Role of melanotransferrin in iron metabolism: studies using targeted gene disruption in vivo

Eric O. Sekyere, Louise L. Dunn, Yohan Suryo Rahmanto, and Des R. Richardson

Melanotransferrin (MTf) or tumor antigen p97 is a transferrin homolog that binds one iron (Fe) atom and has been suggested to play roles in a variety of processes, including Fe metabolism, eosinophil differentiation, and plasminogen activation. Considering the vital role of Fe in many metabolic pathways, such as DNA and heme synthesis, it is important to understand the function of MTf. To define this, a MTf knockout (MTf<sup>−/−</sup>) mouse was generated through targeted disruption of the MTf gene. The MTf<sup>−/−</sup> mice were viable and fertile and developed normally, with no morphologic or histologic abnormalities. Assessment of Fe indices, tissue Fe levels, hematolgy, and serum chemistry parameters demonstrated no differences between MTf<sup>−/−</sup> and wild-type (MTf<sup>+/+</sup>) mice, suggesting MTf was not essential for Fe metabolism. (Blood. 2006;107:2599-2601)

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Introduction

Melanotransferrin (MTf; also known as Mf62) is a membrane-bound transferrin (Tf) homolog<sup>1-4</sup> expressed at low or undetectable levels in normal tissues but in much larger amounts in neoplastic cells (especially melanoma cells).<sup>1</sup> MTf shares a 37% to 39% sequence homology with human Tf, human lactoferrin, and chicken Tf.<sup>4,5</sup> In humans, the MTf gene is located on chromosome 3 as are Tf and the transferrin receptor 1 (TfR1) genes.<sup>6</sup> Additionally, the N-terminal iron (Fe)–binding site of MTf is identical to that of Tf and can bind Fe.<sup>5,7-9</sup> These similarities to Tf suggest that MTf plays a role in Fe metabolism.<sup>1</sup> However, unlike Tf, MTf is bound to the membrane by a glycosyl-phosphatidylinositol anchor.<sup>4</sup> Previous in vitro studies demonstrated that, although MTf could bind Fe, it did not efficiently donate it to the cell.<sup>10-13</sup>

Many roles have been proposed for MTf, including transcytosis of Fe across the blood brain barrier,<sup>14,15</sup> angiogenesis,<sup>16</sup> cell migration,<sup>16,17</sup> plasminogen activation,<sup>17</sup> chondrogenesis,<sup>18</sup> eosinophil differentiation,<sup>19</sup> and Alzheimer’s disease.<sup>9,20</sup> However, proof for the functional role of MTf is lacking. To help define its role(s), we generated MTf knockout (MTf<sup>−/−</sup>) mice.

Study design

Generation of MTf<sup>−/−</sup> mice

MTf<sup>−/−</sup> mice were generated by homologous gene targeting in embryonic stem (ES) cells. The targeting vector was linearized and electroporated into 129/SvJ ES cells grown in medium containing G418 (Invitrogen, Victoria, Australia). The ES cell clones targeted at both the 5' and 3' ends were used to generate chimeras.

High-percentage chimeric mice were mated with C57BL/6 females to obtain germ line–transmitting heterozygote offspring of mixed 129/SvJ × C57BL/6 background. To delete the neo<sup>+</sup>- cassette, male heterozygote (MTf<sup>−/+;neo<sup>+</sup>−</sup>) mice were mated to B6-deleter females to obtain (MTf<sup>−/+;neo<sup>+</sup>−esc−</sup>) offspring. MTf<sup>−/+;esc−</sup> females were crossed with C57BL/6 males to remove cre, generating MTf<sup>−/−</sup> mice free of neo<sup>+</sup> and cre. These heterozygotes were intercrossed to generate MTf<sup>−/−</sup> and MTf<sup>−/+</sup> littermates.

Genotyping was carried out by Southern analysis and polymerase chain reaction (PCR) using genomic tail DNA.

Examination of MTf expression

RNA isolation and reverse transcriptase (RT)–PCR were performed by standard procedures.<sup>21</sup> A peptide sequence at the C-terminal of mouse MTf (Ac-DDHNKNG-FQMFDSSKYHSQDLC-amide) was chosen based on antigenicity and probability of surface exposure. The conjugated peptide was used to immunize rabbits, and the antibody was purified (Quality-Controlled Biochemicals, MA). Western analysis was performed as described.<sup>22</sup>

Blood analysis and Fe estimation

Serum chemistry and hematologic parameters were determined using a Konelab-20i analyzer (Thermo-Electron, Vantaa, Finland) and Sysmex-K-4500 analyzer (TOA Medical Electronics, Kobe, Japan). Non–heme-Fe was measured using inductively coupled plasma atomic emission spectrometry.<sup>23</sup>

Statistics

Data were compared using Student t test. Data were considered significant when the P value was less than .05.

Results and discussion

Generation of MTf<sup>−/−</sup> mice

We generated MTf<sup>−/−</sup> mice by homologous recombination in ES cells. In this procedure, exons 2, 3, and 4 that encode the Fe-binding domain<sup>4</sup> and the intervening introns of the MTf gene were replaced with the neo<sup>+</sup> gene. In addition, exon 1 was cloned in the reverse orientation (Figure

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Supported by a fellowship and project grants from the National Health and Medical Research Council of Australia.
1A). This targeting strategy disrupts the Fe-binding domain, the translation initiation codon, and promoter. Targeted ES cells were used to generate germ line-transmitting chimeric male mice that were mated with C57BL/6 mice to generate heterozygote offspring containing the neor gene. Mouse genotypes were confirmed by Southern analysis of tail DNA digested with NheI and probed with a 3' probe located outside the region of homology (Figure 1A). In MTf+/− mice, a band of 12.6 kilobase (kb) corresponding to the normal allele was detected (Figure 1B). Using MTf+/− animals, a 8.3-kb band representing the targeted allele was observed (Figure 1B). The MTf+/− mice showed bands of 8.3 kb and 12.6 kb, corresponding to the targeted and wild-type alleles (Figure 1B).

The neor- and cre-negative heterozygous males and females were used to generate MTf+/− and MTf+/− littermates. Southern analysis confirmed the generation of homozygote mice (Figure 1C). Genomic DNA from mouse tail was digested with NheI and probed with a 3' probe that detected a 12.6-kb fragment in the null allele and a 19.0-kb fragment in the targeted knockout (KO) allele (Figure 1C). Two bands were detected in MTf+/− mice corresponding to the targeted and wild-type alleles (Figure 1C). Routine genotyping by PCR yielded a 1.2-kb fragment (MTf+/−) and a 0.7-kb fragment (MTf−/−) (Figure 1D).

To confirm the knockout strategy did not result in a gene product, RT-PCR was performed (Figure 1E). Using primers specific for exons 1 and 6 that amplify across the deleted region of MTf, no product was detected in tissues from MTf−/− mice (Figure 1E). In contrast, a product was detected in MTf+/+ and MTf+/− mice (Figure 1E). MTf mRNA expression in heterozygous mice was reduced to 25% to 36% of that in MTf+/− littermates (Figure 1E), indicating no compensatory up-regulation. As a measure of Fe status, expression of Tfr1 (also known as Tfrc) in mice was compared between the genotypes and was found to be the same (Figure 1E). Hence, ablation of MTf expression does not affect Fe pools that control TIR1 expression.

Western analysis was performed to determine whether MTf protein expression was ablated in MTf−/− mice (Figure 1F). We detected an 82-kDa MTf protein in MTf+/− tissues that was absent from MTf+/− mice (Figure 1F). These data confirm successful ablation of MTf. As found for TIR1 mRNA levels, examination of TIR1 protein expression showed no significant difference between the genotypes (Figure 1F).

**Phenotypic characterization**

The MTf−/− mice were viable and fertile, showed no physical abnormalities, and developed normally. Histologic assessment of major tissues, bone marrow, and blood smears showed no differences when sections from MTf−/− and MTf+/− littermates were stained with hematoxylin and eosin, Giemsa, or Prussian-blue. Hematologic indices (red blood cells [RBCs], white blood cells [WBCs], hemoglobin, hematocrit, and mean corpuscular volume) were assessed, and no significant differences were detected between MTf−/− and MTf+/− littermates (data not shown).

Figure 1. Targeted disruption of the mouse melanotransferrin (MTf) gene. (A) Targeting strategy used for deletion of mouse MTf exons 2, 3, and 4 and reversal of part (443 bp) of the MTf promoter region and the translation initiation codon. The genomic structure of the wild-type MTf allele is shown at the top depicting the promoter region, 16 exons, and intervening introns. Primers used for identification of the wild-type allele are denoted P1 and P2, and fragments used for Southern analysis are denoted the 5' and 3' probes. The targeting construct is shown below the wild-type allele. In this construct, the 1.4-kb BamHI fragment containing exon 1, intron 1, and part of the promoter region is reversed. After homologous recombination, the neomycin resistance (neor) targeted allele contains an additional NheI restriction enzyme site introduced into the neor gene cassette (Neo allele). In the Neo allele, exons 2, 3, and 4 have been replaced with the neor gene cassette flanked by lox-P sites and exon 1 is reversed. The neo cassette was deleted from the genome by mating of MTf−/− males to B6-deleter females to obtain the knockout (KO) allele. The P3 and P4 primers detect the targeted allele. (B) Confirmation of MTf ablation in the mouse at the genomic DNA, mRNA, and protein levels. (B) Genotype identification by Southern blot analysis of mice containing the neor cassette. (C) Genotype identification by Southern blot analysis of mice from where there has been cre-mediated deletion of the neor cassette. (D) Genotype identification by PCR analysis of mice with the cre-mediated deletion of the neor cassette. P1 and P2 primers detect a wild-type fragment of 1.2 kb (first lane). Primers P3 and P4 detect a fragment of 0.7 kb in the targeted knockout (KO) allele (second lane) and fragments of 0.7 and 1.2 kb in the MTf−/− mice (third lane). (E) RT-PCR of MTf and transferrin receptor 1 (Tfr1) mRNA transcripts in MTf+/−, MTf+/−, and MTf−/− mice. (F) Western analysis of MTf and Tfr1 in brain and testis from MTf+/− and MTf−/− mice. Results are representative results in a typical experiment from 3 separate experiments.
Table 1. Tissue Fe stores in MTf<sup>–/–</sup> mice compared with their MTf<sup>+/+</sup> littermates at 12 weeks of age on basal Fe (0.02% Fe/wt) and high-Fe (2.00% Fe/wt) diets

<table>
<thead>
<tr>
<th>Fe diet and genotype by sex</th>
<th>Liver</th>
<th>Spleen</th>
<th>Heart</th>
<th>Kidney</th>
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<tbody>
<tr>
<td>0.02%</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td></td>
<td></td>
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<tr>
<td>MTf&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>286 ± 14</td>
<td>2628 ± 223</td>
<td>454 ± 52</td>
<td>210 ± 7</td>
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<tr>
<td>MTf&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>280 ± 34</td>
<td>3426 ± 398</td>
<td>481 ± 47</td>
<td>396 ± 114</td>
</tr>
<tr>
<td>Female</td>
<td></td>
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<tr>
<td>MTf&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>552 ± 66</td>
<td>5565 ± 915</td>
<td>449 ± 43</td>
<td>374 ± 102</td>
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<tr>
<td>MTf&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>451 ± 24</td>
<td>4949 ± 544</td>
<td>512 ± 34</td>
<td>361 ± 58</td>
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<tr>
<td>2.00%</td>
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<td>Male</td>
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<tr>
<td>MTf&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>4018 ± 168</td>
<td>5274 ± 424</td>
<td>590 ± 51</td>
<td>466 ± 160</td>
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<tr>
<td>MTf&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>3599 ± 378</td>
<td>4838 ± 365</td>
<td>702 ± 108</td>
<td>594 ± 164</td>
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<tr>
<td>MTf&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>4317 ± 184</td>
<td>8145 ± 554</td>
<td>683 ± 95</td>
<td>556 ± 129</td>
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<tr>
<td>MTf&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>3872 ± 280</td>
<td>6701 ± 485</td>
<td>579 ± 35</td>
<td>468 ± 16</td>
</tr>
</tbody>
</table>

*Values expressed as mean ± SEM.

To determine whether MTf has a role in Fe metabolism, serum and tissue Fe indices were measured in 12-week-old MTf<sup>–/–</sup> and MTf<sup>+/+</sup> littermates. Serum Fe levels, Tf saturation, total iron-binding capacity (TIBC), and tissue Fe levels were not significantly different (P > .05) in MTf<sup>–/–</sup> animals compared with MTf<sup>+/+</sup> littermates (data not shown).

Considering the lack of any alterations in hematologic indices and Fe levels, the effect of the MTf null allele on Fe homeostasis was assessed by dietary Fe challenge of MTf<sup>–/–</sup> and MTf<sup>+/+</sup> littermates. Mice were maintained on a high-Fe (2.00% Fe wt/wt) or basal Fe diet (0.02% Fe wt/wt) for 4 weeks, after which serum Fe indices and tissue Fe were measured. Animals on a high-Fe diet showed a significant increase (P < .001) in serum Fe (data not shown). When tissue Fe was examined in mice on a high-Fe diet, there was significantly (P < .001) increased Fe loading in the spleen and liver of MTf<sup>–/–</sup> and MTf<sup>+/+</sup> genotypes compared with animals given the basal Fe diet (Table 1). However, there was no difference in the response to the high-Fe diet between MTf<sup>–/–</sup> and MTf<sup>+/+</sup> mice, both genotypes becoming similarly Fe loaded (Table 1). Hence, the MTf null allele did not have any effect on Fe homeostasis.

The absence of any phenotype in MTf<sup>–/–</sup> animals was surprising but not without precedent, because genetic disruption of lactoferrin did not result in an altered phenotype. The fact that disruption of either Tf homolog results in no phenotype suggests these molecules do not play essential roles in Fe metabolism and may have other functions.

The current investigation supports in vitro studies demonstrating that MTf did not play an important role in Fe uptake. Collectively, our results indicate MTf is not essential for Fe metabolism in either normal or Fe-overloaded animals.

Acknowledgments

We thank Prof. Peter Gunning (Westmead Children’s Hospital, Sydney) for discussions relating to vector design and strategy. We thank Dr. David Lovejoy, Dr. Ralph Watts, and Miss Danuta Kalinowski of the Iron Metabolism and Chelation Program for their kind help in reviewing this paper prior to submission. We thank veterinary pathologists Dr. J. Schuh (Applied Veterinary Pathobiology, Bainbridge Island, WA) and Dr. John W. Finney (Institute of Medical and Veterinary Science, Adelaide, South Australia) for their independent assessments of tissues from MTf<sup>–/–</sup> and MTf<sup>+/+</sup> mice. Children’s Cancer Institute Australia for Medical Research is affiliated with the University of New South Wales and Sydney Children’s Hospital.

References

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