

**WHY THYROID HORMONE TRANSPORTERS ARE IMPORTANT**

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**ABSTRACT**

Thyroid hormone (TH) plays an essential role in the proper development of the brain and peripheral tissues. Lack of sufficient TH results in abnormal development, including mental retardation. It has become clear that TH transporters are necessary for proper TH metabolism and action inside the cell. Different specific TH transporters are known to date including MCT8, MCT10 and OATP1C1. MCT8 and MCT10 are widely expressed throughout the body, whereas expression of OATP1C1 is rather restricted to specific areas in the brain and testis. The clinical importance of TH transporters is dramatically shown in patients with mutations in *MCT8*, suffering from severe psychomotor retardation in combination with disturbed TH levels, especially high serum T<sub>3</sub> levels. No patients have been identified yet with mutations in *MCT10* or *OATP1C1*. *Mct8* deficient mice show no overt neurological deficits but have the same marked disturbed TH serum levels. Apparently mice have a different subset of TH transporters important for TH transport into the brain. It is expected that more TH transporters will be identified to explain the cell-specific subsets of TH transporters in normal tissue and brain. (*Hot Thyroidol. 2009: e14*).

**Key-words:** thyroid hormone; transport; MCT8; MCT10; OATP1C1; brain; development.

## Introduction

Thyroid hormone (TH) is important for the foetal growth and development of different tissues, especially the brain, and for the regulation of the basal metabolic rate throughout life. Disturbances in TH supply due to, for example, maternal iodine deficiency during foetal development cause severe neurological abnormalities in the neonate (1). However, also in the postnatal period TH is essential for further development of the brain, and in many countries neonatal screening programs have been instituted to detect congenital hypothyroidism and to prevent mental retardation by early supplementation of TH (2).

The biological effects of TH are mediated by binding of the active form  $T_3$  to its nuclear receptor, resulting in a change of interaction of the receptor with  $T_3$ -responsive elements in regulatory regions of the target genes (3). The thyroid itself produces predominantly  $T_4$ , which is converted to  $T_3$  through outer ring deiodination by the deiodinase D1 or D2.  $T_4$  is also metabolized to receptor-inactive  $rT_3$  or  $T_3$  is inactivated to  $3,3'$ - $T_2$  through inner ring deiodination by D3. TH availability is regulated by these three different deiodinases (4). D1 is expressed in liver, kidney and thyroid and is assumed to contribute to the production of serum  $T_3$  and clearance of serum  $rT_3$ . D2 is important for local production of  $T_3$  in the central nervous system but may also contribute to the production of serum  $T_3$ . D3 is expressed in adult brain and skin, and at high levels in multiple foetal tissues as well as in the placenta and the uterus during pregnancy. D3 is only capable of degrading TH and is thus important for the negative control of both tissue and serum  $T_3$  levels.

The biological activity of TH is determined by the intracellular  $T_3$  concentration available for binding to its nuclear receptor, and this depends on a) the circulating concentrations of  $T_4$  and  $T_3$ , b) the activities of the different deiodinases catalyzing the production or degradation of  $T_3$  and c) the presence of transporters regulating TH specific uptake and/or efflux. Although it has been thought for a long time that the lipophilic THs are capable of crossing the plasma membrane by simple diffusion, it has become increasingly clear that this is impossible without transporters (5).

## Existence of TH transporters

Studies already published in the 1970s by Krenning *et al* (6) and Rao *et al* (7), have shown saturable and energy-dependent transport of T<sub>3</sub> and T<sub>4</sub> into rat hepatocytes. Since then different research groups have reported studies confirming carrier-mediated, mostly energy- and Na<sup>+</sup>-dependent transport of TH into a variety of cells from different species.

Fifteen years ago, we decided to use the *Xenopus laevis* oocytes expression system, at that time the most successful method known for cloning and characterization of plasma membrane transporters, to find the long-sought Na<sup>+</sup>-dependent hepatic TH transporter (8). For this purpose, *X. laevis* oocytes were injected with rat liver mRNA and analyzed for TH transport. Only a modest increase in TH uptake was found in oocytes injected with different mRNA size fractions, but we were not successful in cloning a single TH transporter. The investigations were shifted towards functional screening of already known transporters for homologous ligands still using the oocytes expression system. Using this approach, we were successful this time as we identified different candidates as potential TH transporters within the organic anion, fatty acid and amino acid transporter families (9).

In the last decade, different groups have identified transporters that are capable of transporting TH (9, 10). One specific transporter is the Na<sup>+</sup>-taurocholate cotransporting polypeptide (NTCP) (11). Although NTCP is Na<sup>+</sup>-dependent and exclusively expressed in liver it appears not to be the long-sought hepatic TH transporter as it has a low affinity for TH. Also different members of the Na<sup>+</sup>-independent organic anion transporting polypeptide (OATP) family were characterized as TH transporters although with low affinity (12). Most of the OATP family members are widely expressed and multi-specific accepting a wide variety of ligands. A notable exception is OATP1C1, which is almost exclusively expressed in brain, in particular in capillary endothelial cells and in the choroid plexus, and shows high specificity for only T<sub>4</sub> and rT<sub>3</sub> (13-15). Recently, van der Deure *et al* reported that also T<sub>4</sub> sulphate (T<sub>4</sub>S) is transported by OATP1C1 (16). This transporter is thought to be very important for the transport of T<sub>4</sub> across the blood-brain-barrier (BBB) into the brain. The heterodimeric L-type amino acid transporters, LAT1 and LAT2 are capable as well to facilitate Na<sup>+</sup>-independent cellular entry and efflux of TH, but show restricted tissue distributions (17). We also found that fatty

acid translocase (FAT) expression in oocytes induces TH uptake (18). However, FAT (also known as CD36) is not a true transporter but may facilitate TH transport by forming a complex with neighbouring transporters.

### Identification of MCT8 as TH transporter

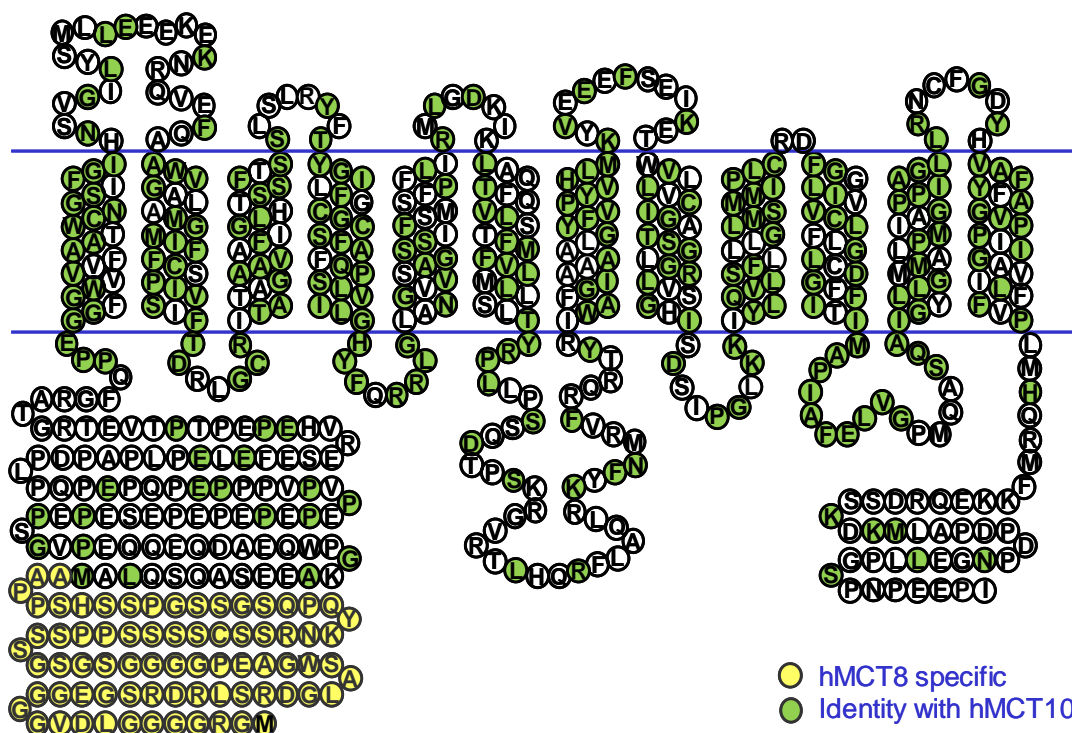
Due to the structural relation between aromatic amino acids and THs, the group of Blondeau and Francon (19, 20) had already suggested in the 1990s the involvement of a T-type amino acid transporter in the uptake of TH. Such a transporter has been cloned and characterized by Kim *et al* in 2001 (rat, (21)) and 2002 (human, (22)), termed T-type amino acid transporter 1 (TAT1), later referred to MCT10 (SLC16A10). This transporter is a member of a larger family of monocarboxylate transporters (23, 24). The family name is derived from the preference of the first 4 members (MCT1-4) for the substrates lactate and pyruvate. Kim *et al* have clearly shown the transport of L-DOPA and the aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) (21). However, they could not find any transport of THs by MCT10. Within the MCT family, MCT10 and MCT8 share the highest level of amino acid sequence identity (49%). So, we hypothesized that MCT8 could be the T-type amino acid transporter that also accepts THs.

In close collaboration with Prof Andrew Halestrap we studied rat MCT8 in our former *X. laevis* oocyte expression system and we found an incredible 10-fold induction in TH transport (25). This was by far the best TH transporter we had studied up till now. Substrates studies revealed that MCT8 showed ligand specificity exclusively towards iodothyronines; sulphated THs, lactate, Leu and the aromatic amino acids Phe, Tyr and Trp were not transported.

The gene coding for human *MCT8* is located on the X-chromosome (Xq13.2) and consist of 6 exons (26). The putative structure of MCT8 consists of 12 transmembrane domains and both the N- and the C-terminus are located intracellular (see Fig 1). In contrast to most species, including the mouse and rat, the human *MCT8* gene contains two possible translation start sites (TLSs). Depending on which of these TLSs is used, proteins are generated of 613 (hMCT8L) or 539 (hMCT8S) amino acids *in vitro*. Preliminary studies using human liver revealed the presence of mRNA species containing both TLSs. The function of the additional N-terminal sequence in hMCT8L

is still under investigation. The N-terminal domain in both hMCT8 isoforms is enriched in Pro (P), Glu (E), Ser (S) and Thr (T), also known as PEST domain (27). PEST domains are often associated with rapid protein degradation, but the function of this domain in MCT8 is yet unknown.

In the meantime we changed our expression system from *X. laevis* oocytes to mammalian cells like COS1 (Green monkey kidney cells) or JEG3 (human choriocarcinoma cells).



**Figure 1.** Putative structure of human MCT8. In yellow, the hMCT8 specific structure is indicated; in green, amino acid identity between hMCT8 and hMCT10 is indicated. Both the N- and C-terminus are located intracellular.

Transfection with hMCT8S results in a significant increase in TH transport but the fold induction was much lower than observed in oocytes (28). Further studies revealed the capacity of hMCT8S to mediate efflux even faster than the uptake of TH. To prevent the rapid efflux of TH in our transport studies we co-transfected our cells with mu-crystallin, a cytosolic TH binding protein. When doing so, we increased the uptake signal to the same level as what we had found earlier in our *X. laevis* oocyte expression system. To demonstrate that MCT8 increases the intracellular availability of TH for metabolism by the different deiodinases, cells were co-transfected with MCT8 and for instance D3 to

measure  $T_3$  metabolism. The results showed that expression of a TH transporter markedly increased intracellular TH metabolism.

MCT8 is widely expressed in liver, heart, intestine, placenta, kidney and brain (29, 30). Detailed studies of the mouse brain by the groups of Heuer *et al* (31) and Roberts *et al* (32) revealed high MCT8 expression in neuronal populations of the cerebral and cerebellar cortex, hippocampus, striatum and hypothalamus. This suggests that MCT8 is involved in neuronal TH transport. MCT8 is also expressed in the choroid plexus and in large capillaries indicating its involvement in the transport of TH across the BBB and/or blood-cerebrospinal fluid (CSF) barrier. Studies from Alkemade *et al* have shown that at the interface of the human hypothalamus and the peripheral circulation, MCT8 protein is present particularly in neurons of the paraventricular and infundibular nuclei (33). Also strong MCT8 expression has been observed in tanycytes that are located in the central lining of the third ventricle. These cells are in close contact with the CSF and the hypothalamus and median eminence. As OATP1C1 and D2 are expressed in these tanycytes, conversion of  $T_4$  to  $T_3$  in these cells plays an important role in the negative feedback of TH at the hypothalamus (31).

### Pathophysiology of human MCT8

In 2001, the group of Grueters in Berlin and our group in Rotterdam investigated two apparently identical severely mentally retarded male patients with abnormally high serum  $T_3$  levels. Since no mutations were found in the genes coding for the  $T_3$  receptors or the different deiodinases, we raised the hypothesis that this syndrome of TH resistance was caused by a defect in cellular TH uptake. Therefore, we screened the *MCT8* gene for mutations. In the first patient we could not amplify the first exon of the *MCT8* gene and later results showed the exact deletion of 24 kb, comprising part of the 5'-UTR, entire exon 1 and also a part of intron 1. Family investigation revealed that the mother was carrier of this deletion and that one of her other sons was also affected with this deleterious mutation in *MCT8*. In the *MCT8* gene of the second patient we found a missense mutation in the second exon resulting in an Ala224Val substitution (34).

Since 2004, more than 40 families have been described with hemizygous affected males carrying mutations in the *MCT8* gene (34-43). Recently, a sporadic case of a female carrying a *de*

*novo* translocation that disrupted the *MCT8* gene in combination with unfavorable nonrandom X-inactivation has been reported (44). The reported mutations range from large deletions, resulting in the loss of one or more exons, smaller frame-shift deletions, triplet (1-amino acid) deletions or insertions, nonsense mutations resulting in a premature truncation of the MCT8 protein, and missense mutations resulting in 1-amino acid substitutions. All patients share the severe neurological deficits and markedly elevated serum T<sub>3</sub> and low T<sub>4</sub> and rT<sub>3</sub> levels. The neurological phenotype includes in most patients central hypotonia, with poor head control; initially peripheral hypotonia, which evolves into spastic quadriplegia; inability to sit, stand or walk independently; severe mental retardation; and absence of speech (45). This severe form of X-linked psychomotor retardation had already been described in 1944 by Allan, Herndon and Dudley (46), since then referred as AHD syndrome (OMIM 300523).

We have tested a variety of the mutations found in patients with AHDS, especially the 1-amino acid deletions, insertions and substitutions as for these mutations it is not clear what the effect will be on the proper function of MCT8 (37, 43, 47). To test the effect of the mutation, we compared the function of the mutated with the wild-type hMCT8S using transiently transfected mammalian cell lines. TH transport was measured in cells transfected with the different variants of MCT8 alone, and metabolism of T<sub>3</sub> was measured in cells co-transfected with the different transporter variants and D3. The results from both tests showed that most mutations resulted in a complete loss of hMCT8S transport function, but significant residual activity was observed with a few MCT8 mutations, associated with a somewhat milder clinical phenotype. Thus, with the functional analysis of MCT8 mutations found in patients with AHDS we found impaired uptake and subsequently impaired metabolism of TH *in vitro*. These results predict that mutations in a TH transporter result in an impaired tissue TH supply especially into the brain and represent a novel mechanism for TH resistance.

### ***Mct8* deficient mice**

To study the pathophysiology of MCT8 deficiency Dumitrescu *et al* (48) and Trajkovic *et al* (49) have generated independently two different *Mct8* knockout mouse strains. Unexpectedly, these

mouse mutants do not show any overt neurological deficits, but they exhibit the same marked increase in serum  $T_3$  and decrease in serum  $T_4$  and  $rT_3$  as found in the AHDS patients with mutations in *MCT8*. Analysis of the mutant mouse liver showed an increased activity of D1 as well as an increased  $T_3$  content. This indicates that the liver is in a hyperthyroid state. In contrast, the  $T_4$  and  $T_3$  content in the brain was diminished and associated with an increase in D2 activity and a decrease in D3 activity, reflecting a hypothyroid state of this tissue. In the mutant mouse brain  $T_4$  entry was not affected as  $T_3$  uptake was almost completely diminished. Recently, comprehensive studies on the *Mct8*-deficient mice revealed several behavioral abnormalities (50) as decreased anxiety-related behavior reported in hyperthyroid mice and, in contrast, reduced grooming and increased latency of grooming reported in hypothyroid rats. This indicates that also certain brain areas may be hyperthyroid, whereas other areas remain hypothyroid in *Mct8* knockout mice.

Apparently, *Oatp1c1* is involved in the specific transport of  $T_4$  via the BBB or blood-CSF barrier into the mouse brain (15, 32). The local conversion of  $T_4$  to  $T_3$  in the mouse brain is sufficient to provide neuronal cells as cerebellar Purkinje cells with enough TH to prevent serious neurological damage, as they show normal dendritic outgrowth and responded normally to  $T_3$  treatment *in vitro*. Studies by Ceballos *et al* (51) reported that the brains of *Mct8* mutant mice do not respond to a low dose of  $T_3$  due to the critical restriction of  $T_3$  transport into the brain via the BBB rather than at the plasma membrane of a neuronal cell. The fact that *Mct8* mutant mice do not show any overt neurological deficits could be explained by a different subset of TH transporters in the mouse brain when compared to the human brain leading to a less severe TH deficiency in mouse brain. Wirth *et al* (50) speculate that the L-type amino acid transporter *Lat2* might compensate in the mouse, but not in the human brain for the lack of *Mct8* as only a low *LAT2* expression was found in developing neurons in the human brain. Also, the rat brain expresses the TH transporter variants *Oatp1a4* and *Oatp1a5* (52), but both transporters have to our knowledge no ortholog in the human brain.

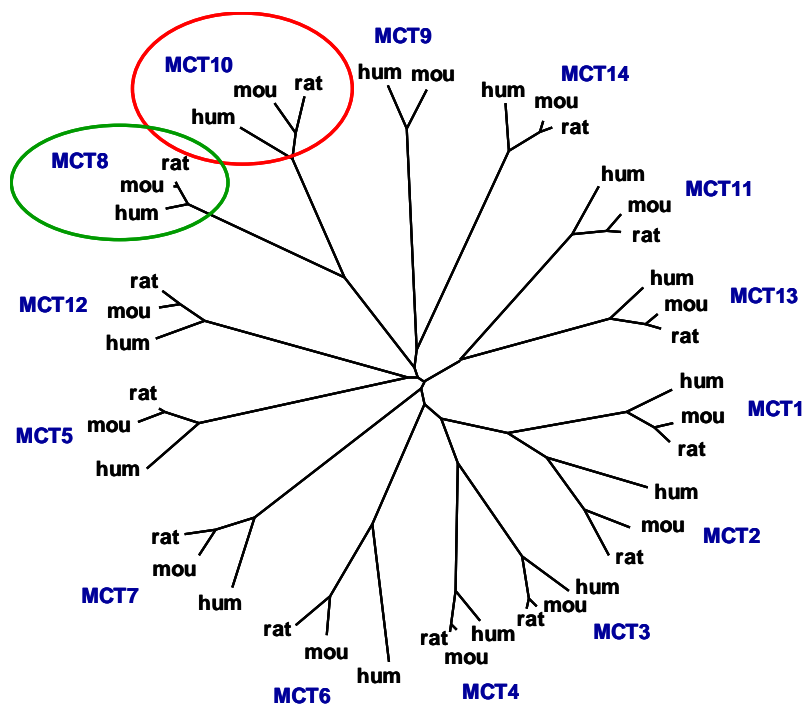
Recently, Di Cosmo *et al* (53) reported the use of the ligand 3,5-diiodothyropropionic acid (DITPA) as available analogue of TH bypassing the involvement of *MCT8* to be transported into different target tissues. Using the *Mct8* mutant mouse they found that DITPA is relatively *MCT8* independent for entry into the hypothyroid brain, and normalizes the thyrotoxic state of the liver,

resulting in the achievement of an overall euthyroid state. According to the authors, the clinical use of DITPA in MCT8 patients needs further studies.

### Identification of human MCT10

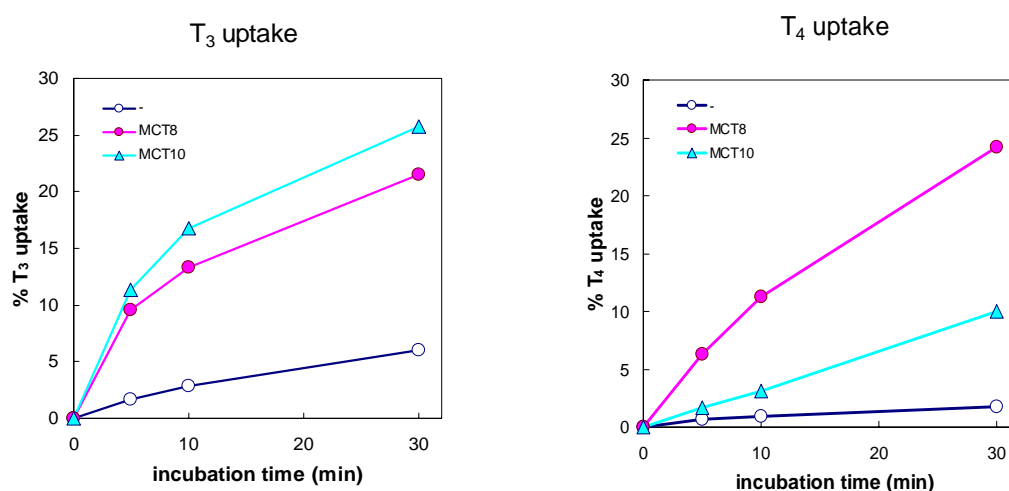
The human *MCT10* gene is located on chromosome 6q21-q22 and has the same structure as the human *MCT8* gene. The mRNA contains one single TLS and codes for a protein of 515 amino acids, containing 12 transmembrane domains (see Fig 1). Also the MCT10 protein contains a PEST domain as described for MCT8. Based on its expression pattern human MCT10 may be important for TH transport specifically in intestine, kidney, liver, skeletal muscle and placenta (21, 22, 30). Ramadan *et al* (54) reported on the function of mouse MCT10 as a net efflux pathway for aromatic amino acids and showed localization of the protein to the basolateral membrane of small intestine and proximal kidney tubule cells.

In view of the involvement of a T-type amino acid transporter in uptake of TH and due to the homology between MCT8 and MCT10 (see Fig 2), we decided to reinvestigate the possible transport of TH by MCT10.



**Figure 2.** Phylogenetic tree of the monocarboxylate transporter (MCT) family. In the green cycle are the MCT8 members located and in the red cycle the closely related MCT10 members.

Our studies showed clear TH transport capacity by MCT10 showing preference of  $T_3$  over  $T_4$  (55). Like MCT8 also MCT10 facilitates TH uptake as well as efflux. As with MCT8, cells co-transfected with MCT10 and one of the deiodinases largely stimulated the intracellular deiodination of TH. Uptake of  $T_3$  in cells transfected with MCT10 was significantly inhibited by the aromatic amino acids. Also the uptake of  $T_3$  was more inhibited by an excess of different iodothyronines in cells transfected with MCT10 than MCT8 (personal observation).



**Figure 3.** Transport of  $T_3$  or  $T_4$  into cells transfected with hMCT8 or hMCT10. Uptake of TH was measured in the presence of mu-crystallin, a cytosolic TH binding protein (55).

Together with its high homology with MCT8, it is very likely that MCT10 could be an important transporter in human physiology by regulating local and tissue TH levels. But so far, no patients with mutations in MCT10 have been identified.

### Concluding Remarks

With the discovery of patients with mutations in MCT8 the physiological importance of transporters for the metabolism and action of TH has been generally recognized. Next to MCT8, also MCT10 and OATP1C1 are characterized as specific TH transporters (see Table 1). It is clear that more TH transporters will be identified in the near future, because the  $Na^+$ -dependent, high affinity TH transporter expressed in liver has not yet been discovered. From mouse and human studies it is also

confirmed that different subsets of TH transporters are present in the two species, especially in specific areas of the brain.

**Table 1.** Important candidates for specific TH transport.

	<b>hMCT8</b>	<b>hMCT10</b>	<b>OATP1C1</b>
Function	Uptake/efflux T <sub>3</sub> + T <sub>4</sub>	Uptake/efflux T <sub>3</sub> >T <sub>4</sub> Export of aromatic amino acids	Uptake T <sub>4</sub> + rT <sub>3</sub> > T <sub>4</sub> Sulphate
Chromosome	X	6	12
Physiological relevance	AHDS: mental retardation + elevated serum T <sub>3</sub> levels	Unknown	Unknown
Tissue expression	Liver, kidney, heart, skeletal muscle, brain, thyroid, placenta	Intestine, liver, kidney, skeletal muscle, placenta	Blood-brain-barrier brain capillaries, testis

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