The function of melanotransferrin: a role in melanoma cell proliferation and tumorigenesis

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Melanotransferrin (MTf) or melanoma tumor antigen p97 is an iron (Fe) binding transferrin homolog expressed highly on melanomas and at lower levels on normal tissues. It has been suggested that MTf is involved in a variety of processes such as Fe metabolism and cellular differentiation. Considering the crucial role of Fe in many metabolic pathways, for example, DNA synthesis, it is important to understand the function of MTf. To define the roles of MTf, two models were developed: (i) an MTf knockout (MTf<sup>−/−</sup>) mouse and (ii) downregulation of MTf expression in melanoma cells by post-transcriptional gene silencing (PTGS). Examination of the MTf<sup>−/−</sup> mice demonstrated no differences compared with wild-type littermates. However, microarray analysis showed differential expression of molecules involved in proliferation such as Mef2a, Tcf4, Gls and Apod in MTf<sup>−/−</sup> mice compared with MTf<sup>+/+</sup> littermates. Considering the role of MTf in melanoma cells, PTGS was used to downregulate MTf mRNA and protein levels by >90% and >80%, respectively. This resulted in inhibition of proliferation and migration. As found in MTf<sup>−/−</sup> mice, in melanoma cells with suppressed MTf expression, hMEF2A and hTFC4 were upregulated compared with parental cells. Furthermore, when melanoma cells with decreased MTf expression were injected into nude mice, tumor growth was markedly reduced, suggesting a role for MTf in proliferation and tumorigenesis.

Introduction

Melanotransferrin (MTf) or melanoma tumor antigen p97 is an iron (Fe) binding transferrin (Tf) homolog originally identified at high levels on melanomas and other tumors, cell lines and fetal tissues (1–3). Initial studies found MTf to be absent or only slightly expressed in normal adult tissues (2), while later investigations demonstrated MTf in a range of normal tissues (4–6). Recent studies showed MTf to be expressed at higher levels in the brain and epithelial surfaces of the salivary gland, pancreas, testis, kidney and sweat gland ducts compared with other normal tissues (7–9). Nonetheless, the highest levels of MTf are found on melanoma cells, in particular the SK-Mel-28 melanoma cell line that expresses 3.0–3.8 × 10<sup>5</sup> MTf molecules per cell (2,3).

The MTf molecule shares many properties in common with the Tf family of proteins, including (i) a 37–39% sequence homology with human serum Tf, human lactotransferrin and chicken ovoTf; (ii) co-localization of the MTf gene on chromosome 3 with Tf and the Tf receptor 1 (TfR1) genes; (iii) many conserved disulfide bonds; (iv) an N-terminal Fe-binding site that is very similar to that found in serum Tf; and (v) the ability of isolated and purified MTf to bind Fe from Fe(III) citrate complexes (3,10–13). These characteristics and the high expression of MTf on melanoma cells suggested that it played a role in Fe transport, possibly assisting these tumor cells with their increased Fe requirements (7,14,15). However, unlike serum Tf, MTf is typically tethered to the cell membrane by a glycosyl phosphatidylinositol (GPI) anchor with only a very small amount of soluble MTf (sMTf) being detectable in serum, saliva, urine and cerebrospinal fluid (2,16,17).

Extensive in vitro studies examining the role of MTf in Fe uptake by SK-Mel-28 melanoma cells demonstrated that both the membrane-bound and soluble forms of MTf do not efficiently donate Fe to the cell, or bind to either TfR1 or transferrin receptor 2 (7,14,15,18–20). Analysis of normal tissues in humans and mice indicates that the expression pattern of MTf is very different to that of Tf and TfR1, and that MTf expression is not regulated by Fe levels (7). Collectively, these findings suggest that MTf has a negligible role in cellular Fe uptake in melanoma cells.

To date, the function of MTf in both normal and neoplastic cells remains unknown. A wide variety of studies have suggested that MTf is involved in physiological and pathological processes, such as (i) Fe transport (21,22); (ii) Alzheimer’s disease (16,23); (iii) eosinophil differentiation (24); (iv) chondrogenesis (25); (v) arthritis (26); (vi) angiogenesis (27); and (vii) plasminogen activation (28). However, there is no definitive evidence for the functional role of MTf.

To determine the role of MTf, we generated MTf knockout (MTf<sup>−/−</sup>) mice by targeted gene disruption. A preliminary report on this work described the development of this mouse model with very limited description of the effects of the null allele on Fe homeostasis (9). In the current paper, we provide a comprehensive assessment of the phenotype of this animal, which is important for defining the function of MTf. Our studies show that lack of MTf expression had no significant

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effect on the growth, development, behavior or metabolism of MTf<sup>-/-</sup> mice compared with wild-type (MTf<sup>+/+</sup>) littermates. However, whole genome microarray analysis showed changes in the expression of genes such as myocyte enhancer factor 2A (Mef2A), transcription factor 4 (Tcf4), glutaminase (Gls) and apolipoprotein D (Apold) when comparing MTf<sup>-/-</sup> mice with MTf<sup>+/+</sup> littermates. To assess the role of MTf in neoplastic cells, we downregulated MTf expression by post-transcriptional gene silencing (PTGS) in SK-Mel-28 melanoma cells. These studies showed that downregulation of MTf expression impaired cellular proliferation, DNA synthesis, cellular migration in vitro and tumor formation in vivo.

Materials and methods

**Animals**

MTf<sup>-/-</sup> mice were generated by homologous gene targeting in embryonic stem (ES) cells as described previously (9). Animal work was conducted in accordance with the University of New South Wales Animal Ethics Committee Guidelines. All mice were housed under a 12 h light–dark cycle, fed routinely with basal rodent chow (0.02% Fe) and watered ad libitum.

**Serum chemistry, hematology and hemochemistry**

Serum chemistry and hematological parameters were determined using a Konelab 20 analyzer (Thermo- Electron Corporation, Finland) and Sysmix K-4500 analyzer (TOA Medical Electronics, Kobe, Japan), respectively. The total Fe-binding capacity (TIBC) was calculated by adding the serum Fe level and the unbound Fe-binding capacity (UIBC). The T saturation was calculated as serum Fe level/TIBC × 100.

**Tissue non-heme Fe, copper and zinc determinations**

Tissue non-heme Fe, copper (Cu) and zinc (Zn) concentrations were measured using inductively coupled plasma atomic emission spectrometry (ICP-AES) via standard techniques (29).

**Microarray processing and analysis**

Six 17.5-week-old female mice comprising four MTf<sup>-/-</sup> and two MTf<sup>+/+</sup> from the same litter were used. Total RNA was isolated by grinding brain tissue in 1 ml of TRIzol reagent (Invitrogen, Sydney, Australia). First-strand cDNA synthesis was performed using a total of 15 μl of TRIzol reagent (Invitrogen, Sydney, Australia). First-strand cDNA synthesis kit and the cDNA products purified using the Affymetrix GeneChip sample clean-up kit (Millenium Sciences, Victoria, Australia). The vector was used to clone four separate transgenes (A–D) of 66 bp each that transcribe 19mer double-stranded hairpin RNAs of the target gene. The four transgenes were specifically targeted to positions 2046–2064 (A), 2031–2049 (B), 2048–2066 (C) and 1416–1434 (D) in the MTf gene (Genbank accession no.: NM_005929). They showed no homology to other human genes as shown using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). As a control, we cloned a scrambled, non-specific transgene into the vector (pS-scrambled) (Ambion). Human SK-Mel-28 melanoma cells were obtained from the American Type Culture Collection (MD) and cultured as described previously (7). Transfections of pS-MTf transgenes or pS-scrambled vectors were performed with Lipofectamine 2000 reagent (Invitrogen) and cells were selected and maintained in 1000 μg/ml of G418 (Invitrogen).

**RT–PCR and western analysis**

RNA was isolated using the TRIzol reagent as described above and RT–PCR analyses of transcripts were carried out by standard procedures (32) using the primers in Table I. Briefly, 0.3–1.0 μg of RNA was incubated with gene-specific oligonucleotides (0.2 μM final primer concentration) in a 50 μl volume containing 25 μl of 2x Reaction Mix (1.6 mM MgSO<sub>4</sub> and 200 μM dNTP) and 2 μl of SuperScript III RT/Platinum Taq Mix for 30 min at 50°C. After reverse transcription the samples were initially denatured for 3 min at 94°C. The reactions were then amplified for 20–35 PCR cycles that included a 90°C denaturation step for 30 s, 55–60°C annealing step for 30–60 s, a 68°C extension step for 60 s, with a final extension time of 5 min. As an

Table I. Primers for amplification of mouse and human mRNA

<table>
<thead>
<tr>
<th>Pair no.</th>
<th>Primer name</th>
<th>Genotype/ accession no.</th>
<th>Oligonucleotides (5’–3’)</th>
<th>Forward Priming sites</th>
<th>Reverse Priming sites</th>
<th>Product size (bp)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>mMTf</td>
<td>NM_013900</td>
<td>AGGCCTCTGAGGCTGATCTTT</td>
<td>121–139</td>
<td>CACCTGTTGCTTCATCGAG</td>
<td>795–813</td>
</tr>
<tr>
<td>2</td>
<td>hMTf</td>
<td>NM_005929</td>
<td>TACTCGGCCGCGGACAGGCTG</td>
<td>266–286</td>
<td>GCGACCGGTTGGGGTACAG</td>
<td>1137–1157</td>
</tr>
<tr>
<td>3</td>
<td>mTfR1</td>
<td>NM_011638</td>
<td>TCCCCGTTTTCCTTCATCGCC</td>
<td>700–717</td>
<td>GCGCAGAATGCTGATGATTGA</td>
<td>1002–1023</td>
</tr>
<tr>
<td>4</td>
<td>hTfR1</td>
<td>NM_003254</td>
<td>TACGCTTAAAGCAGGCTGCA</td>
<td>833–853</td>
<td>TTGGGAAATAGGAGAAGAC</td>
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<tr>
<td>5</td>
<td>m7-actin</td>
<td>NM_007393</td>
<td>CCGCCCGCACCCGCTACAGG</td>
<td>64–84</td>
<td>AAGGTCCTCAACATGCTGTC</td>
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<tr>
<td>6</td>
<td>h7-actin</td>
<td>NM_001101</td>
<td>CCGCCCGCCGAGTCTACAGG</td>
<td>57–77</td>
<td>AAGGTCCTCAACATGCTGTC</td>
<td>433–453</td>
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<tr>
<td>7</td>
<td>hOAS1</td>
<td>NM_016816</td>
<td>AGGGGTTAAGGTTGCTCTC</td>
<td>203–222</td>
<td>ACAACAGGCTGCCTCAG</td>
<td>252–272</td>
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<tr>
<td>8</td>
<td>mMef2a</td>
<td>NM_00103373</td>
<td>GCCCTGATGCTGAGGCTT</td>
<td>629–646</td>
<td>TGGCCGCTTCATCCATCGA</td>
<td>1120–1139</td>
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<tr>
<td>9</td>
<td>hMef2a</td>
<td>NM_005587</td>
<td>TCTACAGATGTTGGTGCTTC</td>
<td>52–77</td>
<td>TCTACAGATGTTGGTGCTTC</td>
<td>260–283</td>
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<tr>
<td>10</td>
<td>mGlS</td>
<td>NM_129846</td>
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<td>1557–1574</td>
<td>TCTCAGATGCTGCTGCTG</td>
<td>2038–2055</td>
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<td>11</td>
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<td>NM_010005</td>
<td>GCCATTAAGGTTGGTCTG</td>
<td>1557–1574</td>
<td>TCTCAGATGCTGCTGCTG</td>
<td>2038–2055</td>
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<tr>
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<td>mtfl</td>
<td>NM_013685</td>
<td>GCCAGGCTCATTACCCTT</td>
<td>843–860</td>
<td>CACGCTATCCTACCTCA</td>
<td>1612–1629</td>
</tr>
<tr>
<td>13</td>
<td>hTcf4</td>
<td>NM_003199</td>
<td>GCCCGTCTTGTGAGGTT</td>
<td>176–194</td>
<td>ATATGTTCCTGAGCGGTTG</td>
<td>393–412</td>
</tr>
<tr>
<td>14</td>
<td>mApod</td>
<td>NM_007470</td>
<td>TTCTTGTGGTGGCTTTG</td>
<td>747–764</td>
<td>CCGGAGCTGAGGATTCTA</td>
<td>1524–1541</td>
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</table>
uptake from $^{59}$Fe–Tf (34,35). Briefly, cells were incubated with $^{59}$Fe–Tf (internalized) $^{59}$Fe in the cell pellet, while the Pronase-sensitive $^{14}$C000 r.p.m. for 1 min. This procedure resulted in the separation of Pronase-sensitive from apo-MTf (from Malcolm Kennard, Synapse Technologies, British Columbia, Canada) with $^{59}$Fe or $^{59}$Fe (Perkin-Elmer, Boston, MA) (19).

Iron uptake assay
Standard techniques in our laboratory were used to determine cellular $^{59}$Fe uptake from $^{59}$Fe–Tf (34,35). Briefly, cells were incubated with $^{59}$Fe–Tf ([$^{59}$Tf] = 0.75 μM; [$^{59}$Fe] = 1.5 μM) for up to 240 min at 37°C. The culture plates were then placed on a tray of ice and the monolayer was washed four times with ice-cold PBS. The cells were subsequently incubated with the general protease, Pronase (1 mg/ml; Sigma), for 30 min at 4°C. The cells were removed from the plates using a plastic spatula and centrifuged at 14,000 r.p.m. for 1 min. This procedure resulted in the separation of Pronase-insensitive (internalized) $^{59}$Fe in the cell pellet, while the Pronase-sensitive (membrane-bound) $^{59}$Fe remained in the supernatant. Previous studies have demonstrated that this latter technique is valid for measuring internalized and membrane-bound $^{59}$Fe–Tf uptake by cells (14,34). The supernatant and pellet were separated and placed in separate counting tubes. Radioactivity was measured on a γ-scintillation counter (Wallac, Compugamma, Finland).

In vitro cell proliferation and migration assays
Cell counts were performed using Trypan blue staining. DNA synthesis was measured via incorporation of $^{3}H$-thymidine (Perkin-Elmer, MA) using standard procedures (36). Cell migration assays were performed over 18 h using Transwell filters in 24-well plates (Corning, NY) pre-coated with 0.15% gelatin (28). As a second measurement of cell migration and proliferation, wound scrape assays were also implemented (37,38).

Tumor biology in nude mice
Male nude mice (BALB/c-nu) were subcutaneously injected at one site on the right flank with $1 \times 10^6$ SK-Mel-28 cells, MTf/B1 or scrambled control cells. Tumor growth was measured by standard procedures (39).

Statistical analysis
Excluding the statistical analysis of the microarray results described above, all data were compared using Student’s t-test. Results were expressed as mean ± standard error of the mean (SEM) or mean ± standard deviation (SD). Data were considered statistically significant when $P < 0.05$.

Results

Phenotypic characterization of MTf$^{−/−}$ mice
Melanotransferrin knockout mice of a mixed 129/SvJ and C57BL/6 background were generated by homologous recombination in ES cells, as described previously (9). In this procedure, exon 1 was cloned in the reverse orientation, while exons 2, 3 and 4 that encode the Fe-binding domain (40) and the intervening introns of the MTf gene were removed. This targeting strategy disrupted the promoter region, the translation initiation codon and the Fe-binding domain, and resulted in no MTf mRNA or protein product (9).

The MTf$^{−/−}$ mice displayed no differences in reproduction, growth, development or histology compared with MTf$^{+/+}$ littersmates. Offspring from MTf$^{+/+}$ matings had a distribution of $26\%$ MTf$^{+/+}$, $48\%$ MTf$^{−/−}$ and $26\%$ MTf$^{+/−}$ ($n = 312$). This was consistent with a normal Mendelian inheritance ratio of 25% wild-types, 50% heterozygotes and 25% homozygotes. Litter sizes of MTf$^{+/+}$ matings were comparable with the average litter sizes of MTf$^{−/−}$ matings. There were no significant differences in mouse body weight from 3 to 15 weeks of age (Figure 1A and B). For example, at 12 weeks of age, the weights of male MTf$^{+/+}$ mice (26.5 ± 0.4 g; mean ± SEM, $n = 22$) were not significantly different to male MTf$^{−/−}$ littersmates (27.5 ± 0.6 g, $n = 27$) (Figure 1A). Similarly, the weights of female MTf$^{+/+}$ mice (21.2 ± 0.4 g, $n = 14$) were not significantly different to female MTf$^{−/−}$ littersmates (20.4 ± 0.5 g, $n = 18$) (Figure 1B).

Examination of organ-to-total body weight ratios at autopsy indicated no gross changes between genotypes (Figure 1C and D), or histological differences in the tissues (data not shown). To address the suggestion that MTf may play a role in brain Fe transport (21,22,44), we assessed brain Fe levels in mice maintained for 4 weeks on either a basal (0.02%) Fe or high (2.00%) Fe diet (Figure 1E and F). Regardless of diet, no significant differences were observed in either male or female MTf$^{−/−}$ mice compared with MTf$^{+/+}$ littersmates. The effect of dietary Fe challenge on Fe levels in the liver, spleen, heart and kidney was reported in our previous study, where no differences were observed between the genotypes (9).

A panel of hematological indices (Table II) and selected serum biochemistry parameters (Table III) were assessed with no significant differences being detected between MTf$^{+/+}$ and MTf$^{−/−}$ littersmates except in alkaline phosphatase (ALP) levels in male animals. However, this significant ($P < 0.05$) decrease in ALP levels was within the normal range (45). Moreover, this difference in ALP levels was not observed when comparing female MTf$^{+/+}$ and MTf$^{−/−}$ mice. All the hematological and biochemical parameters assessed were within physiological limits observed in mice (45,46). A broader serum biochemistry and hematological screen, which included amylose, creatine kinase-MB, cholesterol, glucose, triglycerides, urea, Ca$^{2+}$/Mg$^{2+}$/P0$^{4+}$, prothrombin and partial prothrombin time and differential cell counts (including neutrophils, basophils and eosinophils) also showed no significant differences between MTf$^{+/+}$ and MTf$^{−/−}$ littersmates (data not shown).

Male and female mice were assessed as separate groups owing to the known differences in Fe metabolism between the two sexes (47,48). All of the above-mentioned parameters were also assessed as part of a longevity study when mice were 18 months old, with no significant differences being observed (data not shown).

MTf expression is not essential for normal Fe metabolism
MTf is a Tf homolog and shares many of its characteristics, including the ability to bind Fe (3,40). To determine whether MTf has a role in Fe metabolism, serum and tissue Fe indices were measured in 12-week-old MTf$^{−/−}$ and MTf$^{+/+}$ littersmates. In male or female mice, serum Fe levels, Tf saturation, TIBC and tissue Fe levels were not significantly different ($P > 0.05$) in MTf$^{−/−}$ animals compared with MTf$^{+/+}$ littersmates (Tables IV and V).

Since MTf has an Fe-binding site identical to Tf (1,3,11,13), it could bind other vital metals such as Cu$^{2+}$ and Zn$^{2+}$ (49). Hence, it was important to examine the effects of the MTf null allele on tissue Cu and Zn levels. As found for Fe, no differences were observed comparing MTf$^{−/−}$ animals with their MTf$^{+/+}$ littersmates (data not shown).

Comparative data analysis of the differential gene expression between MTf$^{+/+}$ and MTf$^{−/−}$ mice
Considering that there were no observable phenotypic differences between the MTf$^{−/−}$ and MTf$^{+/+}$ littersmates, we embarked upon microarray analysis to determine if there
were changes in gene expression that may lead to a further understanding of MTf function. The levels of gene expression were determined from brain tissue RNA samples that were hybridized to the Mouse Genome 430 2.0 array GeneChip (Affymetrix). Mouse brain had the highest MTf expression in both sexes (8) and was used to determine differential gene expression between MTf\(^{+/+}\) and MTf\(^{-/-}\) mice. The top 30 genes showing both the largest positive and negative fold changes are summarized in Figure 2A. A positive value of the log\(_2\)-fold change in expression indicates upregulation in MTf\(^{-/-}\) samples relative to the wild-type animals, while a negative value indicates downregulation. Analysis of the genes affected by ablation of MTf (Figure 2A–C) using NetAffx software (http://www.affymetrix.com/analysis/index.affx)
indicated that they have roles in gene transcription, differentiation and development, regulation of cellular metabolism, transport and cell adhesion.

Similar to the mRNA and protein data from the MTf<sup>−/−</sup> animal studies (9), microarray analysis demonstrated no differential expression of TFR1 or any other molecules known to be involved in Fe metabolism between MTf<sup>−/−</sup> and MTf<sup>+</sup/>+ mice. These data relating to TFR1 expression again confirm our previous observations that deletion of MTf did not affect the Fe pools that regulate TFR1 expression (9). In addition, and as expected, gene array assessment of MTf expression showed that it was downregulated in the MTf<sup>−/−</sup> mice compared with their MTf<sup>+</sup/>+ littermates.

To validate the expression of the 30 genes showing the largest changes in the array data (Figure 2A), RT–PCR was used. Increased expression of the following genes was observed in MTf<sup>−/−</sup> mice when compared with their MTf<sup>+</sup/>+ littermates: Me2a, Gls and Tcf4 (Figure 2B). Conversely, expression of the Apod and MTf genes were downregulated in MTf<sup>−/−</sup> mice compared with MTf<sup>+</sup/>+ littermates (Figure 2B). Densitometric analysis of the RT–PCR data was used to quantify the magnitude of change in gene expression. Expression of Me2a mRNA was found to be 4-fold higher (log<sub>2</sub> ratios of +2.03) in MTf<sup>−/−</sup> mice compared with MTf<sup>+</sup/>+ littermates, while Gls and Tcf4 mRNA expression were increased by 2-fold (log<sub>2</sub> ratios of +1.03) and 3-fold (log<sub>2</sub> ratios of +1.63), respectively (Figure 2C). Examination of Apod mRNA expression indicated a 5-fold downregulation (log<sub>2</sub> ratios of −2.39) (Figure 2C) in MTf<sup>−/−</sup> mice compared with MTf<sup>+</sup/>+ littermates. In addition to the samples submitted

### Table II. Hematological indices in MTf<sup>−/−</sup> mice compared with their MTf<sup>+</sup/>+ littermates at 12 weeks of age

<table>
<thead>
<tr>
<th>Sex</th>
<th>n</th>
<th>Genotype</th>
<th>RBC (10&lt;sup&gt;12&lt;/sup&gt;/l)</th>
<th>HGB (g/l)</th>
<th>HCT</th>
<th>MCV (fl)</th>
<th>WBC (10&lt;sup&gt;9&lt;/sup&gt;/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>19</td>
<td>MTf&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>8.6 ± 0.2</td>
<td>134 ± 3</td>
<td>0.45 ± 0.01</td>
<td>52.2 ± 0.5</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>MTf&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>9.0 ± 0.1</td>
<td>138 ± 2</td>
<td>0.47 ± 0.01</td>
<td>52.1 ± 0.4</td>
<td>6.3 ± 0.5</td>
</tr>
<tr>
<td>F</td>
<td>17</td>
<td>MTf&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>9.2 ± 0.1</td>
<td>146 ± 4</td>
<td>0.48 ± 0.01</td>
<td>52.5 ± 0.4</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>MTf&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>9.1 ± 0.1</td>
<td>132 ± 2</td>
<td>0.47 ± 0.01</td>
<td>51.9 ± 0.4</td>
<td>5.5 ± 0.3</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM.

M, male; F, female; HCT, hematocrit; HGB, hemoglobin; MCV, mean corpuscular volume; RBC, red blood cells; WBC, white blood cells.

### Table III. Selected serum chemistry indices in MTf<sup>−/−</sup> mice compared with their MTf<sup>+</sup/>+ littermates at 12 weeks of age

<table>
<thead>
<tr>
<th>Sex</th>
<th>n</th>
<th>Genotype</th>
<th>Creatinine (µmol/l)</th>
<th>Protein (g/l)</th>
<th>AST (U/l)</th>
<th>ALP (U/l)</th>
<th>ALT (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>12</td>
<td>MTf&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>44 ± 1</td>
<td>46 ± 1</td>
<td>108 ± 13</td>
<td>121 ± 6*</td>
<td>43 ± 5</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>MTf&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>43 ± 2</td>
<td>44 ± 1</td>
<td>125 ± 10</td>
<td>98 ± 5*</td>
<td>51 ± 7</td>
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<tr>
<td>F</td>
<td>7</td>
<td>MTf&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>42 ± 1</td>
<td>48 ± 1</td>
<td>111 ± 11</td>
<td>150 ± 6</td>
<td>33 ± 3</td>
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<tr>
<td></td>
<td>6</td>
<td>MTf&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>44 ± 2</td>
<td>45 ± 1</td>
<td>121 ± 6</td>
<td>139 ± 11</td>
<td>45 ± 4</td>
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</table>

Values expressed as mean ± SEM.

M, male; F, female; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

*p< 0.05 as determined by Student’s t-test.

### Table IV. Serum Fe indices in MTf<sup>−/−</sup> mice compared with their MTf<sup>+</sup/>+ littermates at 12 weeks of age

<table>
<thead>
<tr>
<th>Sex</th>
<th>n</th>
<th>Genotype</th>
<th>Serum Fe (µmol/l)</th>
<th>TIBC (µmol/l)</th>
<th>TF saturation (%)</th>
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</thead>
<tbody>
<tr>
<td>M</td>
<td>16</td>
<td>MTf&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>57.5 ± 2.5</td>
<td>105.2 ± 3.8</td>
<td>73 ± 3</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>MTf&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>50.7 ± 2.7</td>
<td>96.0 ± 4.2</td>
<td>67 ± 3</td>
</tr>
<tr>
<td>F</td>
<td>13</td>
<td>MTf&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>65.2 ± 6.5</td>
<td>115.7 ± 8.1</td>
<td>81 ± 6</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>MTf&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>55.3 ± 4.8</td>
<td>113.4 ± 9.9</td>
<td>80 ± 7</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM.

M, male; F, female; TIBC, total iron-binding capacity.

### Table V. Tissue Fe stores in MTf<sup>−/−</sup> mice compared with their MTf<sup>+</sup/>+ littermates at 12 weeks of age

<table>
<thead>
<tr>
<th>Sex</th>
<th>n</th>
<th>Genotype</th>
<th>Tissue Fe level (µg/g) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Male</td>
<td>25</td>
<td>MTf&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>276 ± 14</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>MTf&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>291 ± 17</td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
<td>MTf&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>368 ± 17</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>MTf&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>426 ± 20</td>
</tr>
</tbody>
</table>

The function of melanotransferrin
for gene array analysis, we also confirmed the data independently on RNA samples from other MTf/C0 and MTf/+ mice.

Downregulation of MTf expression in SK-Mel-28 cells by PTGS

While there were some differential gene expression in the microarray study, no observable phenotype was detected in the MTf/C0/littermates. Indeed, the role of MTf may only become apparent in melanoma cells where it is expressed at very high levels (1, 2). To assess this, we stably transfected SK-Mel-28 cells with a pS-MTf vector, which encoded each of the four transgenes that transcribe a 19mer anti-MTf hairpin siRNA. As a control, SK-Mel-28 cells were transfected with the same vector containing a scrambled, non-specific sequence. Of the four siRNAs specifically targeted to the human MTf gene, we found that the vector containing the B transgene (pS-MTf/B1) consistently gave us the most pronounced downregulation of MTf expression and inhibition of cellular proliferation. The other three transgenes resulted in less MTf downregulation and not as marked reduction in proliferation (data not shown). Therefore, all further experiments used clones from the B transgene. The fact that all transgenes reduced MTf expression to some degree suggested that the effect observed was not due to possible off-target effects of the siRNA. The clones obtained from transfection with the pS-MTf/B1 vector demonstrated downregulation of MTf mRNA and protein by >90% (Figure 3A) and >80% (Figure 3B), respectively, compared with cells transfected with the scrambled transgene. In Figure 3B, two MTf protein bands were visible on the western blot at 79- and 82-kDa, which are the result of post-translational modification of MTf (50).

Multiple transfection experiments with the pS-MTf/B1 vector 6 months after the initial study showed similar downregulation of MTf expression in SK-Mel-28 cells, indicating that this was not a clonal effect. Cells transfected with a scrambled, non-specific transgene displayed no reduction in hMTf mRNA or protein expression compared with non-transfected SK-Mel-28 parent cells (data not shown). To exclude the possibility that siRNA was eliciting an interferon-stimulated response that could affect cellular phenotypes, we performed RT–PCR to assess the expression of the interferon-target gene, hOAS1 (51). There were no differences in hOAS1 expression between the scrambled control cell line, MTf/B1 (Figure 3A), and SK-Mel-28 cells (data not shown). As another control, the effect of downregulation of MTf on the expression of the Fe-regulated gene, hTfR1 (52), was assessed. There was no alteration in hTfR1 mRNA expression in the MTf/B1 cell line compared with the scrambled control (Figure 3A). The expression of the transcription factors, hMEF2A and hTCF4, were assessed in MTf/B1 and scrambled...
control cells. As found for the MTf−−/− mice, these transcrip- tion factors were upregulated in the MTf/B1 cell line (Figure 3A). However, there was no significant change in hAPOD expression, while hGDS was not detected (data not shown).

During culture of MTf/B1 cells it became apparent that their growth was slower than cells transfected with the scrambled, non-specific transgene. To quantify this, proliferation was assessed using viable cell counts with Trypan blue staining. Over 4 days, MTf/B1 cells showed decreased growth when compared with cells transfected with the scrambled transgene (Figure 3C), and this was significant on Days 2 (P < 0.05), 3 and 4 (P < 0.001). In contrast, there was no growth inhibition in scrambled control cells when compared with the parent SK-Mel-28 cells (data not shown).

DNA synthesis in MTf/B1 cells was also examined using 3H-thymidine incorporation and was found to be significantly decreased (P < 0.001) on Days 1–4 compared with scrambled
control cells (Figure 3D). Fluorescent staining for cell viability (using acridine orange, propidium iodide and Hoechst 33258) indicated that the depressed proliferation was not due to apoptosis or necrosis (data not shown).

Addition of Fe, apo-MTf or holo-MTf does not rescue depressed proliferation in melanoma cells with decreased MTf expression

To further assess the hypothesis that MTf may potentially act as an Fe transport molecule to aid melanoma proliferation (3), we examined whether exogenous Fe could restore the depressed growth of MTf/B1 cells. To do this, cells were incubated with ferri ammonium citrate (FAC; 100 μg/ml), which is well known to effectively donate Fe to cells (34,53). Previously, we showed that FAC donates Fe to the same cell type used here (SK-Mel-28 cells) and downregulates both hTfR1 mRNA and protein levels (7,34). These studies with FAC resulted in no restoration or increase of proliferation in MTf/B1 cells or cells transfected with the scrambled siRNA transgene (Figure 4A). As a positive control for the ability of FAC to donate Fe to cells, hTfR1 mRNA expression was assessed and shown to be markedly decreased after an incubation of 1–4 days with this Fe source (data not shown). The lack of effect of FAC on proliferation suggested that the cells were Fe-replete probably because they were grown under optimal conditions in media containing 10% FCS. The inability of FAC to restore the slower growth of MTf/B1 cells suggested that its depressed proliferation was not due to Fe deprivation because of decreased MTf expression.

As a measure of Fe status in cells cultured under standard growth conditions, hTfR1 mRNA expression was assessed and found not to be increased in the MTf/B1 cell line compared with cells transfected with the scrambled control vector (Figure 3A). These results showed that the MTf/B1 cells with low MTf expression were not Fe-deprived compared with the scrambled control. When holo-Tf
(\([\text{Fe}] = 1.5 \text{ M}\)) was added as an Fe donor, it also did not stimulate growth (data not shown), again probably because these cells were Fe-replete.

To further examine the relationship between MTf function and Fe metabolism, we performed \(^{59}\text{Fe}\) uptake assays using \(^{59}\text{Fe}\)-Tf (0.75 \text{ M})). These studies showed no significant difference in \(^{59}\text{Fe}\) uptake between MTf/B1 cells and the scrambled control (Figure 4B), suggesting that MTf had a negligible role in Fe uptake from Tf. The fact that \(^{59}\text{Fe}\)-Tf uptake was not greater than the scrambled control agrees with our data indicating no difference in \(hTfR1\) mRNA expression between MTf/B1 cells and the scrambled control (Figure 3A). These results support the finding that MTf/B1 cells were not Fe-deplete owing to low MTf expression. A previous study suggested that sMTf can inhibit the soluble and membrane-bound forms of MTf can regulate SK-Mel-28 cellular migration and that the balance between these molecules known to be involved in Fe transport and cell adhesion. (65), MTf expression remained constant throughout the in vitro and in vivo processes including Fe metabolism and cellular differentiation (22,24,27,40,54). Since MTf possesses a functional Fe-binding site (3,13), a role in Fe metabolism appears plausible, particularly in malignant melanoma cells that express high levels of this tumor antigen (3). In the current study, two models using ablation and downregulation of MTf expression were developed to determine its function. First, a MTf\(^{-/-}\) mouse model was generated by targeted disruption of the MTf gene to assess its function in vivo. Second, to examine the role of MTf in neoplastic cells, we downregulated MTf expression by PTGS using SK-Mel-28 melanoma cells, which express high MTf levels (10).

Our studies demonstrated that MTf\(^{-/-}\) mice were viable, developed normally and showed no obvious morphological, histological, behavioral, hematological or Fe status changes when compared with MTf\(^{+/+}\) littermates. In contrast to other studies indicating a role for MTf in brain Fe metabolism (16,21,22), no obvious changes were observed in brain Fe levels between MTf\(^{-/-}\) and MTf\(^{+/+}\) animals (Figure 1E and F). This suggests the hypothesis that MTf had no essential role in normal Fe metabolism or Fe supply to the brain. In addition, other studies have not confirmed a role for MTf in brain Fe metabolism (55,56).

Interestingly, disruption of another Tf homolog, namely lactoferrin, also led to no phenotype (57), suggesting that these molecules do not play essential roles in Fe metabolism and may have other functions (40). In contrast, loss of function of molecules involved in Fe metabolism (58–63) has dramatic consequences. Indeed, in the case of TIR1, there are lethal effects in utero (59). Clearly, if MTf had an essential role in Fe homeostasis, significant alterations in Fe indices, if not a deleterious phenotype, would have been apparent.

The current investigation supports our previous in vitro studies using melanoma cells that demonstrated that membrane-bound MTf or sMTf did not play an important role in Fe uptake (7,15,18,19). These results were confirmed by others using different cell types (64). Furthermore, sMTf did not bind to TIR1 or TIR2 to donate Fe to cells (19,20).

The current investigations using melanoma cells that demonstrated that membrane-bound MTf or sMTf did not play an important role in Fe uptake (7,15,18,19). These results were confirmed by others using different cell types (64). Furthermore, sMTf did not bind to TIR1 or TIR2 to donate Fe to cells (19,20).

Other studies also suggest that MTf does not play a key role in Fe metabolism. For instance, unlike TIR1, which is upregulated to supply Fe for DNA synthesis in the S phase (65), MTf expression remained constant throughout the cell cycle (66). Moreover, in contrast to TIR1, MTf is not regulated by cellular Fe status (4,7) and is not upregulated in dividing cells (67). Collectively, these data and our previous results (9) demonstrate that MTf is not essential for normal Fe homeostasis.

As there was no obvious phenotype in the MTf knockout mouse, gene array analysis was employed to identify any changes in the gene expression profile of mice with the MTf null allele. These studies showed no changes in the expression of molecules known to be involved in Fe metabolism. However, the analysis did identify genes that were differentially expressed between MTf\(^{-/-}\) and MTf\(^{+/+}\) littermates. The products of these genes have roles in processes such as transcription, differentiation and development, regulation of cellular metabolism, transport and cell adhesion. Subsequent analysis of these changes using RT–PCR confirmed that the genes Mef2a, Tcf4 and Gls were upregulated, while
the Apod gene was downregulated in MTf<sup>−/−</sup> mice compared with their wild-type littermates.

Of these genes, two were identified as transcription factors, namely Mef2a and Tcf4 (70,71). The Mef2a transcription factor is strongly expressed in muscle tissues and is involved in fetal cardiac development, calcium-signaling and the MAP kinase pathways (70,72,73). This transcription factor is suggested to be important in cell proliferation, differentiation

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**Fig. 5.** Downregulation of MTf expression reduces SK-Mel-28 melanoma cell migration in vitro and decreases tumor growth in vivo. (A) Cell migration rates through a transwell chamber over 18 h by SK-Mel-28 melanoma cells stably transfected with anti-MTf siRNA (MTf/B1) or scrambled control siRNA. (B) MTf/B1 cells showed reduced proliferation and migration assessed by the ‘wound’ closure assay when compared with the scrambled control. Control cells closed the wound after 60 h, while MTf/B1 cells begin to close the wound after 72 h. The photograph shows a wound in the scrambled and MTf/B1 monolayers at the 60 h time point. Results in (A) and (B) are expressed as mean ± SEM (three determinations) in a typical experiment from three separate experiments performed. (C) Net tumor growth in nude mice injected subcutaneously with 1 × 10<sup>6</sup> SK-Mel-28 melanoma cells, scrambled control cells or the MTf/B1 cell line. The MTf/B1 cell line with low MTf expression displayed the least net tumor growth after 30 days in vivo. (D) Expression of MTf protein in MTf/B1 cells remains decreased relative to parental SK-Mel-28 cells or scrambled control cell lines after 30 days of in vivo growth in nude mice. (C) Results are mean ± SEM (four to nine animals) in a typical experiment from two separate experiments. (D) Results are from a typical experiment performed as two separate experiments.
and survival (74). The Tcf4 gene product plays a role in the Wnt signaling pathway (75) and the regulation of melanocyte differentiation (76). Tcf4 is expressed at high levels in some cancers, and functional genomic investigations demonstrated that this transcription factor has a role in cell proliferation (71,77). Notably, genes such as solute carrier family 2a member 4 (Slc2a4) and Cd44 are directly regulated by Mef2a and Tcf4 (78,79), and our array data showed that Slc2a4 and Cd44 were affected by MTf deletion. In fact, Cd44 was found in the top 30 genes showing differential regulation after MTf deletion (Figure 2A). These observations suggest some functional links between MTf, Mef2a and Tcf4.

The other two genes showing differential expression, namely Gls and Apod, have also been demonstrated to have a role in proliferation and encode the proteins, glutaminase and apolipoprotein D, respectively. Glutaminase has been found to be highly expressed in the mitochondria of tumor cells immediately before the maximum rate of proliferation is achieved (80–82). Conversely, apolipoprotein D inhibits cellular proliferation and is associated with transporting sterols, steroids and arachidonic acid for tissue repair (83,84). The upregulation of Gls and downregulation of Apod in the knockout mouse compared with the wild-type indicates a direct or indirect response to gene deletion and suggests some role for MTf in proliferation. Under the current experimental conditions, no defect in growth or development was obvious and a phenotype may only be observed when the mouse is exposed to an appropriate stress.

Given these results from the MTf−/− mouse, it should be noted that this is a normal physiological system in a whole organism, which has many existing compensatory mechanisms that may mask deletion of MTf expression. Potentially, in melanoma cells that express high levels of MTf, the function of the protein may become more evident when its expression is decreased. MTf is hyper-expressed in melanoma cells and much less so in other tumors (1,2), suggesting a cell-type-specific function. We used PTGS in human SK-Mel-28 cells and showed that MTf downregulation impaired proliferation, DNA synthesis and migration in vitro and decreased tumor growth in vivo in nude mice. This was not a clonal effect as transfection experiments performed 6 months after the initial study gave similar results. Moreover, the decrease in MTf expression and melanoma proliferation was observed with four different siRNA constructs (see Materials and methods), indicating that these effects could not be explained by the off-target influence of one individual siRNA.

The addition of Fe donors such as FAC or holo-Tf did not rescue the impaired proliferation of MTf/B1 cells with low MTf expression. This indicated that decreased growth of cells with low MTf expression was independent of any effect on Fe metabolism, and this was in agreement with our MTf−/− mouse studies and investigations in vitro (7,9,15,18). Further, there was no difference in Fe uptake from Tf between clones with low MTf levels and the scrambled control.

Considering the role of MTf in proliferation, it is notable that evidence from hyper-expression studies using melanoma cells suggested that increased MTf expression results in accelerated growth (85). In contrast, MTf hyper-expression in CHO cells did not increase proliferation (35). These data support our results suggesting that the role of MTf may only be obvious in melanoma cells where it is highly expressed.

Potentially, the changes in gene expression observed in the MTf−/− mice compared with MTf+/+ littersmates could be involved in the altered proliferation observed in the PTGS experiments using melanoma cells. Assessment of gene expression by RT–PCR indicated that in melanoma cells engineered with suppressed MTf expression, hMEF2A and hTCF4 were similarly upregulated. These data support the relationship between MTf and the transcription factors observed in the MTf−/− mouse. Despite the decrease of Apod expression in MTf−/− mice compared with wild-type animals, there was no change in hAPOD expression in MTf/B1 cells compared with their scrambled control counterparts. Therefore, to rule out the possibility that decreased Apod expression in MTf−/− mice may have resulted from events associated with MTf gene deletion, we examined the expression of a gene at the same chromosomal locus as MTf and Apod. Indeed, TjR1 is located on the same strand as MTf (714 kb apart), and the former gene showed no change in its expression when comparing MTf−/− and MTf+/+ mice (9). Apod is located on the alternate strand as MTf (564 kb apart), and these latter results demonstrating no difference in TjR1 expression between the genotypes (9) suggest that the alteration in Apod expression was not due to a deletion in MTf. However, we cannot rule out the fact that the deleted region of MTf itself may contain distal regulatory elements for nearby genes such as Apod that could affect its expression in the MTf−/− mouse model.

In summary, studies using MTf−/− mice conclusively demonstrate that MTf does not play an essential role in Fe metabolism or homeostasis. We identified differentially expressed genes such as Mef2a, Tcf4, Gls and Apod in the knockout mouse, which suggested that MTf may play a role in growth and proliferation under normal physiological conditions. We also showed using melanoma cells that MTf downregulation resulted in inhibition of proliferation and cell migration in vitro and reduced tumor growth in vivo in nude mice. These data indicate that MTf plays an important role in melanoma cell growth and tumorigenesis.

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References


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