White Rose Research Online

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk
Universities of Leeds, Sheffield and York

Deposited via The University of Sheffield.

White Rose Research Online URL for this paper:
https://eprints.whiterose.ac.uk/id/eprint/188102/

Version: Published Version

Article:
Alsaffar, F.A., Mujamammi, A.H., Aldughaim, M.S.K. et al. (2023) Evidence that all Sorsby's fundus dystrophy mutations cause TIMP3 dimerization resulting in impaired inhibition of VEGFR2. Genes and Diseases, 10 (1). pp. 45-47. ISSN: 2352-4820

https://doi.org/10.1016/j.gendis.2022.03.008

Reuse
This article is distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND) licence. This licence only allows you to download this work and share it with others as long as you credit the authors, but you can't change the article in any way or use it commercially. More information and the full terms of the licence here: https://creativecommons.org/licenses/

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

UNIVERSITY OF LEEDS
University of Sheffield
UNIVERSITY of York

ARTICLE IN PRESS
+ MODEL
Genes & Diseases xxx (xxxx) xxx

Available online at www.sciencedirect.com
ScienceDirect
journal homepage: http://ees.elsevier.com/gendis/default.asp

RAPID COMMUNICATION

Evidence that all Sorsby's fundus dystrophy mutations cause TIMP3 dimerization resulting in impaired inhibition of VEGFR2

Tissue inhibitor of metalloproteinases 3 (TIMP3) regulates turnover of the extracellular matrix (ECM) and is also a potent inhibitor of the vascular endothelial growth factor receptor 2 (VEGFR2), a key mediator of angiogenesis. Mutations in TIMP3 give rise to Sorsby's fundus dystrophy (SFD), a dominantly inherited degenerative disease of the retina that leads to blindness, usually in middle age. To date, fifteen different mutations in TIMP3 have been identified as causing the disease. Conventionally, these were numbered for their positions in the secreted protein, excluding the signal sequence but are referenced in Table S1, with and without including the signal sequence; however, we use the conventional nomenclature in the text. Figure 1A illustrates their relationship to the primary structure. Thirteen of these are missense mutations, one a nonsense mutation and one a mutation in the splice acceptor site at the intron 4/exon 5 boundary. All but one of these mutations (S15C) affect exon 5, which codes for all except the first 3 residues of the carboxyl-terminal domain of the molecule. Notably, eleven mutations result in a change to a cysteine residue, while the nonsense mutation (E139X), also gives rise to an unpaired cysteine residue.[1] It seems unlikely that this is coincidental. Indeed, we have shown many of these Cys residues result in dimerization and/or multimerization of the TIMP3 protein. Moreover, the E139K missense mutation[2] and the E139X nonsense mutation[1] have also been shown to form disulfide bonded dimers, strengthening the hypothesis that TIMP-3 dimerization is a prerequisite for the disease. However, the H158R mutation and the S15C mutation have both been reported to be solely monomeric, challenging this hypothesis.[3,4] Moreover, the molecular consequences of several other mutations, and the novel splice acceptor site mutation have never been examined.

Initially we examined the expression of the H158R mutation, reported to be monomeric when expressed by patient dermal fibroblasts or transfected HEK293 cells,[3] the S15C mutation, also reported to be monomeric when expressed from patient fibroblast cells or by a cell-free expression system[4] and the Y128C, Y154C and Y159C mutants whose dimerization status had not been examined.

cDNAs corresponding to these mutations were stably expressed from ARPE19 retinal pigment epithelial cells, alongside the S181C mutant, which is widely accepted to dimerize. Figure 1B shows that all of these mutant forms of TIMP3 gave rise to bands in the range of 20–30 kDa, corresponding to the expected size of glycosylated and unglycosylated forms of TIMP3. However, in the non-reduced gels additional bands at ~50 kDa were also observed for all the mutants which disappeared upon reduction, indicating that these higher molecular weight forms are disulfide bonded dimers.

Next we examined the splice-acceptor site mutation by creating a synthetic gene construct comprising exons 1–4, intron 4 and the whole of exon 5 with either the wild-type (sWT) -cagATC- or mutant (sM) -caagATC- splice acceptor site at the end of intron 4 (Fig. S1A). These constructs were transfected into COS-7 and ARPE19 cells and total RNA isolated, reverse transcribed and sequenced. Sequencing of TIMP3 cDNA from COS-7 and ARPE19 cells transfected with the sM construct, but not the normal control construct (sWT), gave rise to a number of novel cDNA species (Fig. S1B). In all cases these resulted from mis-splicing of exon 4 into cryptic acceptor sites in the non-coding sequence of exon 5, potentially giving rise to TIMP3 proteins with novel C-terminal domains of different length and numbers of cysteine residues (illustrated in Fig. 1C from the DNA sequences in Fig. S1C).

ARPE19 cells were stably transfected with the sM and sWT constructs (endogenous wild-type TIMP3 is also expressed by these cells) and their extracellular matrix (ECM) isolated and stained with anti-TIMP3 antibody. Novel bands at ~15 kDa and ~30 kDa were observed in the non-

Peer review under responsibility of Chongqing Medical University.

https://doi.org/10.1016/j.gendis.2022.03.008
2352-3042/Copyright © 2022, Chongqing Medical University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Please cite this article as: F.A. Alsaffar, A.H. Mujamammi, M.S.K. Aldughaim et al., Evidence that all Sorsby's fundus dystrophy mutations cause TIMP3 dimerization resulting in impaired inhibition of VEGFR2, Genes & Diseases, https://doi.org/10.1016/j.gendis.2022.03.008

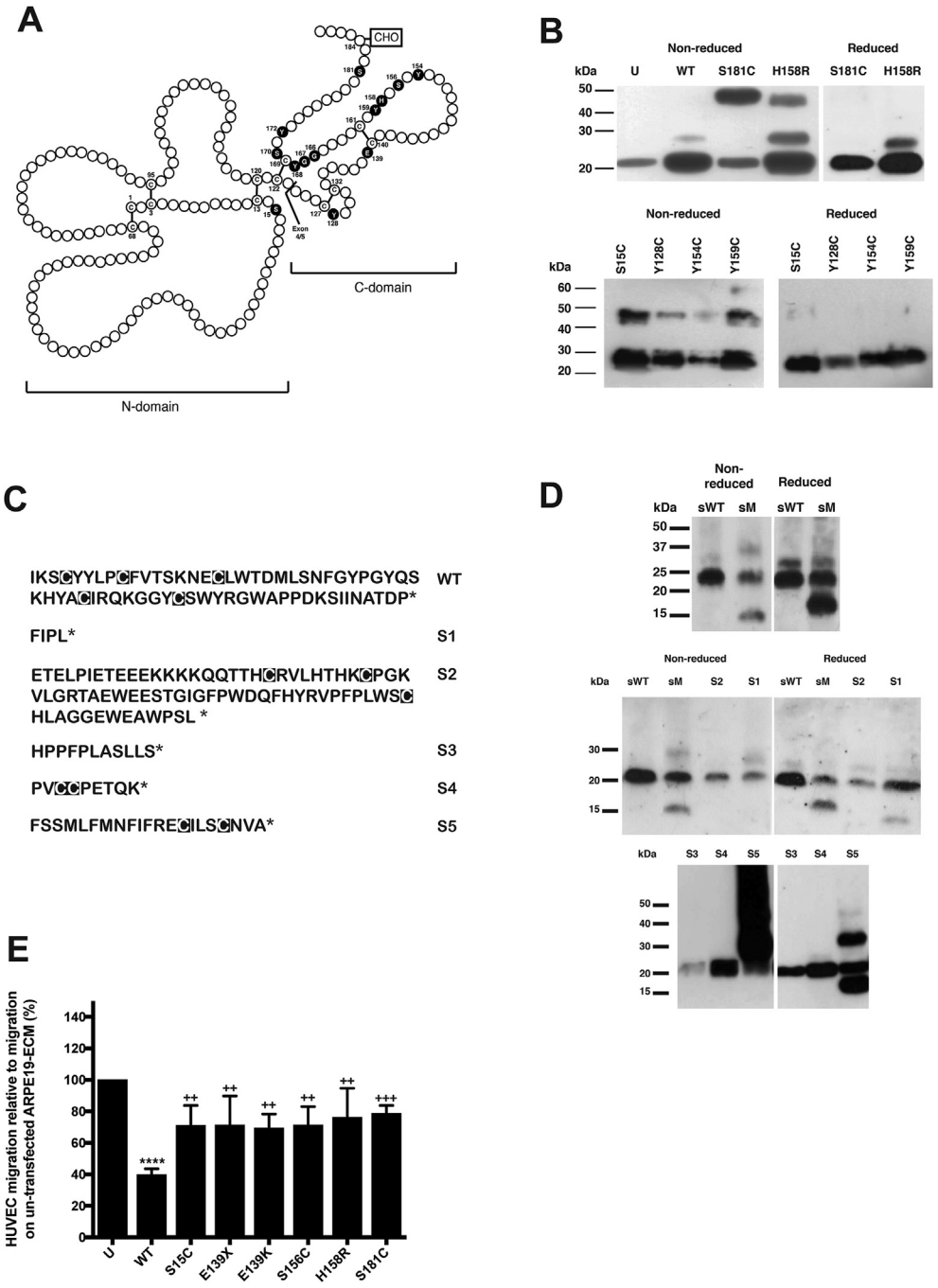

Figure 1 The effects of Sorsby's fundus dystrophy mutations on TIMP3 expression and dimerization and on endothelial cell migration in response to VEGF. **(A)** Schematic representation of the primary structure of TIMP3 illustrating the positions of residues found to be mutated in SFD. Mutated residues are highlighted in black and numbered according to the secreted protein sequence. Other key residues, including cysteines and the N-glycosylation site are also numbered and the exon 4/exon 5 boundary indicated. **(B)** Representative Western blots of non-reduced and reduced ECM from ARPE19 cells stably transfected with different SFD mutant constructs. (U) untransfected, (WT) wild-type TIMP3 or the specified mutant forms of TIMP3. Mutations in the upper panel were probed using an anti-TIMP3 polyclonal antibody, those in the lower panel with an anti-V5 monoclonal antibody. **(C)** Protein sequence of the normal, wild-type (WT), exon 5 and the predicted sequences of the novel mis-spliced products (S1—S5). **(D)** Representative Western blots of non-reduced and reduced ECM from ARPE19 cells stably transfected with splice site SFD mutant constructs. Upper panel, the products of the full-length wild type (sWT) and full-length splice site mutated (sM) splice constructs i.e., including intron 4 and all of exon 5. Lower two panels; sWT and sM construct alongside products of the S1, S3, S4 and S5 splice product cDNA sequences. Membranes were probed with anti-TIMP3 polyclonal antibody. **(E)** Bar chart showing percentage migration relative to ECM from untransfected cells (means ± SE, $n = 3$, one way ANOVA) ****, $P < 0.0001$ relative to untransfected ARPE19; +++, $P < 0.001$ relative to WT TIMP3; ++ $P < 0.01$ relative to WT TIMP3.

reduced gel for sM but not sWT (Fig. 1D, top panel). Upon reduction, the band at ~30 kDa was absent and there was a concomitant increase in the intensity of the ~15 kDa band, which suggests that the ~30 kDa band is a dimer.

This banding pattern is unlikely to be explained by expression of the splice product S2, which would be ~23 kDa, if expressed. However, S1, S3, S4 and S5 would all be expected to yield 15–17 kDa polypeptides with short aberrant C-terminal sequences and all or one of these products might comprise the observed lower band. They would also result in molecules with an odd number of Cys residues, which could contribute to intermolecular disulfide bonds and the ~30 kDa band. In order to determine which, if any, of the cloned mis-spliced cDNA sequences could explain the protein expression pattern, the cDNA sequences were subcloned into the pcDNA3 mammalian expression vector and stably transfected into ARPE19 cells (alongside sM and sWT for comparison). Without reduction, none of the cDNA constructs gave rise to a ~15 kDa band but both S1 and S5 gave rise to bands at ~30 kDa, which could be reduced to bands at ~15–16 kDa (Fig. 1D, bottom two panels).

All of the mis-splicing events identified on expression of the splice mutation would give rise to TIMP3 proteins that lack all but the first three amino acid residues of the carboxyl domain of TIMP3, which has been shown to be both essential and sufficient for VEGFR2 inhibition,[5] making it highly unlikely that these mis-spliced protein products would inhibit the receptor. However, patients with the splice site mutation have a late onset form of the disease and do not show a more pronounced degree of CNV than patients with any other TIMP3 mutation. Similarly, patients with the E139X mutation, which also lacks most of the carboxyl domain, do not exhibit particularly pronounced CNV (referenced in Table S1). This led us to hypothesize that dimerization of TIMP3 about the carboxyl domain may impair the ability of all mutant TIMP3 proteins to inhibit VEGFR2, potentially explaining the CNV which is a hallmark of this disease. We tested this hypothesis by analysing the ability of vascular endothelial cells to migrate through ECM from ARPE19 cells expressing either wild-type or several different SFD mutant TIMP3 proteins in response to VEGF.

The cell matrix from wild-type TIMP3 transfected ARPE19 cells inhibited human umbilical endothelial cell (HUVEC) migration by ~60% relative to that from untransfected cells (Fig. 1E, S2A). While ECM from all the SFD-transfected ARPE19 cells also showed some inhibition, this was significantly less than for the wild-type protein (~30%, relative to untransfected cell matrix). Western blotting of the ECM from these cells for total TIMP3 protein expression, showed some variation in expression levels, however these did not correlate with the degree of inhibition seen with, for example, more TIMP3 protein being expressed by the S156C SFD transfected cells (Fig. S2B).

In summary, our data support the hypothesis that all SFD-mutated forms of TIMP3 result in dimerization/multimerization of the protein which in turn significantly impairs its ability to inhibit the proangiogenic growth factor receptor VEGFR2. However, we cannot categorically dismiss the possibility that SFD mutations have an, as yet unidentified, effect on the ECM which facilitates endothelial cell migration, although this seems unlikely as, in patients at least, mutations result in increased matrix deposition and basement membrane thickening which would be expected to impair, rather than facilitate, migration.

Conflict of interests

The authors declare no conflict of interests.

Funding

This publication is made possible, in part, by studentship support from the Saudi Arabian Cultral Bureau in London to F.A.A., A.H.M. and M.S.K.A.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.03.008.

References

1. Langton KP, McKie N, Curtis A, et al. A novel tissue inhibitor of metalloproteinases-3 mutation reveals a common molecular phenotype in Sorsby's fundus dystrophy. *J Biol Chem*. 2000; 275(35):27027–27031.
2. Saihan Z, Li Z, Rice J, et al. Clinical and biochemical effects of the E139K missense mutation in the TIMP3 gene, associated with Sorsby fundus dystrophy. *Mol Vis*. 2009;15:1218–1230.
3. Lin RJ, Blumenkranz MS, Binkley J, Wu K, Vollrath D. A novel His158Arg mutation in TIMP3 causes a late-onset form of Sorsby fundus dystrophy. *Am J Ophthalmol*. 2006;142(5):839–848.
4. Naessens S, de Zaeytijd J, Syx D, et al. The N-terminal p.(Ser38Cys) TIMP3 mutation underlying Sorsby fundus dystrophy is a founder mutation disrupting an intramolecular disulfide bond. *Hum Mutat*. 2019;40(5):539–551.
5. Hua QJ, Quteba E, Mariya A, et al. Tissue inhibitor of metalloproteinases-3 peptides inhibit angiogenesis and choroidal neovascularization in mice. *PLoS One*. 2013;8(3):e55667.

Fatimah A. Alsaffar [a,1], Ahmed H. Mujamammi [a,2], Mohammed S.K. Aldughaim [a,3], Martin J.H. Nicklin [b], Michael D. Barker [a,*]

[a] *Department of Oncology & Metabolism, University of Sheffield Medical School, Sheffield S10 2RX, UK*
[b] *Department of Infection Immunity & Cardiovascular Disease, University of Sheffield Medical School, Sheffield S10 2RX, UK*

*Corresponding author.
E-mail address: m.barker@sheffield.ac.uk (M.D. Barker)

27 April 2021

[1] Current address: Department of Clinical Laboratory Sciences, Alghad International Colleges for Applied Medical Sciences, Dammam, Kingdom of Saudi Arabia.
[2] Current address: Department of Pathology and Laboratory Medicine, College of Medicine and King Saud University Medical City Hospitals, King Saud University, P.O. Box 2925, Riyadh 11461, Kingdom of Saudi Arabia.
[3] Current address: Research Center, King Fahad Medical City, P.O.Box. 59046, Riyadh 11525, Kingdom of Saudi Arabia.