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THE PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ROLES OF MELANOTRANSFERRIN

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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

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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.**
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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].

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LIST OF INVITED PRESENTATIONS

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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 tumourigenesis. ***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 tumourigenesis. ***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 tumourigenesis – 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
hΔMTf	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
MTf$^{-/-}$	Melanotransferrin knockout mouse
MTf$^{+/+}$	Melanotransferrin wild-type mouse
MTf^{Tg}	Transgenic mouse hyper-expressed MTf
MTf^{WT}	Melanotransferrin wild-type mouse
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
neo^{r}	Neomycin resistance gene cassette
OAS1	2', 5'-oligoadenylate synthetase 1
^{o}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

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