Monocarboxylate Transporter 10 Functions as a Thyroid Hormone Transporter in Chondrocytes

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Thyroid hormone is essential for normal proliferation and differentiation of chondrocytes. Thus, untreated congenital hypothyroidism is marked by severe short stature. The monocarboxylate transporter 8 (MCT8) is a highly specific transporter for thyroid hormone. The hallmarks of Allan-Herndon-Dudley syndrome, caused by MCT8 mutations, are severe psychomotor retardation and elevated T₃ levels. However, growth is mostly normal. We therefore hypothesized that growth plate chondrocytes use transporters other than MCT8 for thyroid hormone uptake. Extensive analysis of thyroid hormone transporter mRNA expression in mouse chondrogenic ATDC5 cells revealed that monocarboxylate transporter 10 (Mct10) was most abundantly expressed among the transporters known to be highly specific for thyroid hormone, namely Mct8, Mct10, and organic anion transporter 1c1. Expression levels of Mct10 mRNA diminished with chondrocyte differentiation in these cells. Accordingly, Mct10 mRNA was expressed most abundantly in the growth plate resting zone chondrocytes in vivo. Small interfering RNA-mediated knockdown of Mct10 mRNA in ATDC5 cells decreased [¹²⁵I]T₃ uptake up to 44% compared with negative control (P < 0.05). Moreover, silencing Mct10 mRNA expression abolished the known effects of T₃, i.e. suppression of proliferation and enhancement of differentiation, in ATDC5 cells. These results suggest that Mct10 functions as a thyroid hormone transporter in chondrocytes and can explain at least in part why Allan-Herndon-Dudley syndrome patients do not exhibit significant growth impairment. (Endocrinology 153: 4049–4058, 2012)

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hyroid hormone (TH) receptors are nuclear receptors.
It is therefore necessary for TH to enter the cell to exert its actions. Until recently, it was thought that TH passively enters the cell. However, from reports since the late 1970s showing that TH uptake is saturable, energy and Na⁺-dependent in various tissues/cells, it was reasoned that TH transport into the cell occurs via certain transporter proteins (1). Several transporter families have been identified at the molecular level including the Na⁺/taurocholate cotransporting polypeptide (2), fatty acid translocase (3), multidrug resistance-associated proteins (4), amino acid transporters (5) and members of the organic anion-transferring polypeptide (6), and monocarboxylate transporter (MCT) (7) families. Although these transporters have been shown to transport TH, with the exception of MCT8 (8), they do not exclusively transport TH and show affinity for other substrates such as amino acids.

However, transmembrane trafficking of thyroid hormone has gained much attention with the discovery that MCT8 is a TH-specific transporter (8) and that mutations

* S.A. and N.N. contributed equally to this work.
Abbreviations: AHDs, Allan-Herndon-Dudley syndrome; BCH, 2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid; BrdU, 5-bromo-2’-deoxy-uridine; Col2, type II collagen; Col10, type X collagen; E, embryonic day; FBS, fetal bovine serum; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; GP, growth plate; HZ, hypertrophic zone; ITS, insulin-transferrin-selenium; Lat, L-type amino acid transporter; MCT, monocarboxylate transporter; Mct10, monocarboxylate transporter 10; Oatp, organic anion transporter; PZ, proliferating zone; qRT, quantitative real-time (PCR); RZ, resting zone; siRNA, small interfering RNA; TH, thyroid hormone; TRα1, thyroid hormone receptor-α1.

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in this transporter result in Allan-Herndon-Dudley syndrome (AHDS), an X-linked syndrome characterized by severe mental and motor retardation (9–12). In AHDS patients, although severe neurological symptoms predominate, growth failure is only mildly retarded (13, 14). In contrast, in patients with untreated congenital hypothyroidism (cretinism), severe short stature results from delayed chondrocyte differentiation with delayed appearance of primary ossification centers. In vitro and in vivo experiments have shown that TH inhibits proliferation and promotes differentiation of chondrocytes and is dispensable for normal growth (15–17). Because AHDS patients grow well compared with their congenital hypothyroidism counterparts, we hypothesized that growth plate (GP) chondrocytes have means other than MCT8 for TH translocation across the plasma membrane. To date, little is known about TH transporters in bone. Williams et al. (18) have shown that Mct8 is expressed in mouse chondrogenic ATDC5 cells, primary mouse osteoblasts, mouse osteoblastic MC3T3-E1 cells, and primary osteoclasts. Capelo et al. (19) have demonstrated expression of L-type amino acid transporter (Lat) 1 and 2 in addition to Mct8 in MC3T3-E1 cells. However, it is not known whether these molecules function as physiologically relevant TH transporters in these cells.

In this paper, we show that monocarboxylate transporter 10 (Mct10) is most abundantly expressed among the transporters with high specificity for TH in ATDC5 cells as well as in murine GP resting zone (RZ) chondrocytes. Silencing of Mct10 mRNA resulted in reduced TH uptake and function in ATDC5 cells.

### Materials and Methods

#### Cell culture

Mouse chondrogenic ATDC5 cells were maintained in a 1:1 mixture of DMEM nutrient mixture F-12 (Sigma, St. Louis, MO) containing 5% fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX) and 1% insulin-transferrin-selenum (ITS) (Life Technologies, Inc., Carlsbad, CA) at 37°C in a humidified atmosphere of 5% CO2 in air. For subculture, 0.05% Trypsin-EDTA (Life Technologies) and PBS without Ca2+ or Mg2+ were used. For differentiation, ATDC5 cells were seeded in six-multiwell plates. After reaching confluency, differentiation was induced by changing the medium to αMEM (Wako, Osaka, Japan) with 5% FBS and 1% ITS and refreshing it every 2 d for up to 6 wk (20).

For the differentiation experiments using small interfering RNA (siRNA) transfected ATDC5 cells, rapid differentiation was induced by culturing the cells encapsulated in alginate beads. Briefly, trypsinized ATDC5 cells were washed once with 150 mM NaCl and 20 mM HEPES (pH 7.4) and suspended in alginate solution (1.25% alginate, 150 mM NaCl, 20 mM HEPES, 1 mM CaCl2) at a density of 1.0 × 10⁶ cells/ml. This mixture was dropped into 25 ml 100 mM CaCl2 and 5 mM HEPES using a disposable syringe and 22-gauge needle. After disposing the

### Table 1. Mouse primers for qRT-PCR (SYBR GREEN) analysis

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer</th>
<th>Name</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mct8 (NM_009197.2)</td>
<td>Forward, GGAGAACAGAAGAAGATGC</td>
<td>Oatp1c1 (NM_021471.2)</td>
<td>Forward, TCCTGGGGAAATCACAGAAC</td>
</tr>
<tr>
<td></td>
<td>Reverse, TCCATGCTACTAGAGGA</td>
<td></td>
<td>Reverse, GATAGGGCTCTCCTCCAAC</td>
</tr>
<tr>
<td>Mct10 (NM_001114332.1)</td>
<td>Forward, CAAAGGACGATGACAACATGG</td>
<td>Oatp2a1 (NM_033314.3)</td>
<td>Reverse, GTGCGTGAAACACTCACAGA</td>
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<tr>
<td></td>
<td>Reverse, GACGACAGAGGAGGAAGA</td>
<td>Reverse, CAGGACACCTGGCTTATTC</td>
<td>Reverse, ACAGCCACAGAGGGAAGA</td>
</tr>
<tr>
<td>Ntcp (NM_001177561.1)</td>
<td>Forward, GACGACGGAGGAGGAAGA</td>
<td>Oatp3a1 (NM_001038643.1)</td>
<td>Reverse, CAGGACACCTGGCTTATTC</td>
</tr>
<tr>
<td>Lat1 (NM_0011404.3)</td>
<td>Forward, GACGACAGAGGAGGAAGA</td>
<td>Oatp4a1 (NM_148933.1)</td>
<td>Reverse, CAGGACACCTGGCTTATTC</td>
</tr>
<tr>
<td>Lat2 (NM_016972.2)</td>
<td>Forward, GACGACAGAGGAGGAAGA</td>
<td>Oatp4c1 (NM_172658.3)</td>
<td>Reverse, CAGGACACCTGGCTTATTC</td>
</tr>
<tr>
<td>Oatp1a1 (NM_013797.5)</td>
<td>Forward, AGAGAAAAAGGTTGCAACAA</td>
<td>Oatp5a1 (NM_172841.2)</td>
<td>Reverse, GGGGATCATCCTCAGTCAGA</td>
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<tr>
<td></td>
<td>Reverse, CAAATCGGCATGATGATTTGG</td>
<td>Oatp5a1 (NM_172841.2)</td>
<td>Reverse, GATGGGATCATCCTCAGTCAGA</td>
</tr>
<tr>
<td>Oatp1a4 (NM_030687.1)</td>
<td>Forward, CAATTTCACGTTGCTGTAAT</td>
<td>Oatp6b1 (NM_01309475.1)</td>
<td>Reverse, TTGGGATCATCCTCAGTCAGA</td>
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<tr>
<td>Oatp1a5 (NM_130861.2)</td>
<td>Reverse, CAGCGACGACGACGAGGAGG</td>
<td>Oatp6c1 (NM_028942.4)</td>
<td>Reverse, CAAATCGGCATGATGATTTGG</td>
</tr>
<tr>
<td>Oatp1a6 (NM_023718.3)</td>
<td>Forward, GACGACAGAGGAGGAAGA</td>
<td>Oatp6d1 (NM_027584.1)</td>
<td>Reverse, TTGGGATCATCCTCAGTCAGA</td>
</tr>
<tr>
<td>Oatp1b2 (NM_020495.1)</td>
<td>Reverse, GACGACAGAGGAGGAAGA</td>
<td>Reverse, GGGGATCATCCTCAGTCAGA</td>
<td>Reverse, TTGGGATCATCCTCAGTCAGA</td>
</tr>
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Ntcp, Na+ /taurocholate cotransporting polypeptide.
CaCl₂ solution, the cells were washed once with DMEM/F12, placed in six-multiwell plates with DMEM/F12 containing 10% FBS and 1% ITS, and cultured for up to 3 d. For recovery, the differentiation medium was discarded and the beads were washed with 150 mm NaCl, 20 mM HEPES (pH 7.4). The beads were then dissociated in releasing buffer (50 mM citric acid, 150 mM NaCl, 20 mM HEPES (pH 7.4) and pelleted by centrifugation (21, 22). The serum used in these experiments was charcoal stripped to eliminate the effects of endogenous TH (23).

Mice

Animal protocols were approved by the Osaka University Graduate School of Medicine Animal Care and Use Committee. C57/BL6 mice (SLC, Hamamatsu, Japan) were maintained under pathogen-free conditions and fed ad libitum. To obtain materials for in situ hybridization studies, pregnant dams on embryonic day (E) 18 were humanely killed by carbon dioxide asphyxiation. Humeri including the GP were excised and were fixed overnight in 4% paraformaldehyde at 4 C. They were then washed three times in PBS and cryoprotected overnight in 30% sucrose/PBS. Subsequently they were embedded in optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan), snap frozen in liquid nitrogen, and then cryosectioned at 14 μm thickness (24).

TH transporter mRNA detection and quantification in ATDC5 cells

Total RNA was extracted from ATDC5 cells using an RNeasy minikit (QIAGEN, Hilden, Germany), and cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Expression of TH transporters reported to date was screened by PCR (Table 1). Detected transporters were quantified by SYBR GREEN quantitative real-time (qRT)-PCR (Roche Applied Science, Mannheim, Germany). All primers for RT-PCR and SYBR GREEN qRT-PCR were designed to span an intron and to result in approximately 100-bp amplicons. The PCR products were cloned into pCR4-TOPO (Invitrogen) and subsequently used to obtain standard curves. The sequences of the clones were confirmed by direct sequencing (Applied Biosystems, Foster City, CA). Gene expression levels were calculated as number of copies per nanogram RNA. In addition, transporters that have been reported to have high specificity for TH, namely Mct8, Mct10, and organic anion transporter (Oatp) 1c1 (25), were quantified by TaqMan qRT-PCR on the 7900 qRT-PCR System (Applied Biosystems). Gene expression levels were quantified by qRT-PCR (TaqMan) and normalized to 18S rRNA. Data from representative experiments (each n = 3) are shown as mean ± sd. A, Knockdown efficiency in 96-well plate cultures. B, Knockdown efficiency in alginate bead cultures. Both Mct10-1 and Mct10-2 siRNA decreased Mct10 mRNA expression to 30% or less compared with negative control siRNA in all the conditions tested.

In situ hybridization

Antisense and sense digoxigenin-labeled riboprobes were generated from pCR4-TOPO plasmids (Invitrogen) carrying mouse cDNA sequences for transporters as follows: Slc16a10 (Mct10; NM_001114332.1) bp 596-1664 (26); Slc16a2 (Mct8; NM_009197.2) bp 109-1882; and Slc7a5 (Lat1; NM_011404.3) bp 1076–2024. Hybridization was performed overnight at 70 C. Anti-digoxigenin antibody (alkaline phosphatase conjugated) (Roche) was preabsorbed with E13.5 embryo powder before overnight incubation of the sections. Precipitating BM purple AP substrate (Roche Applied Science) was used as a substrate. The specificity of each probe was verified by parallel analysis with a corresponding sense probe (24). Image brightness and contrast were adjusted using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA). All modifications were applied equally to the images.

Silencing of Mct10 mRNA expression

Mct10 mRNA expression in ATDC5 cells was suppressed by using two different Mct10 siRNA (Silencer Select predesigned siRNA, product no. 4390771, siRNA ID: s91015, s91016; Ambion, Austin, TX). The positive control was glyceraldehyde-3-phosphate dehydrogenase (Gapdh) siRNA (Silencer Select Gapdh siRNA, product no. 4390849; Ambion). As negative control, Negative Control siRNA (Silencer Select Negative Control no. 1 siRNA, product no. 4390843; Ambion) with limited homology to known genes was used.

ATDC5 cells were transfected using the reverse transfection method according to the manufacturer’s methods. Briefly, 60 nM siRNA and 6 μl/ml siPORT amine transfection agent (Ambion) were added to suspended ATDC5 cells (200,000 cells/ml in DMEM/F12 containing 5% FBS and 1% ITS) and subsequently plated on 10-cm, six- or 96-multiwell plates. For the alginate

FIG. 1. Efficiency of siRNA mediated knockdown. Mct10 or Gapdh expression levels were quantified by qRT-PCR (TaqMan) and normalized to 18S rRNA. Data from representative experiments (each n = 3) are shown as mean ± sd. A, Knockdown efficiency in 96-well plate cultures. B, Knockdown efficiency in alginate bead cultures. Both Mct10-1 and Mct10-2 siRNA decreased Mct10 mRNA expression to 30% or less compared with negative control siRNA in all the conditions tested.
bead cultures, after culturing the transfected cells for 24 h at 37 C, the cells were trypsinized, counted, and treated as described above.

To verify knockdown efficiency in ATDC5 cells transfected with Mct10–1, Mct10–2, and Gapdh siRNA, Mct10 and Gapdh mRNA was quantified by TaqMan qRT-PCR at d 1, 3, and 5 after transfection for the 96-multiwell cultures (Fig. 1A) and d 1–4 after transfection for the alginate bead cultures (Fig. 1B). The values were corrected with amounts 18S rRNA. The primer and probe sets for the genes were as follows: Mct10, described above; Gapdh (Gapdh; NM_008084.2), 4352932E; RN18S1 (Eukaryotic 18S rRNA; NR_003286.2), 4319413E (Applied Biosystems).

**T3 uptake into ATDC5 cells**

Mct10 or negative control siRNA-transfected cells were plated onto six-multiwell plates (200,000 cells/well). Twenty-four hours after transfection, the medium (DMEM/F12 containing 5% FBS) was refreshed, and the cells were cultured for another 18 h at 37 C. After a wash with DMEM/F12 containing 0.1% BSA, the cells were incubated for 30, 60, 90, or 120 min at 37 C with 1.0 × 10−9 M (7.3 × 106 cpm) triiodothyronine [L-(3,5,3′-32I)-(125I)T3] (PerkinElmer, Waltham, MA) in 1.5 ml DMEM/F12 with 0.1% BSA. After incubation, cells were washed twice with DMEM/F12 containing 0.1% BSA and lysed with 0.1 M NaOH, and the radioactivity of the lysate was counted with a scintillation γ-counter (Aloka, Tokyo, Japan) (8, 27).

To inhibit Lat1, 2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid (BCH) (Sigma) was used at 1 mM [three times inhibitory constant (28)] (29). Subconfluent ATCD5 cells in six-multiwell dishes were washed with DMEM/F12 containing 0.1% BSA and incubated for 30, 60, 90, or 120 min at 37 C in DMEM/F12 containing 0.1% BSA, 1.0 × 10−9 M [125I]T3 (PerkinElmer), and 1 mM BCH or vehicle. After incubation, the radioactivity of the cell lysate was measured as described above.

**Cell proliferation assays**

ATDC5 cells were seeded in 96-multiwell plates on d 0 (12,000 cells/well) and maintained in the absence or presence of

**FIG. 2.** Expression of TH transporters in ATDC5 cells. A, Quantification by qRT-PCR (SYBR GREEN). The experiment was performed two times. B, Quantification by qRT-PCR (TaqMan). The experiment was performed four times. Data from representative experiments (each n = 3) are shown as mean ± SD.

**FIG. 3.** Mct10 and Mct8 mRNA expression during ATDC5 differentiation. Expression of Mct10, Mct8, and chondrocyte differentiation markers (Col2 and Col10) were analyzed by qRT-PCR (TaqMan), whereas ATDC5 cells were differentiated for up to 6 wk. Mct10 and Mct8 are presented as copies per nanogram RNA. Col2 and Col10 were normalized to 18S rRNA.
T₃ (10⁻⁸, 10⁻⁹ M) (Sigma) for 5 d. Cell proliferation was examined by counting cell numbers using a hemocytometer and by 5-bromo-2′-deoxyuridine (BrdU) uptake (5-bromo-2′-deoxyuridine Labeling and Detection Kit III; Roche Applied Science) on d 1, 3, and 5 after plating the cells. Charcoal-stripped serum was used for this experiment to eliminate endogenous TH (23).

Analysis of ATDC5 differentiation

ATDC5 cells were differentiated, total RNA was extracted, and cDNA was synthesized with reverse transcriptase according to the methods described above. The change in the levels of Mct10 and Mct8, as well as markers of chondrocyte differentiation, type II collagen (Col2), and type X collagen (Col10), was examined by TaqMan qRT-PCR. Gene expression levels of Mct10 and Mct8 were calculated as number of copies per nanogram RNA. Values of Col2 and Col10 were corrected with amounts 18S rRNA. The primer and probe sets for the genes were as follows: Mct10 and Mct8, described above; Col2a1 (Col2; NM_001113515.2), Mm00491889_m1; Col10a1 (Col10; NM_009925.4), Mm00487041_m1; 18S rRNA, described above (Applied Biosystems).

To evaluate differentiation in siRNA-transfected cells, ATDC5 cells were rapidly differentiated by culturing in alginate beads as described above. Total RNA was extracted and expression of Col10, a marker of hypertrophic chondrocytes, was evaluated by TaqMan qRT-PCR. The values were corrected with amounts 18S rRNA. The primer and probe sets were the same as the ones listed above. The serum used for this experiment was charcoal stripped to eliminate endogenous TH (23).

Statistical analysis

If not stated otherwise, data are expressed as mean ± SD. Statistical significance between groups was determined with the Student’s t test. P < 0.05 was considered as significant. All statistical analyses were conducted using JMP software version 8.0.2 (SAS Institute, Cary, NC).

Results

Mct10 is the most abundantly expressed transporter with high specificity for TH in ATDC5 cells

By comprehensively screening all TH transporters reported up to date with RT-PCR in nondifferentiated ATDC5 cells, we detected Mct8, Mct10, Oatp1a5, Oatp1a6, Oatp2a1, Oatp3a1, Oatp4a1, Oatp4c1, Oatp5a1, Lat1, and Lat2. Quantification by qRT-PCR (SYBR GREEN) demonstrated that the most prominently expressed transporter was Lat1, followed by Mct10 (Fig. 2A). Because Oatp1c1, one of the three TH transporters known to have high specificity for TH and critical for this study, was not detected by SYBR GREEN qRT-PCR, the three [Mct8, Mct10, and Oatp1c1 (25)] were also quantified by TaqMan qRT-PCR. Expression of Mct10 was the highest with mRNA ratios of Mct10 to Mct8 to Oatp1c1 = 46:1:0 (Fig. 2B). The abundance of mRNA expression taken together with the high specificity for TH suggested that Mct10 functions as a TH transporter in chondrocytes.

Mct10 decreases with ATDC5 differentiation

Although nondifferentiated ATDC5 cells resemble chondroprogenitor cells,
it was not clear how chondrocytic differentiation might influence Mct10 expression. To address this question, we differentiated ATDC5 cells and examined Mct10 mRNA expression. Quantification by qRT-PCR (TaqMan) every week up to 6 wk showed that Mct10 mRNA expression was most prominent in chondroprogenitors, which decreased 56% at 6 wk. In contrast, expression of Mct8 mRNA increased with differentiation (Fig. 3).

Mct10 is expressed most strongly in the RZ chondrocytes

We next used in situ hybridization analysis to further characterize Mct10 and Mct8 as well as Lat1 expression in vivo using E18 humeri (Fig. 4). Mct10 mRNA was most abundant in the RZ chondrocytes, whereas chondrocytes differentiating into the secondary ossification center, the proliferating zone (PZ) as well as the hypertrophic zone (HZ) chondrocytes expressed lower levels of Mct10 than the RZ chondrocytes (Fig. 4, top row). Nonspecific staining in the HZ chondrocytes, primary spongyosa, and the bone marrow was observed with four other probes we tested [Slc16a10 (Mct10; NM_001114332.1) bp 222-1757, bp 521-1220, bp 14–1128, and bp 14–1784 (data not shown). In a reciprocal manner, levels of Mct8 mRNA increased with differentiation. The strongest expression was detected in the HZ chondrocytes (Fig. 4, middle row). The analysis also revealed that the Lat1 signal intensified with chondrocyte maturation (Fig. 4, bottom row). The main sites of TH action in the GP are thought to be chondroprogenitor cells, RZ, and PZ chondrocytes because thyroid hormone receptor-α1 (TRα1), the major TH receptor in the GP, is expressed in these cells but not in HZ chondrocytes (15, 16). The above results, taken together with those from the ATDC5 cells, imply that Mct10 mRNA is expressed in early-stage chondrocytes concomitantly with TRα1.

Mct10 transports T₃ into ATDC5 cells

If Mct10 functions as a TH transporter in ATDC5 cells, knocking down Mct10 expression should result in reduced TH uptake into these cells. To examine this, we used siRNA specific to Mct10 (Mct10–1 siRNA, Mct10–2 siRNA) to reduce Mct10 mRNA expression. Addition of 1.0 × 10⁻⁹ m (7.3 × 10⁶ cpm) [¹²⁵I]T₃, the physiological concentration of this hormone followed by TH uptake measurements at 30-min intervals revealed that T₃ influx into Mct10 siRNA-transfected ATDC5 cells was decreased up to 44% compared with that of cells transfected with negative control siRNA (Fig. 5A). In contrast, 1 mM BCH had no effect on TH intake (Fig. 5B) demonstrating that, although more abundantly expressed than Mct10, in the presence of physiological levels of amino acids, Lat1 plays a minimal role in transporting TH into ATDC5 cells. Taken together, these results indicate that Mct10 mediates a significant portion of TH transport in ATDC5 cells. To further investigate whether Mct10 actually plays a physiological role in these cells, the effect of silencing Mct10 on TH action was investigated.

Knocking down Mct10 mRNA abrogates T₃-mediated inhibition of ATDC5 proliferation

TH is known to inhibit chondrocyte proliferation and promote its differentiation (15, 16, 30, 31). If Mct10 acts as a physiological TH transporter, attenuation of TH action via suppression of Mct10 expression should antagonize these effects of TH in ATDC5 cells. As shown in previous studies, 5 d incubation with 10⁻⁸ or 10⁻⁹ m T₃ significantly decreased ATDC5 proliferation assessed by counting cell numbers compared with cultures without T₃ (Fig. 6A). Similar to the control experiments, in Gapdh siRNA or negative control siRNA-ATDC5 cells, T₃ treatment significantly attenuated cell proliferation. In contrast, in Mct10 siRNA-treated ATDC5 cells, 10⁻⁹ m T₃ did not decrease cell proliferation (Fig. 6B). Similar results were obtained with 10⁻⁸ m T₃ (data not shown). Evaluation with BrdU uptake also demonstrated comparable results (Fig. 6C).
Silencing Mct10 mRNA abolishes T3-mediated promotion of ATDC5 differentiation

To accommodate the siRNA experiments, rapid differentiation of ATDC5 cells was induced by suspending the cells in alginate beads and culturing for up to 3 d. The cells cultured in the presence of $10^{-9}$ M T3 increased expression of Col10 mRNA earlier and to a greater extent than those cultured without T3 (Fig. 7, top row). In Gapdh or negative control siRNA-transfected cells, addition of T3 increased Col10 mRNA expression at d 3 (Fig. 7, middle row), in accordance with nontransfected cells. However, in Mct10 siRNA-treated ATDC5 cells, regardless of T3, Col10 mRNA expression resembled cultures without T3 (Fig. 7, bottom row). Taken together, the results demonstrate that silencing Mct10 mRNA mostly abolishes T3 action in ATDC5 cells and indicates that Mct10 plays a significant role in mediating TH action in these cells.

Discussion

In this paper, we demonstrated that Mct10 is the most prominently expressed transporter with high specificity for TH in the early stages of chondrocyte differentiation. The study also showed that disrupting Mct10 function results in derangement of the endochondral ossification program.

Using murine chondrogenic ATDC5 cells, we first comprehensively examined mRNA expression of 20 transporters previously reported to transport TH. After initial screening with RT-PCR, quantification with SYBR GREEN qRT-PCR revealed that among the 11 transporters detected initially, the most prominently expressed transporter was Lat1, followed by Mct10 in ATDC5 cells. Although the Michaelis constant of Lat1 for T4 and T3 (7.9 and 0.8 M, respectively) is lower compared with large neutral amino acids (30–94 M) (32), physiological concentrations of these amino acids are in the $10^{-5}$ to $10^{-4}$ M range, vastly exceeding the physiological concentrations of T4 and T3 ($10^{-8}$ to $10^{-7}$ M and $10^{-9}$ M range, respectively). Moreover, Friesema et al. (33) have shown that $10^{-4}$ M of Leu, Trp, Tyr, or Phe completely inhibits Lat1-mediated uptake of $10^{-7}$ M T3. This led us to reason that in the in vivo environment, Lat1 would be saturated with amino acids. Addition of 1 mM [three times inhibitory constant (28)] BCH (29) to the medium induced no change in thyroid hormone uptake into ATDC5 cells, confirming that Lat1 does not contribute significantly as a thyroid hormone transporter under the...
conditions we used, i.e. in the presence of physiological levels of amino acids.

We thus decided to focus on the three known transporters with high specificity for TH, namely Mct10, Mct8, and Oatp1c1 (25). Quantification using qRT-PCR (TaqMan) confirmed abundance of Mct10 over Mct8 in ATDC5 cells. MCT10 is a transporter most homologous to MCT8 within the MCT family (34). Although originally characterized as a T-type amino acid transporter that facilitates uptake of aromatic amino acids (35–37), a recent study has demonstrated that MCT10 transports TH as efficiently as MCT8 (27). Although the physiological relevance of MCT10 is still unknown, tissue expression of mRNA differs from that of MCT8 (38), suggesting distinct properties between these transporters.

Initial attempts to quantify Mct10 in vivo in the GP chondrocytes using laser microdissection/qRT-PCR failed to demonstrate abundance of Mct10 over Mct8 (data not shown). We therefore turned to in situ hybridization studies, which demonstrated that Mct10 expression is strongest in the RZ chondrocytes and that mRNA levels of Mct10 and Mct8 vary reciprocally as the GP chondrocytes differentiate. In particular, compared with the RZ chondrocytes, the cells beginning to differentiate into the secondary ossification center exhibited weaker Mct10 signal, whereas that of Mct8 was stronger. Because we were not able to distinguish these cells from the RZ chondrocytes when performing the laser microdissection/qRT-PCR experiments, we believe this is most likely the reason why discordant expression of Mct10 and Mct8 was initially observed.

From the in situ hybridization data, taken together with the fact that Mct10 expression is strongest in nondifferentiated ATDC5 cells and decreases with differentiation, we judged that ATDC5 cells recapitulate the in vivo situation and subsequently analyzed Mct10 function in these cells.

Silencing Mct10 in ATDC5 cells resulted in a significant decrease in T3 influx and reduced effects of T3. These results demonstrate that Mct10 serves as a TH transporter in these cells. The abundance of Mct10 combined with these results makes it a good candidate as a major functional TH transporter in chondroprogenitors/chondrocytes in the early stages of differentiation. However, one must keep in mind that the suppression of T3 intake into Mct10-silenced cells was partial, indicating that other yet-unknown transporters also function as TH transporters in these cells. In addition, because the experiments were carried out in DMEM/F12 containing 0.1% BSA, which is closer to physiological conditions in the GP compared with buffers such as PBS that do not contain amino acids but nonetheless does not completely replicate the in vivo environment, these results must be interpreted with care. Interestingly, silencing Mct10 reduced T3 action on ATDC5 cells to a greater extent. This suggests that without Mct10, these cells lack the threshold amount of intracellular T3 required to sustain normal proliferation and differentiation. The fact that we used minimal amounts of T3 (actual levels in the medium, total T3, 89 ng/dl; free T3, 1.52 pg/ml: equivalent to levels in cord blood) may also have contributed to this phenomenon. Collectively, our results indicate that absence of stunted growth in AHDS patients, which stands in contrast to severe growth retardation in patients with untreated cretinism, is most probably due to compensation by MCT10 as well as by other yet-unidentified TH transporters. The

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**FIG. 7.** ATDC5 cell differentiation assays. ATDC5 cells were cultured for up to 3 d in alginate beads to expedite differentiation. Levels of Col10 were analyzed by qRT-PCR (TaqMan) and corrected for amounts of 18S rRNA. Data from representative experiments (each n = 3) are shown as mean ± SD. P < 0.05 was considered as significant. All experiments were performed four times. Nontransfected cells treated with T3 (10⁻⁹ M) differentiated more rapidly than those without T3 (top row). *, P < 0.05. Similarly, T3 promoted expression of Col10 in Gapdh or negative control siRNA-transfected ATDC5 cells (middle row). *, P < 0.05. In contrast, T3 did not accelerate Col10 expression in Mct10-1 or Mct10-2 siRNA-transfected ATDC5 cells (bottom row). —, No significant difference.
same most likely applies for the Mct8 null mice, which grow normally (39, 40).

In recent years, numerous genome-wide association studies for various human traits including height have been conducted (41). The SLC16A10 gene, which encodes MCT10, localizes to 6q21-q22 (36). A literature search identified several studies demonstrating loci associated with height in this region (42–45). Because MCT10 is prominently expressed in early-stage chondrocytes, it is possible that DNA sequence variants that affect the SLC16A10 gene might influence chondrocyte function and ultimately human height.

In summary, we hypothesized that chondrocytes express TH transporters other than MCT8 for TH intake. Using qRT-PCR, we have demonstrated that Mct10 is the most prominently expressed TH transporter among those highly specific for TH in chondrogenic ATDC5 cells. In the GP, Mct10 is most strongly expressed in the RZ chondrocytes in which TH exerts its actions via TRa1. By silencing Mct10 mRNA expression in ATDC5 cells, we have provided evidence that Mct10 is a functional TH transporter and regulates proliferation and differentiation of these cells. The finding that Mct10 might be involved in bone growth warrants further investigation, with special attention to unresolved causes of short stature and skeletal dysplasias.

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