

THE PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ROLES OF MELANOTRANSFERRIN

YOHAN SURYO RAHMANTO

歐陽耀吉

B.Sc. (Hons I) Biotech, Dip. Innov Man

*A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy (Medicine)*



DEPARTMENT OF PATHOLOGY
FACULTY OF MEDICINE
UNIVERSITY OF SYDNEY

July 2007

STATEMENT OF ORIGINALITY

I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at the University of Sydney or any other educational institution, except where due acknowledgment is made in the thesis. Any contribution made to the research by others, with whom I have worked at the University of Sydney or elsewhere, is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

Yohan Suryo Rahmanto

Dedicated to my parents,

Suryo Yusuf and Renny Rahmanto

TABLE OF CONTENTS

STATEMENT OF ORIGINALITY	i
TABLE OF CONTENTS.....	iii
ACKNOWLEDGEMENTS.....	x
ABSTRACT	xiii
LIST OF PUBLICATIONS.....	xvii
LIST OF AWARDS	xix
LIST OF INVITED PRESENTATIONS	xx
ABBREVIATIONS	xxiii
LIST OF FIGURES	xxviii
LIST OF TABLES	xxxi
CHAPTER 1: MELANOTRANSFERRIN: A 25-YEAR HALLMARK	1
1.1. General introduction.....	2
1.2. Genomic and molecular organisation of melanotransferrin.....	5
1.2.1. Chromosomal localisation of melanotransferrin.....	9
1.2.2. Melanotransferrin gene structure	9
1.2.2.1. Human <i>melanotransferrin</i> gene	9
1.2.2.2. Mouse <i>melanotransferrin</i> gene	11
1.2.3. Melanotransferrin gene transcription	11
1.3. Protein structure of melanotransferrin	13
1.4. Expression and localisation of melanotransferrin	18
1.4.1. GPI-anchored melanotransferrin.....	22
1.4.2. Soluble melanotransferrin	23
1.5. Functional role of melanotransferrin: iron transport and metabolism	24
1.6. Other possible roles of melanotransferrin	27
1.6.1. Cell migration and proliferation, plasminogen activation and angiogenesis	27
1.6.2. Blood brain barrier transport.....	32

1.6.3. Alzheimer's disease	33
1.6.4. Eosinophil differentiation	34
1.6.5. Chondrogenic differentiation and arthritis	34
1.7. Melanotransferrin as therapeutic target	35
1.8. Summary and aims of the study	37
CHAPTER 2: MATERIALS AND METHODS	38
2.1. Materials	39
2.1.1. Chemical and general reagents	39
2.1.2. Cell culture media and transfection reagents	41
2.1.3. Buffers and solutions	42
2.2. Mammalian cell culture	42
2.2.1. Cell lines	42
2.2.2. Growth of cell cultures	43
2.2.3. Harvesting and passaging cell cultures	43
2.2.4. Cryopreservation and thawing of cell cultures	44
2.2.5. Stable transfection	44
2.3. Measurement of cell proliferation	45
2.3.1. MTT cell proliferation assays	45
2.3.2. Cell proliferation assays	45
2.4. DNA processing	46
2.4.1. Agarose gel electrophoresis	46
2.4.2. Restriction digest and DNA recovery	46
2.4.3. Ligation	47
2.4.4. Bacterial strains and transformation	47
2.4.5. Miniprep plasmid DNA purification	47
2.4.6. Sequencing of cDNA	48
2.5. RNA processing	48
2.5.1. Isolation of RNA from whole cells and animal tissues	48

2.5.2. Reverse transcriptase-polymerase chain reaction	49
2.6. Western blotting.....	50
2.6.1. Antibodies	50
2.6.2. Protein extraction from whole cells and animal tissues.....	50
2.6.3. Immunoblotting.....	51
2.7. Densitometry	52
2.8. Animals	52
2.8.1. Animal handling.....	52
2.8.2. Necroscopy.....	52
2.9. Statistical analysis	53

CHAPTER 3: PHENOTYPIC CHARACTERISATION OF MELANOTRANSFERIN KNOCKOUT MOUSE AND DOWN-REGULATION OF MELANOTRANSFERRIN IN MELANOMA CELLS	54
3.1. Introduction.....	55
3.2. Materials and Methods.....	58
3.2.1. Animals	58
3.2.2. Generation of MTF ^{-/-} mice	58
3.2.3. Isolation and Genotyping of Genomic DNA from Animal Tissues.....	58
3.2.4. Serum chemistry, haematology and histochemistry	59
3.2.5. Total tissue iron, copper and zinc determinations.....	60
3.2.6. Microarray processing and analysis.....	60
3.2.7. Construction of siRNA vectors, cell culture and transfections.....	61
3.2.8. RT-PCR and Western analysis.....	62
3.2.9. Labelling of transferrin with Fe	65
3.2.10. Iron uptake assay.....	65
3.2.11. In vitro cell proliferation and migration assays	65
3.2.12. Tumour biology in nude mice.....	66
3.2.13. Statistical analysis	66
3.3. Results.....	67

3.3.1. Generation of MTf ^{-/-} mice	67
3.3.2. Phenotypic characterisation of MTf ^{-/-} mice	71
3.3.3. MTf expression is not essential for normal Fe metabolism	78
3.3.4. Comparative data analysis of the differential gene expression between MTf ^{+/+} and MTf ^{-/-} mice	83
3.3.5. Down-regulation of MTf expression in SK-Mel-28 cells by PTGS	87
3.3.6. Addition of Fe, apo-MTf or holo-MTf does not rescue depressed proliferation in melanoma cells with decreased MTf expression	92
3.3.7. Inhibition of MTf expression decreases cell migration	95
3.3.8. Inhibition of MTf expression by PTGS results in depressed tumour growth in nude mice	97
3.4. Discussion	99

CHAPTER 4: IDENTIFICATION OF DISTINCT CHANGES IN GENE EXPRESSION AFTER MODULATION OF MELANOMA TUMOUR ANTIGEN p97 (MELANOTRANSFERRIN) IN MULTIPLE MODELS <i>IN VITRO</i> AND <i>IN VIVO</i>	106
4.1. Introduction	107
4.2. Materials and Methods	110
4.2.1. Construction of vectors	110
4.2.2. Cell culture, transfection and proliferation assay	110
4.2.3. Animals	111
4.2.4. RNA isolation, RT-PCR and Western analysis	111
4.2.5. Microarray processing	113
4.2.6. Microarray data analysis	113
4.2.7. Statistical analysis	114
4.3. Results	118
4.3.1. Generation of a melanoma cell model of MTf down-regulation	118
4.3.2. Characterisation of cellular models hyper-expressing MTf	122

4.3.3. Identification of new target genes associated with MTF and their potential biological significance	123
4.3.4. Validation of gene expression identified in the microarrays using RT-PCR	128
4.4. Discussion	134
4.4.1. Tcf4 and Thtpa	136
4.4.2. Abcb5	137
4.4.3. Glis, Mef2a and Ptpdc1	138

CHAPTER 5: GENERATION AND CHARACTERISATION OF MELANOTRANSFERRIN TRANSGENIC MICE: IDENTIFICATION OF A MILD HAEMATOLOGICAL PHENOTYPE	140
5.1. Introduction	141
5.2. Materials and Methods	145
5.2.1. Animals	145
5.2.2. Plasmid construction and generation of MTF ^{Tg} transgenic mice.....	145
5.2.3. Verification of the MTF transgene at the genomic, mRNA and protein level	147
5.2.4. Determination of haematological and serum chemistry indices	149
5.2.5. Histopathological analysis	149
5.2.6. Total tissue iron, copper and zinc determinations.....	149
5.2.7. Statistical analysis	149
5.3. Results	150
5.3.1. Generation of MTF ^{Tg} mice.....	150
5.3.2. Assessment of MTF transgene hyper-expression at the mRNA and protein levels	153
5.3.3. Analysis of reproductive capabilities of MTF ^{Tg} mice.....	156
5.3.4. Offspring analysis from heterozygous breeding pairs	158
5.3.5. Body weights and relative organ weights of MTF ^{Tg} mice.....	158
5.3.6. Haematological and biochemical parameters in the blood of MTF ^{Tg} mice	161

5.3.7. Histopathological findings in tissue sections from M Tf ^{Tg} mice	164
5.3.8. M Tf hyper-expression does not affect normal Fe metabolism	164
5.4. Discussion	166

CHAPTER 6: GENERAL CONCLUSIONS AND FUTURE PROJECT

DIRECTIONS	170
6.1. Prelude.....	171
6.2. General conclusions of <i>in vivo</i> studies: M Tf knockout and transgenic mice.....	171
6.2.1. M Tf does not play a role in Fe transport and metabolism	171
6.2.2. Melanotransferrin and its role in melanoma tumourigenesis.....	174
6.2.3. Hyper-expression of melanotransferrin leads to a mild haematological phenotype	174
6.2.4. Future studies utilising the M Tf ^{-/-} and M Tf ^{Tg} mice.....	175
6.2.4.1. Generation and characterisation of double knockouts	175
6.2.4.2. Phenotypic characterisation of the homozygous <i>MTf</i> ^{Tg} mouse	176
6.2.4.3. Other iron-depletion strategies.....	176
6.2.4.4. Immunological investigations	177
6.2.4.5. Examination of the role of M Tf in wound repair	178
6.2.4.6. Further assessment of the role of M Tf in tumour biology	180
6.2.4.7. Identification of M Tf associated key molecules and pathways	182
6.3. Modulation of melanotransferrin expression <i>in vitro</i>	183
6.3.1. Melanotransferrin and its role in cellular proliferation and migration.....	183
6.3.2. Future studies examining the effects of modulating the melanotransferrin expression in vitro models	184
6.4. Changes in gene expression affected by melanotransferrin.....	186
6.4.1. Molecular targets directly or indirectly regulated by melanotransferrin expression.....	186
6.4.2. Future directions for assessing melanotransferrin molecular pathways	188

6.5. Conclusions	189
CHAPTER 7: BIBLIOGRAPHY	190

ACKNOWLEDGEMENTS

First, I wish to thank my supervisor, Professor Des R Richardson, for the opportunity he offered me to undertake my PhD project in the Iron Metabolism and Chelation Program. I am very grateful for his care and endless encouragement, expert guidance and for providing me with an enthusiastic and critical atmosphere for this exciting project. I would also like to give my sincere gratitude to Des for his support in the preparation of manuscripts and for the opportunity to present my work at local and international conferences.

Thanks to my co-supervisor, Dr. Eric O. Sekyere, for sharing his time in this study, his knowledge on molecular techniques and the creation of the MTF knockout mouse. Thank you as well for his assistance with the transgenic MTF hyper-expression model. I must also acknowledge my colleague, Miss Louise Dunn, for sharing the “Ph.D. experience”. I would also like to acknowledge the help and expertise that Louise provided in the various animal and molecular techniques/methods used in this study, particularly: (i) for the help in characterising the MTF knockout mouse and (ii) the development and phenotypic characterisation of siRNA-mediated MTF down-regulation in SK-Mel-28 and SK-Mel-2 cell line models.

Special thanks also go to fellow Ph.D. candidates in the Iron Metabolism and Chelation Program, Miss Danuta S. Kalinowski and Mr. Xiangcong Xu, for their friendship and encouragement. I also acknowledge all the other members at Iron Metabolism and Chelation Program whom I interacted with, especially Dr. David Lovejoy, Dr. Dong Fu,

Dr. Jonathan Howard, Dr. Ralph Watts, Miss Rosemary Siafakas and Miss Megan L. Whitnall for the advice and help during my candidature.

The following people's expertise assisted in the characterisation of the *MTf* knockout mouse, namely: Veterinarian Dr. Susan Maastricht, Veterinary Pathologists Dr. Terry Rothwell and Dr. JoAnn Schuh, and the wonderful technical staff of the Faculty of Veterinary Science at the University of Sydney. This includes: David Griffin, George Tsoulakis and Elaine Chew. Dr. Mervyn Thomas and Kelly Fleetwood (Emphron Informatics) are thanked for their assistance with statistical analysis of the gene array. Mrs. Rabeya Akter (University of New South Wales) is kindly acknowledged for the measurement of Fe, Cu and Zn levels using Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES). Dr. Frank Koentgen and Mr. Eric Hardy from Ozgene Pty. Ltd. are thanked for the help and development of both *MTf* knockout and transgenic hyper-expression models.

I would like to acknowledge the Department of Pathology and its entire staff for a wonderful environment to work and it has really been a joy to spend time with them. I would like to acknowledge funding from the NHMRC for Project Grant support. I also thank the University of Sydney and Children's Cancer Institute Australia for the Australian Postgraduate Award scholarship and financial assistance.

I am very grateful for my girlfriend, Yu Yu, for her patience, kindness and emotional and loving support during the last year of my Ph.D. studies.

Finally, and most importantly, to my family: Dad (Suryo Yusuf), Mum (Renny Rahmanto) and brothers (Aldwin and Bryan), I really couldn't have done this without your endless vision, encouragement, emotional and loving support and your willingness to be there whenever I have needed you.

ABSTRACT

Melanotransferrin or melanoma tumour antigen p97 (MTf) is a transferrin homologue that is found predominantly bound to the cell membrane *via* a glycosyl phosphatidylinositol anchor. The molecule is a member of the transferrin super-family that binds iron through a single high affinity iron(III)-binding site. Melanotransferrin was originally identified at high levels in melanoma cells and other tumours, but at lower levels in normal tissues. Since its discovery, the function of MTf has remained intriguing, particularly regarding its role in cancer cell iron transport. In fact, considering the crucial role of iron in many metabolic pathways *e.g.*, DNA and haem synthesis, it is important to understand the function of melanotransferrin in the transport of this vital nutrient. Melanotransferrin has also been implicated in diverse physiological processes, such as plasminogen activation, angiogenesis, cell migration and eosinophil differentiation. Despite these previous findings, the exact biological and molecular function(s) of MTf remain elusive. Therefore, it was important to investigate the function of this molecule in order to clarify its role in biology.

To define the roles of MTf, six models were developed during this investigation. These included: the first MTf knockout (*MTf*^{-/-}) mouse; down-regulation of MTf expression by post-transcriptional gene silencing (PTGS) in SK-Mel-28 and SK-Mel-2 melanoma cells; hyper-expression of MTf expression in SK-N-MC neuroepithelioma cells and LMTK⁻ fibroblasts cells; and a MTf transgenic mouse (*MTf*^{Tg}) with MTf hyper-expression.

The *MTf*^{-/-} mouse was generated through targeted disruption of the *MTf* gene. These animals were viable, fertile and developed normally, with no morphological or histological abnormalities. Assessment of Fe indices, tissue Fe levels, haematology and serum chemistry parameters demonstrated no differences between *MTf*^{-/-} and wild-type (*MTf*^{+/+}) littermates, suggesting MTf was not essential for Fe metabolism. However, microarray analysis showed differential expression of molecules involved in proliferation such as *myocyte enhancer factor 2a* (*Mef2a*), *transcription factor 4* (*Tcf4*), *glutaminase* (*Gls*) and *apolipoprotein d* (*Apod*) in *MTf*^{-/-} mice compared with *MTf*^{+/+} littermates.

Considering the role of MTf in melanoma cells, PTGS was used to down-regulate *MTf* mRNA and protein levels by >90% and >80%, respectively. This resulted in inhibition of cellular proliferation and migration. As found in *MTf*^{-/-} mice, melanoma cells with suppressed MTf expression demonstrated up-regulation of *MEF2A* and *TCF4* in comparison with parental cells. Furthermore, injection of melanoma cells with decreased MTf expression into nude mice resulted in a marked reduction of tumour initiation and growth. This strongly suggested a role for MTf in proliferation and tumourigenesis.

To further understand the function of MTf, a whole-genome microarray analysis was utilised to examine the gene expression profile of five models of modulated MTf expression. These included two stably transfected MTf hyper-expression models (*i.e.*, SK-N-MC neuroepithelioma and LMTK⁻ fibroblasts) and one cell type with down-regulated MTf expression (*i.e.*, SK-Mel-28 melanoma). These findings were then

compared with alterations in gene expression identified using the *MTf*^{-/-} mouse. In addition, the changes identified from the microarray data were also assessed in another model of MTf down-regulation in SK-Mel-2 melanoma cells. In the cell line models, MTf hyper-expression led to increased proliferation, while MTf down-regulation resulted in decreased proliferation. Across all five models of MTf down- and up-regulation, three genes were identified as commonly modulated by MTf. These included *ATP-binding cassette sub-family B member 5 (Abcb5)*, whose change in expression mirrored MTf down- or up-regulation. In addition, *thiamine triphosphatase (Thtpa)* and *Tcf4* were inversely expressed relative to MTf levels across all five models. The products of these three genes are involved in membrane transport, thiamine phosphorylation and proliferation/survival, respectively. Hence, this study identifies novel molecular targets directly or indirectly regulated by MTf and the potential pathways involved in its function, including modulation of proliferation.

To further understand the function of MTf, transgenic mice bearing the *MTf* gene under the control of the human *ubiquitin-c* promoter were generated and characterised. In *MTf*^{Tg} mice, *MTf* mRNA and protein levels were hyper-expressed in a variety of tissues compared with control mice. Similar to the *MTf*^{-/-} mice, these animals exhibited no gross morphological, histological, nor Fe status changes when compared with wild-type littermates. The *MTf*^{Tg} mice were also born in accordance with classical Mendelian ratios. However, haematological data suggested that hyper-expression of MTf leads to a mild, but significant decrease in erythrocyte count.

In conclusion, the investigations described within this thesis clearly demonstrate no essential role for MTF in Fe metabolism both *in vitro* and *in vivo*. In addition, this study generates novel *in vitro* and *in vivo* models for further investigating MTF function. Significantly, the work presented has identified novel role(s) for MTF in cell proliferation, migration and melanoma tumourigenesis.

LIST OF PUBLICATIONS

Publications in support to this thesis:

1. **Suryo Rahmanto, Y.**, Dunn, L.L., and Richardson, D.R. (2007) The melanoma tumor antigen, melanotransferrin (p97): a 25-year hallmark – from iron metabolism to tumorigenesis. *Oncogene*. 26(42):6113-24. **IF 2006: 6.5.**
2. Dunn, L.L., **Suryo Rahmanto, Y.**, and Richardson, D.R. (2007) Iron in the new millenium. *Trends Cell Biol.* 17(2):93-100. **IF 2006: 12.4.**
3. **Suryo Rahmanto, Y.**, Sekyere, E.O., Dunn, L.L., and Richardson, D.R. (2007) The function of the membrane-bound transferrin homologue, melanotransferrin (melanoma tumour antigen p97). In: *Iron Metabolism and Disease*, Chapter 9 (Fuchs, H. ed.). Transworld Research Network. [Invited Book Chapter].
4. Sekyere, E.O., Dunn, L.L., **Suryo Rahmanto, Y.**, and Richardson, D.R. (2006) Role of melanotransferrin in iron metabolism: studies using targeted gene disruption *in vivo*. *Blood*. 107(7):2599-601. **IF 2006: 10.4.**
**Selected by the Editors of Blood as a Plenary Paper which this journal describes as “papers of exceptional scientific importance”.*
5. Dunn, L.L., Sekyere, E.O., **Suryo Rahmanto, Y.**, and Richardson, D.R. (2006) The function of melanotransferrin: a role in melanoma cell proliferation and tumorigenesis. *Carcinogenesis*. 27(11):2157-69. **IF 2006: 5.3.**
6. **Suryo Rahmanto, Y.**, Dunn, L.L., and Richardson, D.R. (2007) Identification of distinct changes in gene expression after modulation of melanoma tumor antigen p97 (melanotransferrin) in multiple models *in vitro* and *in vivo*. *Carcinogenesis*. 28(10):2172-83. **IF 2006: 5.3.**

-
7. **Suryo Rahmanto, Y.**, and Richardson, D.R. (2007) Generation and characterization of transgenic mice hyper-expressing melanoma tumor antigen p97 (melanotransferrin): Identification of a mild hematological phenotype. [To be submitted].

Other publications during my Ph.D. candidature:

8. Richardson, D.R., and **Suryo Rahmanto, Y.** (2007) Differential regulation of the Menkes and Wilson disease copper transporters by hormones: an integrated model of metal transport in the placenta. *Biochem J.* 402(2):e1-3. **IF 2006: 4.1.**
9. Davies, N.P., **Suryo Rahmanto, Y.**, Chitambar, C.R., and Richardson, D.R. (2006) Resistance to the anti-neoplastic agent gallium nitrate results in marked alterations in intracellular iron and gallium trafficking: identification of novel intermediates. *J Pharmacol Exp Ther.* 317(1):153-62. **IF 2006: 3.9.**
10. Whitnall, M.L., **Suryo Rahmanto, Y.**, Xu, X., Sutak, R., Koenig, M., Puccio, M., Ponka, P., and Richardson, D.R. (2007) Reversal of iron loading in the frataxin knockout mouse (MCK) and the elucidation of molecular pathways involved in frataxin function. [Manuscript in preparation].

LIST OF AWARDS

1. *Australian Postgraduate Award Research Scholarship (2006-2007) University of Sydney*. Sydney, Australia.
2. *Postgraduate Research Support Scheme, Travel Scholarship (2007) Department of Pathology, University of Sydney*. Sydney, Australia. AU\$ 852.
3. *Competitive Fellowship for Young Investigators (2006) The 13th Biennial Congress of the International Society of Free Radical Research*. Davos, Switzerland. US\$ 1,000.
4. *Competitive Travel Award (2006) Society for Free Radical Research (Australasia)*. Sydney, Australia. AU\$ 2,000.
5. *Postgraduate Research Support Scheme, Travel Scholarship (2006) Department of Pathology, University of Sydney*. Sydney, Australia. AU\$ 429.
6. *Competitive Young Investigator Award (2004), The 13th Annual Conference of the Society for Free Radical Research (Australasia)*. Christchurch, New Zealand. AU\$ 1,000.
7. *Australia Postgraduate Supplementary Award (2004-2005) Children's Cancer Institute Australia for Medical Research*. Sydney, Australia. AU\$ 5,200 per annum.

LIST OF INVITED PRESENTATIONS

1. **Suryo Rahmanto, Y.**, and Richardson, D.R. (2008) Melanoma tumor antigen p97 (melanotransferrin): a role in melanoma cell proliferation and tumorigenesis. *1st International Conference Recent Advances in Health and Medical Sciences*. Paphos, Cyprus. March 7-12, 2008.
2. **Suryo Rahmanto, Y.**, and Richardson, D.R. (2007) Melanoma tumor antigen p97 (melanotransferrin): a role in melanoma cell proliferation and tumorigenesis. *4th Joint Meeting of the Society for Free Radical Research Australasia and Japan*. Kyoto, Japan. December 1-5, 2007.
3. **Suryo Rahmanto, Y.**, Dunn, L.L., and Richardson, D.R. (2006) Identification of differential gene expression after modulation of melanoma tumour antigen p97 (melanotransferrin). *The Bosch Institute Young Investigator Symposium*. Sydney, Australia. December 15, 2006.
4. **Suryo Rahmanto, Y.**, Dunn, L.L., and Richardson, D.R. (2006) The melanoma tumour antigen, melanotransferrin: in vivo and in vitro studies – a shift from iron metabolism to tumorigenesis. *The Australian Health and Medical Research Congress*. Melbourne, Australia. November 26 – December 1, 2006.
5. Dunn, L.L., **Suryo Rahmanto, Y.**, and Richardson, D.R. (2006) To be or not to be: the melanotransferrin knockout mouse and melanoma tumorigenesis. *The Australian Health and Medical Research Congress*. Melbourne, Australia. November 26 – December 1, 2006.
6. **Suryo Rahmanto, Y.**, Dunn, L.L., and Richardson, D.R. (2006) Role of melanotransferrin in tumorigenesis – a gene array study. *Health@Sydney, Health Research Conference 2006 “From Cell to Society 5”*. Leura, Australia. November 9-10, 2006.

-
7. Dunn, L.L., **Suryo Rahmanto, Y.**, and Richardson, D.R. (2006) To be or not to be: the melanotransferrin knockout mouse and melanoma tumourigenesis. *Health@Sydney, Health Research Conference 2006 "From Cell to Society 5"*. Leura, Australia. November 9-10, 2006.
 8. **Suryo Rahmanto, Y.**, Sekyere, E.O., Dunn, L.L., and Richardson, D.R. (2006) Identification of melanotransferrin (p97) associated genes using in vitro and in vivo models. *13th Biennial Congress of the International Society of Free Radical Research*. Davos, Switzerland. August 15-19, 2006.
 9. Dunn, L.L., Sekyere, E.O., **Suryo Rahmanto, Y.**, and Richardson, D.R. (2005) The function of melanotransferrin (tumour antigen p97). *3rd Joint Meeting of the Society for Free Radical Research (Australasia & Japan)*. Gold Coast, Australia. December 2-4, 2005.
 10. Dunn, L., Sekyere, E., **Suryo, Y.**, Gunning, P., and Richardson, D.R. (2005) Function of the melanoma tumour antigen p97 (melanotransferrin): a role in the growth and proliferation of malignant melanoma cells. *ASMR (NSW) Scientific Meeting*. Sydney, Australia. June 4-11, 2005.
 11. Sekyere, E., Dunn, L., **Suryo Rahmanto, Y.**, and Richardson, D.R. (2005) Generation on the melanotransferrin knockout mouse: effect on iron metabolism and homeostasis. *First Congress of the International BioIron Society*. Prague, Czech Republic. May 22-27, 2005.
 12. Sekyere, E.O., Dunn, L.L., **Suryo Rahmanto, Y.**, and Richardson, D.R. (2005) Generation of the melanotransferrin knockout mouse: no effect on iron homeostasis. *The 15th International Conference on Oral Chelation in the*

Treatment of Thalassemia and Other Diseases. Taichung, Taiwan. April 22-26, 2005.

13. Dunn, L.L., **Suryo Rahmanto, Y.**, Sekyere, E.O., and Richardson, D.R. (2004) Effect of melanotransferrin (p97) down-regulation on cell proliferation and migration. *The 13th Annual Conference of the Society for Free Radical Research (Australasia)*. Christchurch, New Zealand. December 3–5, 2004.

ABBREVIATIONS

Abbreviation	Full Text
Abcb5	ATP-binding cassette sub-family B member 5
AD	Alzheimer's disease
ALP	Alkaline phosphatase
ALT	Aspartate aminotransferase
Apod	Apolipoprotein d
AST	Alanine aminotransferase
BBB	Blood brain barrier
Bp	Base pair
BSA	Bovine serum albumin
Btg2	Btg family member 2
Cd44	Cd44 antigen
cDNA	Complementary deoxyribonucleic acid
CDS	Coding region
CHO	Chinese hamster ovary
CK-MB	Creatine kinase
CMV	Cytomegalovirus
Cntn4	Contactin 4
Cu	Copper
Ddr1	Discoidin domain receptor family member 1
DEPC	Diethylpyrocarbonate
Dkk1	Dickkopf homologue 1
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraaceticacid

Abbreviation	Full Text
EOS47	Eosinophil-specific cell surface antigen
ER	Endoplasmic reticulum
ES	Embryonic stem cells
EtOH	Ethanol
FAC	Ferric ammonium citrate
FBS	Foetal bovine serum
Fe	Iron
FGF-2	Fibroblast growth factor-2
Fyb-120/130	Fyb binding protein
G	Gram
G418	Geneticin
G418	Geneticin
Gls	Glutaminase
GPI	Glycosyl phosphatidylinositol
GPI-PLD	GPI-specific phospholipase D
H	Hour
HCl	Hydrochloric acid
HCT	Haematocrit
HGB	Haemoglobin
HGPT	Hypoxanthine guanine phosphoribosyl transferase
hMef2a	Human myocyte enhancer factor 2a
hMTf	Human melanotransferrin
hTcf4	Human transcription factor 4
h Δ MTf	Human short melanotransferrin
IgG	Immunoglobulin G
Il2rg	Interleukin 2 receptor gamma
IRES	Internal ribosome entry site

Abbreviation	Full Text
Kb	Kilobase
kDa	Kilodalton
Lf	Lactoferrin
LRP	Low-density lipoprotein receptor-related protein
LTR	Long terminal repeat
μ	Micro
M	Milli
M	Molar (moles litre-1)
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
Mef2a	Myocyte enhancer factor 2a
MEM	Minimum essential medium
Min	Minute
mMTf	Mouse melanotransferrin
MoAb	Monoclonal antibody
mRNA	Messenger RNA
MTf	Melanotransferrin
<i>MTf^{-/-}</i>	Melanotransferrin knockout mouse
<i>MTf^{+/+}</i>	Melanotransferrin wild-type mouse
<i>MTf^{Tg}</i>	Transgenic mouse hyper-expressed MTf
<i>MTf^{WT}</i>	Melanotransferrin wild-type mouse
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
neo ^f	Neomycin resistance gene cassette
OAS1	2', 5'-oligoadenylate synthetase 1
°C	Degrees celsius

Abbreviation	Full Text
ovoTf	Ovotransferrin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI-PLC	Phosphatidylinositol-specific phospholipase C
PLT	Platelet
PMN	Polymorphonuclear neutrophils
pS	pSilencer 3.1-H1 neo vector
PTGS	Post-transcriptional gene silencing
Ptpdc1	Protein tyrosine phosphatase domain containing 1
RBC	Red blood cell
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT-PCR	Reverse transcription - polymerase chain reaction
S	Second
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
siRNA	Small interference ribonucleic acid
sMTf	Soluble MTf
TBS	Tris buffered saline
Tcf4	Transcription factor 4
Tf	Transferrin
TfR1	Transferrin receptor 1
TfR2	Transferrin receptor 2
Tfrc	Mouse transferrin receptor 1
Thtpa	Thiamine triphosphatase

Abbreviation	Full Text
TIBC	Total iron-binding capacity
tPA	Tissue plasminogen activator
TTBS	TBS containing 0.1% Tween-20
U	International unit of enzyme activity
UbiC	Ubiquitin c
UIBC	Unsaturated iron-binding capacity
uPA	Urokinase-type plasminogen activator
VEGF1	Vascular endothelial growth factor-1
WBC	White blood cell
Zn	Zinc

LIST OF FIGURES

Figure 1.1. The phylogenetic tree of MTfs and the other Tf family members.	8
Figure 1.2. BLAT analysis arising from the human <i>MTf</i> (<i>hMTf</i>) and mouse <i>MTf</i> (<i>mMTf</i>) genes.....	10
Figure 1.3. Amino acid sequence and other motifs in human MTf.	17
Figure 1.4. Mouse mRNA (poly A+) Master Blot™ sequentially hybridised to mouse melanotransferrin (mMTf) cDNA, mouse transferrin receptor 1 (TfR1) cDNA and hypoxanthine guanine phosphoribosyl transferase (HGPT) cDNA (loading control).	20
Figure 1.5. Immunohistochemistry using anti-hMTf MoAb L235 compared with a relevant control without the antibody demonstrating the distribution of hMTf in: (A) skin, (B) liver, (C) kidney and (D) salivary gland.	21
Figure 1.6. Overview of the postulated role of MTf in plasminogen activation and angiogenesis.	30
Figure 3.1. Targeted disruption of the mouse melanotransferrin (<i>mMTf</i>) gene.	68
Figure 3.2. Confirmation of mMTf ablation in the mouse at the genomic DNA, mRNA and protein levels.	69
Figure 3.3. Examination of growth rates, organ to body weight ratios and brain Fe levels of <i>MTf</i> ^{-/-} compared with <i>MTf</i> ^{+/+} littermates.....	72
Figure 3.4. Comparative data analysis and RT-PCR of genes showing differential expression between <i>MTf</i> ^{-/-} and <i>MTf</i> ^{+/+} mice.	86
Figure 3.5. Post-transcriptional gene silencing (PTGS) of MTf in melanoma cells leads to decreased <i>MTf</i> mRNA and protein levels.	88

Figure 3.6. Post-transcriptional gene silencing (PTGS) of MTf in melanoma cells leads to a significant decrease in cellular proliferation and DNA synthesis.	91
Figure 3.7. Addition of Fe, soluble apo-MTf or soluble holo-MTf does not rescue decreased melanoma cell proliferation induced by MTf down-regulation.	93
Figure 3.8. Down-regulation of MTf expression reduces SK-Mel-28 melanoma cell migration <i>in vitro</i>	96
Figure 3.9. Down-regulation of MTf expression decreases tumour growth <i>in vivo</i>	98
Figure 4.1. The effect of MTf down-regulation or hyper-expression on proliferation across a variety of cell lines.	120
Figure 4.2. The effect of MTf expression on cellular proliferation.	121
Figure 4.3. Biological processes associated with differentially expressed genes.	125
Figure 4.4. Specific biological processes associated with down- and up-regulated genes.	126
Figure 4.5. RT-PCR analysis of genes showing differential expression across four different MTf models assessed using Affymetrix GeneChips [®]	129
Figure 4.6. Comparative data analysis of genes showing differential expression between MTf down-regulation/ablation and hyper-expression compared with controls.	132
Figure 5.1. Schematic diagram and verification of the <i>melanotransferrin</i> (<i>MTf</i>) lentiviral transgene.	152
Figure 5.2. MTf hyper-expression in the mouse at the mRNA and protein levels.	155
Figure 5.3. Post-natal growth rate and relative organ weights of the <i>MTf^{Tg}</i> and <i>MTf^{WT}</i> mice.	160

Figure 6.1. The MTF knockout mouse and gene array studies indicate that MTF has no essential role in iron metabolism, but could be necessary for cellular growth and proliferation. 173

LIST OF TABLES

Table 1.1. Comparison of known MTf gene and protein sequences between species.....	6
Table 1.2. Comparison of the Fe- and anion-coordinating amino acids present in MTfs and other Tf homologues.	15
Table 2.1. Chemicals and general reagents.....	39
Table 2.2. Cell culture reagents	41
Table 2.3. Buffers and solutions	42
Table 3.1. Primers for amplification of mouse and human mRNA.	64
Table 3.2. Haematological indices in <i>MTf^{-/-}</i> mice compared with their <i>MTf^{+/+}</i> littermates at 12 weeks of age.	75
Table 3.3. Selected serum chemistry indices in <i>MTf^{-/-}</i> mice compared with their <i>MTf^{+/+}</i> littermates at 12 weeks of age.	76
Table 3.4. Differential cell counts in <i>MTf^{-/-}</i> mice compared with their <i>MTf^{+/+}</i> littermates at 12 weeks of age.	77
Table 3.5. Serum Fe indices in <i>MTf^{-/-}</i> mice compared with their <i>MTf^{+/+}</i> littermates at 12 weeks of age.....	79
Table 3.6. Tissue Fe stores in <i>MTf^{-/-}</i> mice compared with their <i>MTf^{+/+}</i> littermates at 12 weeks of age.....	79
Table 3.7. Tissue copper (Cu) stores in <i>MTf^{-/-}</i> mice compared with their <i>MTf^{+/+}</i> littermates at 12 weeks of age.	80
Table 3.8. Tissue zinc (Zn) stores in <i>MTf^{-/-}</i> mice compared with their <i>MTf^{+/+}</i> littermates at 12 weeks of age.	80
Table 3.9. Tissue-Fe stores in <i>MTf^{-/-}</i> mice compared with their <i>MTf^{+/+}</i> littermates at 12 weeks of age on basal-Fe (0.02% w/w) and high-Fe (2.00% w/w) diets....	82

Table 3.10. The 30 genes showing the most significant differential expression between <i>MTf</i> ^{-/-} and <i>MTf</i> ^{+/+} genotypes.	84
Table 4.1. Primers for amplification of mouse and human mRNA	112
Table 4.2. Summary of differential gene expression across all models assessed using Affymetrix GeneChips®	115
Table 5.1. Primers for genotyping and amplification of mouse mRNA.	148
Table 5.2. Offspring from multiple matings [§]	157
Table 5.3. Differential blood count and haematological indices in <i>MTf</i> ^{Tg} mice compared with their <i>MTf</i> ^{WT} littermates at 8 weeks of age [§]	162
Table 5.4. Serum clinical chemistry indices in <i>MTf</i> ^{Tg} mice compared with their <i>MTf</i> ^{WT} littermates at 8 weeks of age [§]	163
Table 5.5. Selected metal stores in the organs of <i>MTf</i> ^{Tg} mice compared with their <i>MTf</i> ^{WT} littermates at 8 weeks of age [§]	165
Table 6.1. Summary of the functional roles of genes affected by modulation of melanotransferrin gene expression.	187
