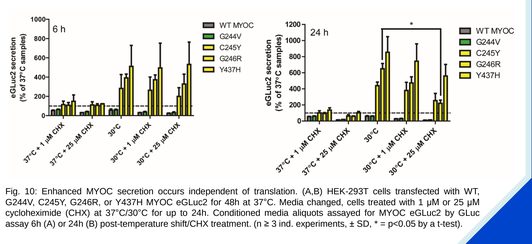


Fig. 10: Enhanced MYOC secretion occurs independent of translation. (A,B) HEK-293T cells transfected with WT, G244V, C245Y, G246R, or Y437H MYOC eGLuc2 for 48h at 37°C. Media changed, cells treated with 1 µM or 25 µM cycloheximide (CHX) at 37°C/30°C for up to 24h. Conditioned media aliquots assayed for MYOC eGLuc2 by GLuc assay 6h (A) or 24h (B) post-temperature shift/CHX treatment. (n ≥ 3 ind. experiments, ± SD, * = p<0.05 by a t-test).

CONCLUSIONS

- WT & mutant MYOC eGLuc2 variants resemble conventionally tagged forms of MYOC in terms of responsiveness to ER stressors, secretion levels, soluble intracellular levels & disulfide bonding ability
- Major advantages of the GLuc assay:
  - Speed – routinely completed within 5 min
  - Sensitivity – can detect 'non-secreted' Y437H MYOC within 30 min of media change
  - Cost – single assay in a 96 well plate costs ~$0.08 and can be scaled down to 384/1586 well plates
- Envisioned true utility of this luciferase-based MYOC assay system is twofold:
  - Performing kinetic experiments on mutant MYOC, requires quick measurement approach and sensitivity
  - Use in chemical/genetic high-throughput screens to ID compounds/genes that modulate MYOC secretion
  - Small molecules or genes could be used as possible therapeutic candidates for MYOC-associated POAG
- Limitations of GLuc assay:
  - Cannot monitor insoluble MYOC – denaturing agents used to solubilize aggregated MYOC
  - Cannot be easily used for determining subcellular localization, in contrast to other reporters like GFP/RFP
  - All findings using eGLuc2-tagged MYOC must be validated in orthogonal assays
- Nonetheless, using this assay, we were able to glean insight into new facets of MYOC folding/secretion:
  - 1st study to demonstrate secretion propensities of D208E, G244V, E300K, G434S & Y471C MYOC
    - D208E, G244V, G434S and Y471C variants secreted at similar levels compared to WT MYOC
      - D208E mutation has been found in control and POAG patients – likely that it is a polymorphism, in agreement with the prediction made by www.myocilin.com
      - G244V variant found in one patient with mild POAG who was compound heterozygous for a G252R MYOC mutation - possible that the G244V mutation is a polymorphic variant that does not affect MYOC secretion, while true disease-causing mutation is the G252R mutation
    - Prediction software suggested that G434S and Y471C are actually pathogenic
      - Secretion data in HEK-293T and NTM-5 cells would suggest otherwise: well tolerated in both HEK-293T and NTM-5 cells (>56% of WT MYOC secretion in all instances)
      - G434S MYOC has been found in one POAG patient, while Y471C MYOC has been found in two POAG patients and is listed as having 'unknown pathologic significance' (www.uniprot.org)
      - Combination of small patient cohorts combined with our secretion results does not suggest Y471C 25 and G434S MYOC are pathogenic
      - Cannot rule out that these mutations may abrogate MYOC function in some manner, but do not necessarily affect secretion
- Enhanced mutant MYOC secretion at permissive temperature independent of newly translated protein – suggests MYOC protein secreted at lower temperature originates from intracellular stores
  - Previous work suggests ER-associated degradation machinery is unable to effectively degrade misfolded, mutant MYOC which creates a buildup of MYOC
  - ER protein folding machinery may reengage and fold pool of mutant MYOC at permissive temp.
  - Cannot rule out possibility that lowered growth temperature directly affects MYOC folding and secretion
- Surprising finding: no detectable differences in cell viability or overt activation of the UPR after expression of mutant MYOC
  - MYOC expression by transient transfection may be less than studies using adenovirus/cell lines
  - Majority of experiments used HEK-293T cells, not human TM cells – mutant MYOC expression may selectively kill TM cells
  - Low transfection efficiency of NTM-5 cells (10-20%), unlikely to detect cell death post-transfection
- Future efforts: miniaturization of MYOC eGLuc2 assay in a physiologically relevant, inducible cell-based model to identify chemical and/or genetic modulators of MYOC folding/secretion which could serve as much-needed treatments for MYOC-associated glaucoma

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