CHARACTERISATION OF STRUCTURAL AND FUNCTIONAL CHANGES OF HUMAN HEAT SHOCK PROTEIN 47 WILD-TYPE AND HIS-TO-ALA MUTANTS

SITI MASTURAH ZAKERIA

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DEDICATION

"My dearest family"

I dedicate this thesis to all of you

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ABSTRACT

Heat shock protein 47 (HSP47) is a collagen-specific molecular chaperone that localises in the endoplasmic reticulum (ER). The HSP47 is essential for a proper formation of the collagen triple helix. The HSP47 binds to the completely-folded collagen molecule and accompanies it from the ER to the Golgi apparatus (GA), where dissociation occurs and HSP47 is recycled back to the ER. The binding and release behaviour are triggered by the lower pH in the GA or the ER-Golgi intermediate compartment (ERGIC). Histidine has been suggested to be the trigger residue due to the side chain pK_a value of around 6.1, similar to the HSP47-collagen dissociation pH. In this study, Histidine-to-alanine (HA) mutants of human HSP47 were constructed to elucidate the specific mechanism that governs HSP47 release from collagen at the molecular level, known as the "pH-switch mechanism". The binding of three mutants were found to be affected, namely H220A, H335A and H368A. This study investigated the effects of these mutations to the human HSP47 binding strength, using collagen type I extracted from rat tail tendon and modified enzyme-linked immunosorbent assay (ELISA)-based binding assay. The secondary structure of mutant proteins was investigated using circular dichroism (CD) spectroscopy. The result showed that mutants were found to retain their binding ability to collagen, except for H220A. The H335A and H368A were found to have slightly lower binding affinity to collagen relative to the wild-type (WT) (K_D = 50.76 nM), with a dissociation constant (K_D) of 152.9 nM and 131.3 nM respectively. In addition to the lost in collagen binding, H220A was also significantly more thermostable compared to the other mutants, that have similar thermostability to WT. The secondary structure of the HA mutants at alkaline pH was found to differ slightly from WT, with H368A showed a perturbed pH-induced secondary structural changes. In conclusion, the binding strength of the human HSP47 HA mutants was successfully elucidated and the structural changes caused by the mutations were described. The H220 was suggested to be important for binding with collagen while H368 was important for the release mechanism.

ABSTRAK

Protein Kejutan Haba 47 (HSP47) adalah protein pendamping kolagen dan dijumpai di dalam retikulum endoplasma (ER). HSP47 sangat penting untuk pembentukan heliks tiga serangkai yang berfungsi sebagai peneman dari ER ke badan Golgi sebelum pemisahan berlaku dan kembali semula ke ER. Tingkah laku pengikatan dan pelepasan ini dicetuskan oleh pH rendah di dalam GA atau di ruang perantaraan ER-Golgi (ERGIC). Histidina telah dicadangkan sebagai residu pencetus kerana nilai pK_a rantai sisinya iaitu sekitar 6.1 yang sama seperti nilai pH di mana pemisahan kolagen daripada HSP47 berlaku. Dalam kajian ini, mutan histidina-ke-alanina telah dibangunkan untuk HSP47 manusia bagi menjelaskan mekanisma khusus yang mengatur pelepasan HSP47 dari kolagen pada tahap molekul, yang dikenali sebagai "mekanisma pertukaran pH". Tiga mutan sebelum ini didapati mempunyai pengikatan lebih rendah dengan kolagen, iaitu H220A, H335A dan H368A. Kajian ini bertujuan menyelidik kesan mutasi ini kepada kekuatan pengikatan HSP47 manusia. Kekuatan pengikatan dikaji menggunakan kolagen jenis I yang diekstrak daripada tendon ekor tikus dan kaedah berasaskan Imunoserap Berkait Enzim (ELISA). Struktur sekunder protin mutan seterusnya dikaji menggunakan spektroskopi dwikroisme membulat (CD). Keputusan menunjukkan semua mutan didapati masih mampu mengikat kepada kolagen, kecuali H220A. H368A dan H335A didapati mempunyai kemampuan mengikat kolagen yang lebih rendah, dengan pemalar pengasingan (K_D) bernilai 131.3 nM dan 152.9 nM, berbanding protein jenis asal (WT) (K_D = 50.76 nM). H220A bukan sahaja hilang kemampuan mengikat kolagen malah mempunyai tahap kestabilan haba yang lebih tinggi berbanding WT dan mutan yang lain. Struktur sekunder pada pH alkali untuk semua mutan didapati sedikit berbeza daripada WT, dengan H368A berbeza dari segi perubahan pertukaran struktur yang dicetuskan oleh pH. Kesimpulannya, kekuatan pengikatan di antara mutan HSP47 manusia dan kolagen telah berjaya dikaji dan perubahan struktur disebabkan oleh mutasi telah dicadangkan di dalam kajian ini. Kajian ini mencadangkan yang H220 adalah penting bagi pengikatan HSP47 dengan kolagen, manakala H368 penting bagi mekanisma pemisahan.

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LIST OF ABBREVIATIONS

OI	-	osteogenesis imperfecta
HA	-	His-to-Ala
ER	-	endoplasmic reticulum
CD	-	circular dichroism
ELISA	-	enzyme-linked immunosorbent assay
WT	-	wild type
ECM	-	extracellular matrix
FACIT	-	fibril-associated collagens with interrupted triple helices
PTM	-	post-translational modifications
BiP	-	immunoglobulin heavy-chain binding protein
Pro	-	proline
Hyp	-	hydroxyproline
GRP94	-	glucose-regulated protein
CNX	-	calnexin
CRT	-	calreticulin
PDI	-	protein disulfide isomerase
PPIases	-	peptidyl-prolyl cis-trans isomerases
P4H	-	prolyl 4-hydroxylase
SERPIN	-	serine protease inhibitor
RDEL	-	Arg-Asp-Glu-Leu
ERGIC	-	*cis*-Golgi or the ER-Golgi intermediate compartment
HSCs	-	hepatic stellate cells
ESCC	-	esophageal squamous cell carcinoma
siRNA	-	Small interfering RNA
HNSC	-	head and neck squamous cell carcinoma
LB	-	Luria Bertani
BCIP/NBT	-	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
IPTG	-	isopropyl-β-D-thiogalactopyranoside
SDS-PAGE	-	sodium dodecyl sulphate polyacrylamide gel electrophoresis

AP	-	alkaline phosphatase
IMAC	-	immobilized metal-ion affinity chromatography
CBB	-	coomassie brilliant blue
SEC	-	size-exclusion chromatography
Asn	-	asparagine
Ala	-	alanine
PAI-1	-	plasminogen activator inhibitor
p*K*a	-	acid dissociation constant
*k*d	-	dissociation constant
NMR	-	nuclear magnetic resonance
CpHMD	-	constant pH molecular dynamics
mrm	-	mature recombinant mouse
hPCI	-	human protein C inhibitor
Trp	-	tryptophan
Phe	-	phenylalanine

LIST OF SYMBOLS

cm	-	centimetre
Da	-	dalton
g	-	gramme
H	-	hour
kDa	-	kilodalton
L	-	litre
M	-	molar
mg	-	milligram
min	-	minute(s)
mL	-	millilitre
mM	-	millimolar
Mw	-	molecular weight
R^2	-	coefficient of determination
rpm	-	revolutions per minute
U	-	unit of enzyme activity
V	-	volt
v/v	-	volume per volume
w/v	-	weight per volume
µg	-	microgram
µL	-	microlitre
µmol	-	micromole
%	-	percentage
xg	-	times gravity
~	-	approximately
°C	-	degree celcius
$\Delta\Delta A_{222}$	-	change in CD signal intensity at 222 nm
ΔA	-	difference in absorbance between CD L and R components
Abs_{280}, A_{280}	-	absorbance at 280 nm
mdeg	-	milidegrees
T_m	-	melting temperature

mAu	-	mili absorbance unit

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Heat shock protein 47 (HSP47) is a collagen-specific molecular chaperone that assists the maturation and transport of procollagens in collagen biosynthesis. This chaperone can be found in all vertebrates, where collagen is synthesised by the cell. Nowadays, there are a growing body of evidence on the involvement of HSP47 in various types of diseases, such as in osteogenesis imperfecta (OI), fibrosis, and cancer (Syx et al., 2021; Westra et al., 2016; Fan et al., 2020). A close relation between the increased expression of HSP47 and the excessive deposition of collagen has been reported in human, in vivo and in vitro fibrotic disease models (Kim et al., 2019). The increased expression in fibrotic diseases is known to help in the increased assembly of procollagen and thus lead to the excessive accumulation of collagen in fibrotic area (Bellaye et al., 2020). HSP47 is also being reported to be upregulated in various types of cancers such as stomach cancer, lung cancer, head and neck cancer, pancreatic ductal adenocarcinoma and ulcerative colitis-associated carcinomas (Chern et al., 2020; Fan et al., 2020; Zhao et al., 2014). This urges the need to completely elucidate the molecular mechanisms of this chaperone.

Previously, Abdul Wahab and colleagues (2013) have successfully constructed a series of His-to-Ala (HA) mutants targeting all fourteen histidine residues of mouse HSP47, to elucidate the so-called "pH-switch mechanism". This is the mechanism known to govern the release of HSP47 from collagen. This relates to its cellular behaviour, where HSP47 is recycled back to the endoplasmic reticulum (ER) after assisting the transport of procollagen from the ER to the cis-Golgi. From the study, some HA mutations do not impose significant perturbations in HSP47 function, but several others were found to impair the binding to collagen, which is an unexpected effect.

In 2014, fourteen histidine residues of human HSP47 were successfully mutated to Alanine (unpublished data). This project was therefore proposed to investigate the effects of the selected HA mutations in human HSP47, and to further investigate the pH-switch mechanism. Human HSP47 was used for more physiologically-relevant results as it has 98% of similarities with mouse species. The mutants investigated were H220A, H335A and H368A, based on the perturbed behaviour observed previously (unpublished data). Mutations to alanine abolished the histidine side chain characteristics and the possible bonding with the neighbouring residues, allowing for the assessment of the importance of these target residues.

1.2 Problem Statement

It was found that the HA mutations at specific locations (particularly H220A, H335A, H368A) of HSP47 has significantly affected the binding behaviour based on gelatin agarose pull down assay (unpublished data). These mutations could potentially affect the release mechanism too. The binding study using gelatin pull down assay was only able to predict the conservation of structure and binding interface of the mutants, but unfortunately it does not provide further information on the binding strength. Thus, to investigate further the effects of the mutation on the binding strength, an enzyme-linked immunosorbent assay (ELISA)-based binding assay was proposed. This assay allowed quantification of the binding strength of the mutants, relative to wild type (WT). Consequently, the effects of mutations on HSP47 were investigated based on the secondary structural changes with pH, using circular dichroism (CD) spectroscopy. This technique allowed a more detailed understanding of the pH-induced structural transition of the mutants.

1.3 Research Objectives

The aim of this research project was to study the binding strength and pH-release behaviour of human HSP47 and target mutants (H220A, H335A, H368A) relative to the WT. The specific objectives are as follows:

1. To express and purify the recombinant human HSP47 WT and target mutants.
2. To determine the binding affinity of human HSP47 WT and target mutants using ELISA-based binding assay.
3. To determine the pH-induced structural transition of human HSP47 WT and target mutants using circular dichroism spectroscopy.

1.4 Scope of Research

This project covers a few aspects:

(a) Preparation of competent cell by using $CaCl_2$ method and transformation of competent cells using heat shock protocol.

(b) Expression and purification of recombinant HSP47 wild type (WT) and target mutants (H220A, H335A, H368A). The purification was done using immobilised nickel affinity column as the first purification step and then using HiTrap^{TM} HP desalting column as the second purification step.

(c) Determination of binding affinity of human HSP47 WT and target mutants by using ELISA-based binding assay.

(d) Investigation on the changes to pH-induced structural transition of HSP47 WT and target mutants by using circular dichroism spectroscopy.

1.5 Significance of Research

This research is important to discover the key His residue(s) that controls HSP47 pH-switch release, and the involvement in binding or structural integrity. Methods used in this research can directly be applied in collagen-related disease model studies. Therefore, this will increase the understanding on these diseases thus can move towards designing suitable therapeutic strategies.

REFERENCES

Abdul-Wahab, M. F., Homma, T., Wright, M., Olerenshaw, D., Dafforn, T. R., Nagata, K., and Miller, A. D. (2013). 'The pH sensitivity of murine heat shock protein 47 (HSP47) binding to collagen is affected by mutations in the breach histidine cluster', *Journal of Biological Chemistry*, 288(6), pp. 4452–4461.

Araki, K., T. Mikami, T. Yoshida, M. Kikuchi, Y. Sato, M. Oh-ishi, Y. Kodera, T. Maeda, and I. Okayasu. (2009). 'High expression of HSP47 in ulcerative colitis-associated carcinomas: proteomic approach', *British Journal of Cancer*, 101(3), pp. 492-497.

Bolanos-Garcia, V. M., and Davies, O. R. (2006). 'Structural analysis and classification of native proteins from E. coli commonly co-purified by immobilised metal affinity chromatography', *Biochimica et Biophysica Acta - General Subjects*, 1760(9), pp. 1304–1313.

Brown, K. E., Broadhurst, K. A., Mathahs, M. M., Brunt, E. M., and Schmidt, W. N. (2005). 'Expression of HSP47, a collagen-specific chaperone, in normal and diseased human liver', *Laboratory Investigation*, 85(6), pp. 789–797.

Cates, G. A., Brickenden, A. M., and Sanwal, B. D. (1984). 'Possible involvement of a cell surface glycoprotein in the differentiation of skeletal myoblasts', *Journal of Biological Chemistry*, 259(4), pp. 2646-2650.

Cesa, L.C., Shao, H., Srinivasan, S.R., Tse, E., Jain, C., Zuiderweg, E.R., Southworth, D.R., Mapp, A.K. and Gestwicki, J.E., (2018). 'X-linked inhibitor of apoptosis protein (XIAP) is a client of heat shock protein 70 (Hsp70) and a biomarker of its inhibition', *Journal of Biological Chemistry*, *293*(7), pp. 2370-2380.

Chern, Y., Zhang, P., Ju, H., and Tai, I. T. (2020). 'Heat shock protein 47 promotes tumor survival and therapy resistance by modulating AKT signaling via PHLPP1 in colorectal cancer', *Cancer Biology and Medicine*, 17(2), pp. 343–356.

Clarke, E. P., and Sanwal, B. D. (1992). 'Cloning of a human collagen-binding protein, and its homology with rat gp46, chick hsp47 and mouse J6 proteins', *BBA - Gene Structure and Expression*, 1129(2), pp. 246–248.

Dafforn, T. R., Della, M., and Miller, A. D. (2001). 'The molecular interactions of heat shock protein 47 (Hsp47) and their implications for collagen biosynthesis', *Journal of Biological Chemistry*, 276(52), pp. 49310–49319.

Dafforn, T. R., Pike, R. N., and Bottomley, S. P. (2004). 'Physical characterization of serpin conformations', *Methods*, 32(2), pp. 150–158.

Davids, J.W., S.H. El-Thaher, A. Nakai, K. Nagata, and A.D. Miller. (1995). 'Modeling the three-dimensional structure of serpin/molecular chaperone HSP47', *Bioorganic Chemistry,* 23(4), pp. 427.

Drain, A.P. and Weaver, V.M., (2020). Matrix molecules and their ligands. In *Principles of Tissue Engineering* (pp. 119-132). Academic Press.

Duarte, B. D. P., and Bonatto, D. (2018). 'The heat shock protein 47 as a potential biomarker and a therapeutic agent in cancer research', *Journal of Cancer Research and Clinical Oncology*. 144(12), pp. 2319-2328.

Elmallah, M.I., Cordonnier, M., Vautrot, V., Chanteloup, G., Garrido, C. and Gobbo, J. (2020). 'Membrane-anchored heat-shock protein 70 (Hsp70) in cancer', *Cancer Letters*, 469, pp.134-141.

El-Thaher, S.H., A.F. Drake, S. Yokota, A. Nakai, K. Nagata, and A.D. Miller. (1996). 'The pH-dependent, ATP-independent interaction of collagen specific serpin/stress protein HSP47', *Protein Peptide Letters.* 3(1), pp. 1-8.

Fan, G., Fan, G., Tu, Y., Wu, N., and Xiao, H. (2020). 'The expression profiles and prognostic values of HSPs family members in head and neck cancer', *Cancer Cell International*, 20(1), pp. 1–12.

Freise, C., Erben, U., Muche, M., Farndale, R., Zeitz, M., Somasundaram, R. and Ruehl, M. (2009). 'The alpha 2 chain of collagen type VI sequesters latent proforms of matrix-metalloproteinases and modulates their activation and activity. *Matrix Biology*', 28(8), pp. 480-489.

Gelse, K., Pöschl, E., and Aigner, T. (2003). 'Collagens - Structure, function, and biosynthesis', *Advanced Drug Delivery Reviews*, 55(12), pp. 1531–1546.

Gordon, M. K., and Hahn, R. A. (2010). 'Collagens', *Cell and Tissue Research*, 339(1), pp. 247–257.

Grodberg, J., and Dunn, J. J. (1988). 'ompT encodes the Escherichia coli outer membrane protease that cleaves T7 RNA polymerase during purification', *Journal of Bacteriology*, 170(3), pp. 1245–1253.

Hagel, L. (1998). 'Gel-filtration chromatography. Current *protocols in molecular*

biology', 44(1), pp. 10.9.1-10.9.2.

Hartl, F.U., and M. Hayer-Hartl. (2009). 'Converging concepts of protein folding in vitro and in vivo', *Nature Structural and Molecular Biology,* 16(6), pp. 574-581.

Hendershot, L. M., and Bulleid, N. J. (2000). 'Protein-specific chaperones: the role of hsp47 begins to gel', *Current Biology*, 10(24), pp. 912–915.

Hosokawa, N. and Nagata, K. (2000). 'Procollagen binds to both prolyl 4-hydroxylase/protein disulfide isomerase and HSP47 within the endoplasmic reticulum in the absence of ascorbate', *FEBS Letters*, 466(1), pp. 19-25.

Ibrahim, F. H., Abd Latip, N., and Abdul-Wahab, M. F. (2018). 'Heat shock protein 47 (HSP47)', *ELSEVIER*, 47, pp. 1–7.

Ishida, Y., and Nagata, K. (2011). 'Hsp47 as a collagen-specific molecular chaperone', *Methods in Enzymology*, *499*, pp. 167–182.

Ishida, Y., Kubota, H., Yamamoto, A., Kitamura, A., Bächinger, H. P., and Nagata, K. (2006). 'Type I collagen in Hsp47-null cells is aggregated in endoplasmic reticulum and deficient in N-propeptide processing and fibrillogenesis', *Molecular Biology of the Cell*, 17(5), pp. 2346–2355.

Ishikawa, Y., Ito, S., Nagata, K., Sakai, L. Y., and Bächinger, H. P. (2016). 'Intracellular mechanisms of molecular recognition and sorting for transport of large extracellular matrix molecules', *Proceedings of the National Academy of Sciences*, 113(41), pp. E6036-E6044.

Ito, S. and Nagata, K., 2019. 'Roles of the endoplasmic reticulum–resident, collagen-specific molecular chaperone Hsp47 in vertebrate cells and human disease', *Journal of Biological Chemistry*, 294(6), pp. 2133-2141.

Ito, S., Saito, M., Yoshida, M., Takeuchi, K., Doi, T., and Nagata, K. (2019). 'A BRET-based assay reveals collagen-Hsp47 interaction dynamics in the endoplasmic reticulum and small-molecule inhibition of this interaction', *Journal of Biological Chemistry*, 294(44), pp. 15962–15972.

Jiang, W., Graham, B., Spiccia, L., and Hearn, M. T. (1998). 'Protein selectivity with immobilized metal ion-tacn sorbents: chromatographic studies with human serum proteins and several other globular proteins', *Analytical Biochemistry*, 255(1), pp. 47-58.

Kadler, K. E., Baldock, C., Bella, J., and Boot-Handford, R. P. (2007). 'Collagens at a glance', *Journal of Cell Science*, 120(12), pp. 1955–1958.

Kawasaki, K., Ushioda, R., Ito, S., Ikeda, K., Masago, Y., and Nagata, K. (2015). 'Deletion of the collagen-specific molecular chaperone Hsp47 causes endoplasmic reticulum stress-mediated apoptosis of hepatic stellate cells', *Journal of Biological Chemistry*, 290(6), pp. 3639–3646.

Kim, HJ., Park, JH., Shin, JM., Yang, HW., Lee, HM., and (2019). 'TGF-β1-induced HSP47 regulates extracellular matrix accumulation via Smad2/3 signaling pathways in nasal fibroblasts', *Scientific Reports* , 9, 15563.

Kitamura, A., Ishida, Y., Kubota, H., Pack, C. G., Homma, T., Ito, S., Araki, K., Kinjo, M., and Nagata, K. (2018). 'Detection of substrate binding of a collagen-specific molecular chaperone HSP47 in solution using fluorescence correlation spectroscopy', *Biochemical and Biophysical Research Communications*, 497(1), pp. 279–284.

Köhler, A., Mörgelin, M., Gebauer, J.M., Öcal, S., Imhof, T., Koch, M., Nagata, K., Paulsson, M., Aumailley, M., Baumann, U. and Zaucke, F., 2020. 'New specific HSP47 functions in collagen subfamily chaperoning', *The FASEB Journal*, 34(9), pp. 12040-12052.

Koide, T., and Nagata, K. (2005). 'Collagen biosynthesis', *Topics in Current Chemistry*, 247, pp. 85–114.

Koide, T., Aso, A., Yorihuzi, T., and Nagata, K. (2000). 'Conformational requirements of collagenous peptides for recognition by the chaperone protein HSP47', *Journal of Biological Chemistry*, 275(36), pp. 27957 – 27963.

Komsa-Penkova, R., Spirova, R., and Bechev, B. (1996). 'Modification of Lowry's method for collagen concentration measurement', *Journal of Biochemical and Biophysical Methods*, 32(1), pp. 33–43.

Kumar, A., Sharma, S., Chunduri, V., Kaur, A., Kaur, S., Malhotra, N., and Garg, M. (2020). 'Genome-wide Identification and Characterization of Heat Shock Protein Family Reveals Role in Development and Stress Conditions in Triticum aestivum L', *Scientific reports*, 10(1), pp. 1-12.

Kurkinen, M., Taylor, A., Garrels, J. I., and Hogan, B. L. M. (1984). 'Cell surface-associated proteins which bind native type IV collagen or gelatin', *Journal of Biological Chemistry*, 259(9), pp. 5915–5922.

Laemmli, U. K. (1970). 'Cleavage of structural proteins during the assembly of the head of bacteriophage T4' *Nature*, 227(5259), pp. 680−685.

Lindert, U., Weis, M. A., Rai, J., Seeliger, F., Hausser, I., Leeb, T., Eyre, D., Rohrbach,

M., and Giunta, C. (2015). 'Molecular consequences of the SERPINH1/HSP47 mutation in the dachshund natural model of osteogenesis imperfecta', *Journal of Biological Chemistry*, 290(29), pp. 17679–17689.

Lee, H.W., Kwon, J., Kang, M.C., Noh, M.K., Koh, J.S., Kim, J.H. and Park, J.H., 2016. 'Overexpression of HSP47 in esophageal squamous cell carcinoma: clinical implications and functional analysis', *Diseases of the Esophagus*, 29(7), pp.848-855.

Macdonald, J. R., and Bächinger, H. P. (2001). 'HSP47 binds cooperatively to triple helical type i collagen but has little effect on the thermal stability or rate of refolding', *Journal of Biological Chemistry*, 276(27), pp. 25399–25403.

Martínez-Laorden, E., Navarro-Zaragoza, J., Milanés, M. V., Laorden, M. L., and Almela, P. (2020). 'Cardiac protective role of heat shock protein 27 in the stress induced by drugs of abuse', *International Journal of Molecular Sciences*, 21(10), pp. 3623.

Matsuoka, Y., Kubota, H., Adachi, E., Nagai, N., Marutani, T., Hosokawa, N., and Nagata, K. (2004). 'Insufficient folding of type IV collagen and formation of abnormal basement membrane-like structure in embryoid bodies derived from Hsp47-null embryonic stem cells', *Molecular Biology of the Cell*. 15(10), pp. 4467-4475.

Miyamura, T., Sakamoto, N., Kakugawa, T., Taniguchi, H., Akiyama, Y., Okuno, D., Moriyama, S., Hara, A., Kido, T., Ishimoto, H. and Yamaguchi, H. (2020). 'Small molecule inhibitor of HSP47 prevents pro-fibrotic mechanisms of fibroblasts in vitro', *Biochemical and Biophysical Research Communications*, 530(3), pp. 561-565.

Mori, K., Toiyama, Y., Otake, K., Fujikawa, H., Saigusa, S., Hiro, J., Kobayashi, M., Ohi, M., Tanaka, K., Inoue, Y. and Kobayashi, Y., (2017). 'Proteomics analysis of differential protein expression identifies heat shock protein 47 as a predictive marker for lymph node metastasis in patients with colorectal cancer', *International Journal of Cancer*, 140(6), pp.1425-1435.

Nagai, N., Hosokawa, M., Itohara, S., Adachi, E., Matsushita, T., Hosokawa, N., and Nagata, K. (2000). 'Embryonic lethality of molecular chaperone Hsp47 knockout mice is associated with defects in collagen biosynthesis', *Journal of Cell Biology*, 150(6), pp. 1499–1505.

Nagata, K., S. Saga, and K.M. Yamada. (1986). 'A major collagen-binding protein of

chick embryo fibroblasts is a novel heat shock protein', *Journal of Cell Biology,* 103:223.

Nakai, A., Satoh, M., Hirayoshi, K., and Nagata, K. (1992). 'Involvement of the stress protein HSP47 in procollagen processing in the endoplasmic reticulum', *Journal of Cell Biology*, 117(4), pp. 903–914.

Natsume, T., Koide, T., Yokota, S. I., Hirayoshi, K., and Nagata, K. (1994). 'Interactions between collagen-binding stress protein HSP47 and collagen. Analysis of kinetic parameters by surface plasmon resonance biosensor', *Journal of Biological Chemistry*, 269(49), pp. 31224–31228.

Oecal, S., Socher, E., Uthoff, M., Ernst, C., Zaucke, F., Sticht, H., Baumann, U., and Gebauer, J. M. (2016). 'The pH-dependent client release from the collagen-specific chaperone HSP47 is triggered by a tandem histidine pair', *Journal of Biological Chemistry*, 291(24), pp. 12612–12626.

Ono, T., Miyazaki, T., Ishida, Y., Uehata, M., and Nagata, K. (2012). 'Direct in vitro and in vivo evidence for interaction between Hsp47 protein and collagen triple helix', *Journal of Biological Chemistry*, 287(9), pp. 6810–6818.

Otsuka, M., Shiratori, M., Chiba, H., Kuronuma, K., Sato, Y., Niitsu, Y. and Takahashi, H., 2017. 'Treatment of pulmonary fibrosis with siRNA against a collagen-specific chaperone HSP47 in vitamin A-coupled liposomes', *Experimental Lung Research*, 43(6-7), pp. -282.

Ponomarenko M., Stepanenko, I., and N. Kolchanov, Heat Shock Proteins, In: Maloy, S. and Hughes, K. (2013). *Brenner's Encyclopedia of Genetics*. 2nd ed. USA: Academic Press. pp. 402-405.

Prockop, D.J., K.I. Kivirikko, L. Tuderman, and N.A. Guzman. (1979). 'The biosynthesis of collagen and its disorders (first of two parts)', *New England Journal of Medcine,* 301, pp. 13-23.

Ragg, H. (2007). 'The role of serpins in the surveillance of the secretory pathway', *Cellular and Molecular Life Sciences*, 64(21), pp. 2763–2770.

Rajan, N., Habermehl, J., Coté, M. F., Doillon, C. J., & Mantovani, D. (2007). 'Preparation of ready-to-use, storable and reconstituted type I collagen from rat tail tendon for tissue engineering applications', *Nature Protocols*, 1(6), pp. 2753–2758.

Rajesh, Y., Biswas, A., and Mandal, M. (2017). 'Glioma progression through the prism of heat shock protein mediated extracellular matrix remodeling and epithelial

to mesenchymal transition', *Experimental Cell Research*, 359(2), pp. 299–311.

Rizk, F. H., Sarhan, N. I., Soliman, N. A., Ibrahim, M. A., Abd-Elsalam, M., and Abd-Elsalam, S. (2018). 'Heat shock protein 47 as indispensible participant in liver fibrosis: possible protective effect of lactoferrin', *IUBMB life*, 70(8), pp. 795-805.

Satoh, M., Hirayoshi, K., Yokota, S. I., Hosokawa, N., and Nagata, K. (1996). 'Intracellular interaction of collagen-specific stress protein HSP47 with newly synthesized procollagen', *The Journal of Cell Biology*, 133(2), pp. 469-483.

Sawano, A., and Miyawaki, A. (2000). 'Directed evolution of green fluorescent protein by a new versatile PCR strategy for site-directed and semi-random mutagenesis', *Nucleic Acids Research*, 28(16), pp. 78.

Smith, T., Ferreira, L. R., Hebert, C., Norris, K., Sauk, J. J., and Smith, T. (1995). 'Hsp47 and cyclophilin b traverse the endoplasmic reticulum with procollagen into pre-golgi intermediate vesicles', *Journal of Biological Chemistry*. 270(31), pp. 18323–18328.

Song, X., Liao, Z., Zhou, C., Lin, R., Lu, J., Cai, L., Tan, X., Zeng, W., Lu, X., Zheng, W., Chen, J., and Su, Z. (2017). 'HSP47 is associated with the prognosis of laryngeal squamous cell carcinoma by inhibiting cell viability and invasion and promoting apoptosis', *Oncology Reports*, 38(4), pp. 2444-2452.

Stachel, I., Schwarzenbolz, U., Henle, T., and Meyer, M. (2010). 'Cross-linking of type I collagen with microbial transglutaminase: Identification of cross-linking sites', *Biomacromolecules*, 11(3), pp. 698–705.

Syx, D., Ishikawa, Y., Gebauer, J., Boudko, S. P., Guillemyn, B., Van Damme, T., D'hondt, S., Symoens, S., Nampoothiri, S., Gould, D. B., Baumann, U., Bächinger, H. P., and Malfait, F. (2021). 'Aberrant binding of mutant HSP47 affects posttranslational modification of type I collagen and leads to osteogenesis imperfecta', *PLOS Genetics*, 17(2), e1009339.

Taguchi, T., and Razzaque, M. S. (2007). 'The collagen-specific molecular chaperone HSP47: is there a role in fibrosis?', *Trends in Molecular Medicine*, 13(2), pp. 45–53.

Tasab, M., Batten, M. R., and Bulleid, N. J. (2000). Hsp47: 'A molecular chaperone that interacts with and stabilizes correctly-folded procollagen', *EMBO Journal*, 19(10), pp. 2204–2211.

Thomson, C. A., and Ananthanarayanan, V. S. (2000). 'Structure–function studies on

Hsp47: pH-dependent inhibition of collagen fibril formation in vitro', *Society*, 883, pp. 877–883.

Tissiéres, A., Mitchell, H. K., and Tracy, U. M. (1974). 'Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs', *Journal of Molecular Biology*, 84(3), pp. 389-398.

Tung, Y.T., Tang, T.Y., Chen, H.L., Yang, S.H., Chong, K.Y., Cheng, W.T. and Chen, C.M., 2014. 'Lactoferrin protects against chemical-induced rat liver fibrosis by inhibiting stellate cell activation', *Journal of Dairy Science*, 97(6), pp.3281-3291.

Wahab, A., and Firdaus, M. (2012). 'Mechanistic behaviour and molecular interactions of heat shock protein 47 (HSP47)', 47(November).

Westra, D. F., Welling, G. W., Koedijk, D. G. A. M., Scheffer, A. J., Theb, T. H., and Welling-Westera, S. (2001). 'Immobilised metal-ion affinity chromatography purification of histidine-tagged recombinant proteins: A wash step with a low concentration of EDTA', *Journal of Chromatography*. 760, pp. 129-136.

Whitmore, L., and Wallace, B. A. (2004). 'DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data', *Nucleic Acids Research*, pp. 668–673.

Whitmore, L., and Wallace, B. A. (2008). 'Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases', *Biopolymers*, 89(5), pp. 392–400.

Widmera, C., Gebauerb, J. M., Brunsteinb, E., Rosenbaumc, S., Zauckec, F., Drogemüllerd, C., Leebd, T., and Baumannb, U. (2012). 'Molecular basis for the action of the collagen-specific chaperone Hsp47/SERPINH1 and its structure-specific client recognition', *Proceedings of the National Academy of Sciences of the United States of America*, 109(33), pp. 13243–13247.

Williams, B. R., Gelman, R. A., Poppke, D. C., and Piez, K. A. (1978). 'Collagen fibril formation. Optimal in vitro conditions and preliminary kinetic results', *Journal of Biological Chemistry*, 253(18), pp. 6578-6585.

Wood, G.C. 1960. 'The formation of fibrils from collagen solutions', 2. 'A mechanism of collagen-fibril formation', *Biochemical Journal,* 75(3), pp. 598–605.

Wynn, T. A., and Ramalingam, T. R. (2012). 'Mechanisms of fibrosis: Therapeutic translation for fibrotic disease', *Nature Medicine*, 18(7), pp. 1028–1040.

Yamakawa, T., Ohigashi, H., Hashimoto, D., Hayase, E., Takahashi, S., Miyazaki, M., Minomi, K., Onozawa, M., Niitsu, Y. and Teshima, T., 2018. 'Vitamin A–coupled liposomes containing siRNA against HSP47 ameliorate skin fibrosis in chronic graft-versus-host disease', *Blood, The Journal of the American Society of Hematology*, 131(13), pp. 1476-1485.

Zhang, J., Shen, H., Xu, J., Liu, L., Tan, J., Li, M., Xu, N., Luo, S., Wang, J., Yang, F. and Tang, J., 2020. 'Liver-targeted siRNA lipid nanoparticles treat hepatic cirrhosis by dual antifibrotic and anti-inflammatory activities', *ACS nano*, 14(5), pp. 6305-6322.

Zhao, D., Jiang, X., Yao, C., Zhang, L., Liu, H., Xia, H., and Wang, Y. (2014). 'Heat shock protein 47 regulated by miR-29a to enhance glioma tumor growth and invasion', *Journal of Neuro-Oncology*, 118(1), pp. 39–47.