CHAPTER 6: GENERAL

CONCLUSIONS AND FUTURE

PROJECT DIRECTIONS

“Once you eliminate the impossible, whatever remains, no matter how improbable, must be the truth.”

Sherlock Holmes by Sir Arthur Conan Doyle, 1859-1930
6.1. Prelude

The work presented in this thesis examined the function(s) of MTf using a variety of newly generated in vitro and in vivo models. Indeed, studies using a knockout mouse and post-transcriptional gene silencing have demonstrated MTf does not have an essential function in Fe metabolism (Chapter 3; [Dunn et al. 2006; Sekyere et al. 2006]). However, the results reported herein show that MTf does play a role in melanoma cell proliferation, migration and tumourigenesis (Chapter 3; [Dunn et al. 2006; Sekyere et al. 2006]). This has been recently verified in a subsequent study by others [Bertrand et al. 2007]. In Chapter 4, using microarray technology, novel signalling pathways were identified through which MTf may act to modulate cellular proliferation [Suryo Rahmanto et al. 2007b]. Finally, Chapter 5 demonstrated that MTf does not have an essential function in Fe metabolism using a transgenic mouse model hyper-expressing MTf (MTf$^{Tg}$). Haematological data from the MTf$^{Tg}$ mouse suggested that hyper-expression of MTf leads to a mild, but significant, decrease in erythrocyte count.

In addition to clarifying MTf’s biological role, the studies reported in this thesis have provided multiple novel in vitro and in vivo models which set a clear direction for future investigation of MTf function.

6.2. General conclusions of in vivo studies: MTf knockout and transgenic mice

6.2.1. MTf does not play a role in Fe transport and metabolism

The resulting data from the characterisation of a MTf$^{-/-}$ mouse showed that these mice were viable, fertile and developed normally, with no morphological or histological
abnormalities (Chapter 3; [Dunn et al. 2006; Sekyere et al. 2006]). Direct assessment of tissue-Fe levels, haematology and serum Fe parameters demonstrated no differences between $MTf^{-/-}$ and $MTf^{+/+}$ mice, suggesting MTf was not essential for Fe homeostasis and metabolism [Dunn et al. 2006; Sekyere et al. 2006]. Furthermore, Fe status was indirectly measured in $MTf^{-/-}$ mice by examining the Fe-regulated gene, $TfR1$ [Hentze & Kuhn 1996; Richardson & Ponka 1997], at the mRNA and protein levels and was found to be the same when compared with wild-type counterparts ($MTf^{+/+}$; [Sekyere et al. 2006]). Hence, ablation of $MTf$ expression does not affect Fe pools that control $TfR1$ expression, nor were there any marked alterations in the expression of other genes associated with Fe metabolism [Dunn et al. 2006].

The absence of a phenotype and particularly any neurological changes in the $MTf^{-/-}$ mice, question the suggestion that MTf plays a significant role in Fe transport across the blood brain barrier [Moroo et al. 2003]. Results obtained from the $MTf^{-/-}$ mouse support other studies demonstrating that brain Fe uptake from MTf is not appreciable when compared with that from serum Tf [Richardson & Morgan 2004]. In fact, recent studies have demonstrated that transcytosis of sMTf across the mouse blood brain barrier is not significant [Pan et al. 2004; Richardson & Morgan 2004], which is in contrast to earlier work by others [Demeule et al. 2002; Demeule et al. 2003]. Hence, serum Tf but not sMTf, is the primary source of Fe for the brain, in accordance with earlier literature [Beard et al. 1993; Morgan 1981].

Collectively, a wide variety of studies in vitro and in vivo have demonstrated that MTf does not play a role in Fe transport and metabolism (Figure 6.1), with most Fe being
assimilated through the binding of Tf to the Tfr1 [Dunn et al. 2006; Richardson & Baker 1991a; Richardson & Baker 1990; Richardson & Baker 1991b; Richardson 2000; Richardson & Morgan 2004; Sekyere et al. 2006].

**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.

Although MTf is a Tf homologue that binds Fe with high affinity, the MTf$^{-/-}$ mouse exhibits no phenotypic changes that are typical of alterations of Fe metabolism [Sekyere et al. 2006]. In addition, there is no change in the expression of genes associated with Fe metabolism nor homeostasis, suggesting that MTf has other roles [Dunn et al. 2006]. Gene array studies on the MTf$^{-/-}$ mouse compared with wild-type littermates indicate that the genes, *myocyte enhancer factor 2a* (*Mef2a*), *transcription factor 4* (*Tcf4*) and *glutaminase* (*Gls*) were up-regulated, while the *apolipoprotein d* (*Apod*) gene was down-regulated [Dunn et al. 2006]. The products of these genes are known to have roles in cell survival, differentiation and proliferation. While their exact interactions with MTf remain unclear, these changes in gene expression could be compensating for loss and/or gain of function induced by MTf ablation. Adapted from [Suryo Rahmanto et al. 2007c].
6.2.2. Melanotransferrin and its role in melanoma tumourigenesis

Although there was no obvious phenotype in the $MTf^{-/-}$ mouse [Dunn et al. 2006; Sekyere et al. 2006], the function of MTf may only become apparent in cell types where it is highly expressed, such as melanoma [Brown et al. 1981a; Woodbury et al. 1980]. Recently, studies were performed in order to understand the function of MTf using two different models, namely the $MTf^{-/-}$ mouse and post-transcriptional gene silencing in the melanoma cell line, SK-Mel-28 [Dunn et al. 2006].

Microarray analysis was carried out on brain tissues from $MTf^{-/-}$ and $MTf^{+/+}$ littermates [Dunn et al. 2006]. Following genome wide microarray screening, semi-quantitative RT-PCR analysis confirmed that the genes: *myocyte enhancer factor-2a* (*Mef2a*), *transcription factor 4* (*Tcf4*) and *glutaminase* (*Gls*) were up-regulated, while the *apolipoprotein d* (*Apod*) gene was down-regulated compared with their wild-type littermates (Figure 1.4; [Dunn et al. 2006]). The products of these genes have roles in transcription, differentiation and development, regulation of cellular metabolism, transport, proliferation and cell adhesion [Black & Olson 1998; Naya et al. 2002; Zhao et al. 1999]. Hence, the regulation of these genes in the $MTf^{-/-}$ mouse compared with the wild-type littermates indicates a direct or indirect response to gene deletion and suggests a role for MTf in proliferation (Figure 6.1).

6.2.3. Hyper-expression of melanotransferrin leads to a mild haematological phenotype

Similar to the $MTf^{-/-}$ mice, the $MTf^{Tg}$ mice were born healthy and can breed normally. The $MTf^{Tg}$ mice did not exhibit any obvious phenotype and remained normal for over
18 months with no abnormalities. Histological assessment also demonstrated no significant differences between MTf\textsuperscript{Tg} and MTf\textsuperscript{WT} mice. Thus, clearly, MTf hyperexpression is not lethal. In the heterozygous MTf\textsuperscript{Tg} mice, we observed a mild haematological phenotype consisting of decreased haemoglobin concentration, decreased haematocrit and significantly (p<0.05) decreased RBC counts (Table 5.3). The mild but significant decrease of erythrocyte number in MTf\textsuperscript{Tg} mice (Table 5.3) suggested MTf hyper-expression resulted in slight alterations of erythropoiesis and/or erythrocyte turnover. However, there was no increase in splenic Fe in MTf\textsuperscript{Tg} mice and the mechanism responsible for the decreased erythrocyte count remains unclear.

6.2.4. Future studies utilising the MTf\textsuperscript{-/-} and MTf\textsuperscript{Tg} mice

6.2.4.1. Generation and characterisation of double knockouts

Other prospective studies for the MTf\textsuperscript{-/-} mouse include inter-crossing with different transgenic strains to examine possible redundancy and compensatory mechanisms. For instance, the Lf knockout (Lf\textsuperscript{-/-}) mouse generated by Ward and colleagues displayed a minimal phenotype when assessed for perturbations in Fe metabolism [Ward et al. 2003]. In terms of evolution, Lf is a relatively new member of the Tf family of proteins being found only in placental mammals, whereas MTf is a relatively ancient molecule found across a range of organisms [Lambert et al. 2005a]. As no studies have been published to the contrary, it could be suggested that MTf or other members of the Tf super-family may compensate for loss of Lf function in the Lf\textsuperscript{-/-} mouse [Ward et al. 2003]. Indeed, the high homology shared by the primary members of the Tf super-family [Lambert et al. 2005a; Lambert et al. 2005b] supports the possibility of redundant functions between these proteins. Even though MTf is typically membrane-
bound and Lf is not, a double-cross of the MTf\(^{-/-}\) mouse and Lf\(^{-/-}\) mouse may be the only way to elucidate the function of these proteins in relation to Fe metabolism and immune function. However, it is of interest to note that there was no increase in expression of Lf in our gene array studies modulating MTf gene expression. Hence, the relationship of these Tf homologues remains unclear [Dunn et al. 2007].

6.2.4.2. Phenotypic characterisation of the homozygous MTf\(^{Tg}\) mouse

MTf hyper-expression is strongly associated with melanoma and characterisation of homozygous MTf\(^{Tg}\) mice that hyper-express MTf would provide vital new clues to its function. Clearly, the hyper-expression of MTf in the transgenic mouse is not lethal (see Chapter 5). However, in the heterozygous MTf\(^{Tg}\) mouse line, a mild haematological phenotype was observed. It is anticipated that this phenotype would be more pronounced in the homozygous MTf\(^{Tg}\) mouse. Therefore, a thorough analysis of the phenotype (as per Chapters 3 and 5) in this homozygous MTf\(^{Tg}\) mouse should be performed, so that any potential MTf functions are not ignored. Such studies involving the intercross of the MTf\(^{Tg}\) heterozygous mice are currently in progress and should provide new information related to MTf function.

6.2.4.3. Other iron-depletion strategies

Although MTf was not found to play any essential role in Fe metabolism under the conditions studied, it is feasible that the molecule could be involved in other aspects of Fe processing which were not explored. For instance, investigations that would complement the present study include the response of MTf\(^{-/-}\) and MTf\(^{Tg}\) mice to
anaemia induced by phlebotomy [Taconic 2004] or phenylhydrazine treatment [Long 1926].

6.2.4.4. Immunological investigations

An avenue which remains to be comprehensively explored in both the $MTf^{-/-}$ and $MTf^{Tg}$ mice is the role of MTf in immunological responses. Although studies over 18 months in the longevity cohort indicated no changes in the incidence of infection or cancer between the knockout, transgenic and their wild-type littermates, the effect of various immunological stressors were not examined as part of the present study due to time limitations. Previous studies using other knockout animals have clearly shown that a phenotype does not emerge until they are immunologically challenged [Flo et al. 2004]. For instance, the lipocalin-2 knockout mouse does not display a phenotype until it is challenged with enterochelin-generating *Escherichia coli* [Flo et al. 2004].

Considering this, a function for MTf may only become apparent when mice are immunologically challenged. These studies are vital to address in $MTf^{-/-}$ and $MTf^{Tg}$ mice since MTf binds Fe [Baker et al. 1992], which is a vital nutrient for bacterial growth [Flo et al. 2004]. Like Lf, MTf could be a participant in the Fe-depletion strategy of the innate immune system [Legrand et al. 2005]. Perhaps MTf may bind excess labile Fe pool as the need arises, reducing its bioavailability to bacteria and parasites. Alternatively, GPI-anchored MTf may induce immune activation or signalling cascades, since many GPI-anchored molecules are involved in these processes [Brown & Waneck 1992; Brown 1993; Loertscher & Lavery 2002].
The first step in any study examining the immunological roles of MTf would be to examine whether there are any phenotypic differences between $\text{MTf}^{-/-}$, $\text{MTf}^{Tg}$ and their wild-type littermates in their response to viruses, bacteria, yeasts and parasitic infections [Crotty et al. 2003; Schaible et al. 2002; Vonk et al. 2002]. In addition to gross observation, a whole body haematological and histological screen would also need to be performed with a particular focus on lymphoid tissue. Any differences could then be investigated and confirmed at the molecular level. For instance, to measure the bacterial burden, blood samples and homogenised liver and spleen would be plated on LB agar to determine bacterial colony forming units. Blood samples would be used for RNA and protein extraction. The measurements of known mRNA and proteins that are involve in immunological response(s) could then be assayed (e.g., IL-6 [Franceschi et al. 2005], TLR-6 [Takeuchi et al. 1999] and NF-κB [Chien & Hammarskjold 2000]). Tissue samples would also be fixed for immunohistochemistry staining, to examine change in the expression or distribution of MTf and other antigens that are associated with the immunological response to infection.

6.2.4.5. Examination of the role of MTf in wound repair

Down-regulation of MTf expression in the SK-Mel-28 melanoma cell line was shown to result in decreased cell proliferation and migration in vitro [Dunn et al. 2006] and also reduced melanoma tumour initiation, tumour xenograft growth, tumour invasiveness and metastasis in vivo [Bertrand et al. 2007; Dunn et al. 2006]. In light of these findings, it could be suggested that MTf may be involved in wound repair. Indeed, this is supported by the fact that MTf is: (i) highly expressed in the epidermis [Sekyere et al.
2005], (ii) assists in endothelial cell migration and angiogenesis [Demeule et al. 2003; Sala et al. 2002] and (iii) plays a role in plasminogen activation [Bertrand et al. 2007].

Initially, to examine the role of MTf in wound repair, cell proliferation and cell migration, studies could be performed in vitro. This would entail deriving primary murine embryonic fibroblast cultures from embryonic day 13.5 MTf^{−/−}, MTf^{Tg} and wild-type littermate mouse embryos [Kopecki et al. 2007]. The proliferative rates of these primary cell cultures could then be assessed using the procedures described in Chapters 3 and 4. In addition, cell migration assays could be performed utilising a transwell chamber [Demeule et al. 2003; Dunn et al. 2006]. Finally, the primary murine embryonic fibroblast cultures could be grown to confluence and wound scrape assays performed to assess the rate of wound closure in vitro [Dunn et al. 2006]. These studies would clearly demonstrate the role of MTf in proliferation and migration in vitro using primary murine embryonic fibroblast cultures from the embryos of MTf^{−/−} and MTf^{Tg} animal models.

To complement these in vitro investigations, the difference in wound repair could be assessed in vivo using MTf^{−/−}, MTf^{Tg} and their wild-type control littermates. Briefly, mice would be anaesthetised, shaved and wounded. The wound would be made by 2 equi-distant 1 cm full-thickness incisions through the skin and panniculus carnosus on the flanks extending 3.5-4.5 cm from the skull, 1 cm on either side of the spine [Cowin et al. 2006; Kopecki et al. 2007]. Wounds would be left to heal without suturing. The wound area and gap across the wound mid-section would then be visually observed and measured with callipers at various time points over a two week period. The wounds
could be harvested at each time point and fixed in 10% formalin for histological assessment with haematoxylin and eosin, Masson’s Trichrome stain for collagen or immunohistochemical assessment. The latter evaluation could involve examination of the proliferating nuclear antigen that is associated with dermal cell proliferation, as well as assessment of type I collagen [Kopecki et al. 2007], epidermal growth factor [Kwon et al. 2006], fibroblast growth factor-binding protein [Kurtz et al. 2004] and vascular endothelial growth factor-1 [Rossiter et al. 2004]. Furthermore, harvested wounds could also be utilised to examine any change in the mRNA or protein expression of these latter molecules that are involved in wound repair [Andreasen et al. 2000; Lund et al. 2006]. Collectively, these studies could determine the role MTf plays in proliferation and wound healing in vivo using the novel MTf<sup>−/−</sup> and MTf<sup>Tg</sup> mouse models.

6.2.4.6. Further assessment of the role of MTf in tumour biology

Another approach to understand MTf function could involve crosses between the MTf<sup>−/−</sup> and MTf<sup>Tg</sup> mice with transgenic models of melanoma tumour biology. The development of melanoma in mice, spontaneous or induced, is not easily achieved, as exposure to UV light leads to a host of other skin cancer types not only melanoma [Gallagher et al. 1984]. Recently, knockout mice models have been developed which target the gene cyclin-dependent kinase 2A (CDK2A), which encodes two gene products, namely p16<sup>INK4a</sup> and p19<sup>ARF</sup> [Krimpenfort et al. 2001; You et al. 2002]. Mice deficient in p16<sup>INK4a</sup> and at least one allele of p19<sup>ARF</sup> (Ink4a<sup>−/−</sup>Δ) spontaneously develop a range of lesions varying from benign tumours to metastatic melanoma that is clearly relevant to human melanoma [Orlow et al. 2007; Ruas & Peters 1998; You et al. 2002]. Indeed, mutations in CDK2A are well known to be associated with human
melanoma [Orlow et al. 2007]. In these studies, $MTf^{-/-}$ and $MTf^{Tg}$ mice could be crossed with $Ink4a^{+/\Delta2,3}$ mice to generate two heterozygote chimeras ($MTf^{-/-}$ $Ink4a^{+/\Delta2,3}$ and $MTf^{Tg}$ $Ink4a^{+/\Delta2,3}$) and their wild-type control littermates.

Studies in $Ink4a^{+/\Delta2,3}$ mice have shown these animals die between 7-17 months from spontaneous tumour development [Krimpenfort et al. 2001]. The tumours are in the skin (e.g., melanoma) and internal organs (e.g., lung etc). Potentially, MTf may markedly affect tumour growth and invasiveness [Bertrand et al. 2007; Dunn et al. 2006]. Considering this, mice could be examined weekly for tumours by palpation, weighing the animal (weight loss is an indicator of cancer-induced cauchexia), observation of respiratory patterns (altered breathing may indicate lung tumours) and general body condition. Initially, we could sacrifice mice at 3, 6 and 9 months of age and compare tumour incidence type between chimeras and their wild-type control littermates. Confirmation of tumour types would be performed by histological assessment.

The use of chemical carcinogens could also be investigated, such as 7,12-dimethylbenzathracene (DMBA), which has been shown to induce melanoma tumours when applied to the skin of the $Ink4a^{+/\Delta2,3}$ mouse [Krimpenfort et al. 2001]. This carcinogen results in 50% of mice developing cutaneous melanocytic tumours within 3-9 months of age [Krimpenfort et al. 2001]. DMBA would be applied to $MTf^{-/-}$, $MTf^{Tg}$ and their wild-type littermates. Similarly, tumour formation, incidence and type would be compared to those with $Ink4a^{+/\Delta2,3}$ at 3, 6 and 9 months of age. Tumour growth would be assessed using Vernier callipers [Balsari et al. 2004].
A more thorough assessment of the role of MTf in the immune response and tumour biology is also warranted. It would be interesting to examine the effect of the MTf knockout and hyper-expression mouse models on the tumour biology of the B16F10 mouse melanoma that has endogenous MTf expression and is syngeneic for the C57BL/6J strain [Eberting et al. 2004]. Other cell lines could be examined such as the murine Lewis lung carcinoma that is an established model of metastasis in mice and which would function as a non-melanoma model of metastasis [Funakoshi et al. 2000; Hatakawa et al. 2002]. Collectively, these studies may demonstrate a role or pathway for MTf in melanoma tumourigenesis and whether this molecule plays a role in the genesis of other cancers in vivo.

6.2.4.7. Identification of MTf associated key molecules and pathways

To further examine the potential biological role(s) of MTf, microarray analysis would also need to be conducted with brain tissues obtained from MTf$^{Tr}$ and MTf$^{WT}$ littermates using the Affymetrix® chips previously implemented (see Chapters 3 and 4; Dunn et al. 2006; Suryo Rahmanto et al. 2007b). Statistical analysis, based on the “false discovery rate corrected empirical Bayes method” for differential expression, would be performed on the microarray data to identify candidate genes. Additionally, pathway analysis using the Kegg database could also be performed [Dennis et al. 2003; Liu et al. 2003]. This would provide a short list of statistically significant genes involved in MTf function. Confirmation of alterations in expression could be obtained using RT-PCR and Western blot analyses. These data would nicely complement the array data from the MTf$^{-/-}$ mouse and other MTf expression models (see Chapters 3 and 4 [Dunn et al. 2006; Suryo Rahmanto et al. 2007b]).
6.3. Modulation of melanotransferrin expression in vitro

6.3.1. Melanotransferrin and its role in cellular proliferation and migration

In the current study, it was conclusively demonstrated using post-transcriptional gene silencing to down-regulate MTf in melanoma cells, that inhibition of proliferation and cell migration occurred \textit{in vitro}, while melanoma tumourigenesis was suppressed \textit{in vivo} (Chapter 3; [Dunn et al. 2006]). In support of these data, a subsequent independent study by others has demonstrated that siRNA-induced down-regulation of MTf expression in the SK-Mel-28 melanoma cell resulted in reduced invasion and metastasis in mice [Bertrand et al. 2007]. On the other hand, hyper-expression of MTf in LMTK− mouse fibroblasts and SK-N-MC neuroepithelioma cells resulted in increased cell proliferation compared to controls (Chapter 4; [Suryo Rahmanto et al. 2007b]). Indeed, it is notable that previous evidence from hyper-expression studies using melanoma cells suggested increased MTf expression results in accelerated growth [Estin et al. 1989]. These exciting findings defined a role for MTf in melanoma tumourigenesis that was independent of cellular Fe status and indicated that a novel signalling pathway remained to be identified (see Chapters 3 and 5; [Dunn et al. 2006]).

Potentially, the MTf-induced changes in cellular proliferation could be explained by the alterations in gene expression observed in the \textit{MTf}^{−/−} mice compared with \textit{MTf}^{+/+} littermates (see Section 6.2.2). In fact, as found in the \textit{MTf}^{−/−} mouse, \textit{hMef2a} and \textit{hTcf4} were up-regulated in melanoma cells with decreased MTf expression. These transcription factors have important roles in cell signalling, development, cell survival and proliferation [Furumura et al. 2001; Mann et al. 1999; Zhao et al. 2004; Zhao et al.
Furthermore, *in vivo* studies using MTf down-regulated melanoma cells resulted in significantly reduced initiation and tumour growth in nude mice, indicating that MTf plays a role in melanoma tumourigenesis [Dunn et al. 2006]. To conclude, it is intriguing that MTf plays a role in tumourigenesis, but further work is necessary to examine the precise molecular pathways involved. Such studies are described below.

### 6.3.2. Future studies examining the effects of modulating the melanotransferrin expression in vitro models

*In vivo* studies using SK-Mel-28 melanoma cells with decreased MTf expression showed significantly reduced initiation and tumour growth in nude mice compared with melanoma cells expressing high MTf levels (Chapter 3; [Dunn et al. 2006]). Hence, it would be interesting to perform complementary experiments using the hyper-expression models of MTf in LMTK’ mouse fibroblasts and SK-N-MC neuroepithelioma cells (see Chapter 4). It can be speculated that these two hyper-expression xenograft models would show a significant increase in both the initiation and growth of tumours in nude mice.

Following on from the above studies, the specific cellular and tissue changes occurring *in vivo* when melanoma cells with down-regulated or up-regulated MTf expression are injected into nude mice for tumourigenesis studies needs to be examined. As demonstrated in this thesis, PTGS of MTf expression was stable in SK-Mel-28 melanoma cells injected *in vivo* into nude mice after one month (Figure 3.9; [Dunn et al. 2006]). This could allow examination of the function of MTf in tumourigenesis and metastases and also the role of MTf on angiogenesis.
Melanomas are highly vascularised and aggressive tumours [Denijn & Ruiter 1993; Neitzel et al. 1999; Ribatti et al. 1992] and it has been reported that endogenous MTf expression in SK-Mel-28 melanoma cells may play a role in plasminogen activation which facilitates angiogenesis [Bertrand et al. 2007]. This could be examined \textit{in vivo} by macroscopic quantification of vascularisation after sub-cutaneous injection of melanoma cells. This would be performed using power Doppler sonography combined with image analysis as described previously [Denis et al. 2003].

Histological sectioning of tumours and the underlying connective tissue would also be performed using standard methods [Dunn et al. 2006; Whitnall et al. 2006]. For instance, sections could be stained immunohistochemically for proteins that play critical roles in angiogenesis such as vascular endothelial growth factor-1 [Demeule et al. 2003; Michaud-Levesque et al. 2007; Rossiter et al. 2004] and CD31 [Gyorffy et al. 2001]. CD31 is a cell adhesion molecule expressed on endothelial cell intercellular junctions that can be used as endothelial cell marker for vessel quantification, as per standard methods [Gyorffy et al. 2001]. Briefly, the frozen tumour is cryosectioned and treated to remove endogenous peroxidases [Gyorffy et al. 2001] and then stained with anti-CD31 antibody (BD PharMingen, San Diego, CA). Following the staining process, vessels could be quantified using either a visual microscopic count or imaging software (Empix Imaging, Ontario, Canada; [Gyorffy et al. 2001]).
6.4. Changes in gene expression affected by melanotransferrin

6.4.1. Molecular targets directly or indirectly regulated by melanotransferrin expression

In this study, differential changes in gene expression were identified across five models of MTf down-regulation and hyper-expression across a range of cell lines and in the MTf$^{-/-}$ mouse. In these five models, three genes, namely $Abcb5$, $Thtpa$ and $Tcf4$, were commonly modulated by MTf expression. Four other notable genes included $Apod$, $Gls$, $Mef2a$ and $Ptpdc1$ which were commonly regulated by MTf expression in at least two of the models (see Chapter 4). The functional roles of the products of these genes are summarised in Table 6.1. However, the precise relationship between each of these molecules and MTf is unclear and could be further examined using the experimental protocols below.
Table 6.1. Summary of the functional roles of genes affected by modulation of melanotransferrin gene expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Reference</th>
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| \( Abcb5 \) | Cell fusion  
Membrane transport  
Tumour biology | [Frank et al. 2003]  
[Frank et al. 2005] |
| \( Apod \) | Cell proliferation  
Tumour biology | [Sugimoto et al. 1994] |
| \( Gls \) | Differentiation  
Signalling  
Tumour biology | [Medina et al. 1992]  
[McKinsey et al. 2002] |
| \( Ptpdc1 \) | Cell proliferation  
Differentiation  
Signalling  
Tumour biology | [Easty et al. 2006] |
| \( Tcf4 \) | Cell proliferation  
Differentiation  
Signalling  
Tumour biology | [Furumura et al. 2001]  
[Graham et al. 2001] |
| \( Thtpa \) | Energy metabolism | [Makarchikov et al. 2003] |
6.4.2. Future directions for assessing melanotransferrin molecular pathways

Considering the results from microarray studies (see Chapters 3 and 4; [Dunn et al. 2006; Suryo Rahmanto et al. 2007b]), it is critical to examine the protein interactions occurring between MTf and other molecules. To determine their relationship to MTf at the protein level, both Western blot and proteomic analyses could be utilised. Proteomic analysis could be performed using 2-dimension gel electrophoresis. Differentially expressed proteins could then be excised, digested and identified by mass spectrometry using standard techniques [Kashem et al. 2007]. Together with microarray analysis, this approach would also allow determination of whether MTf modulates gene expression at the mRNA or protein level or both. Furthermore, it could possibly identify molecules that may be post-transcriptionally modulated by changes in endogenous MTf expression which were not detected at the genomic level.

Another consideration when examining the signalling pathways through which MTf may act is by examining the physiological role of the soluble form of the protein that is found at very low levels in body fluids [Desrosiers et al. 2003; Food et al. 2002; Richardson & Morgan 2004]. Indeed, *in vitro* studies using high levels of recombinant soluble hMTf have shown that it may antagonise cell migration and plasminogen activation of endothelial and melanoma cells [Demeule et al. 2003; Michaud-Levesque et al. 2005a; Michaud-Levesque et al. 2005b; Michaud-Levesque et al. 2007; Rolland et al. 2006]. However, these studies did not examine both the apo- and holo-forms of the recombinant protein and since Fe-binding results in marked conformational changes in Tf family of proteins [Baker & Lindley 1992], Fe status must be investigated and addressed. Although the physiological relevance of the soluble form of MTf remains
unclear, recombinant soluble MTf [Demeule et al. 2003; Richardson & Morgan 2004] could be utilised to investigate interactions between MTf and the molecules identified from the microarray studies.

6.5. Conclusions

In summary, the results presented in this thesis provides clear evidence that MTf does not play an essential role in Fe metabolism, but identifies an important function for this molecule in melanoma tumourigenesis [Dunn et al. 2006; Sekyere et al. 2006]. The microarray data in combination with studies *in vivo* using melanoma xenografts strongly suggests novel roles for MTf in cell proliferation, migration and melanoma tumourigenesis. Additionally, a broad outline for future experiments has been detailed in this Chapter which can extend the current findings. Finally, the results presented in this thesis have the potential to further our understanding of the role of MTf in health and disease. In view of this potential outcome, the continued investigation of MTf function is essential and may lead to the development of novel therapies for the management of melanoma.