Thyroid Hormone Transporters MCT8 and OATP1C1 Control Skeletal Muscle Regeneration

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SUMMARY

Thyroid hormone (TH) transporters are required for the transmembrane passage of TH in target cells. In humans, inactivating mutations in the TH transporter MCT8 cause the Allan-Herndon-Dudley syndrome, characterized by severe neuromuscular symptoms and an abnormal TH serum profile, which is fully replicated in Mct8 knockout mice and Mct8/Oatp1c1 double-knockout (M/O DKO) mice. Analysis of tissue TH content and expression of TH-regulated genes indicate a thyrotoxic state in Mct8-deficient skeletal muscles. Both TH transporters are upregulated in activated satellite cells (SCs). In M/O DKO mice, we observed a strongly reduced number of differentiated SCs, suggesting an impaired stem cell function. Moreover, M/O DKO mice and mice lacking both transporters exclusively in SCs showed impaired skeletal muscle regeneration. Our data provide solid evidence for a unique gate-keeper function of MCT8 and OATP1C1 in SC activation, underscored the importance of a finely tuned TH signaling during myogenesis.

INTRODUCTION

Cellular entry and efflux of thyroid hormones (THs) are facilitated by transmembrane transporters such as the monocarboxylate transporters 8 (MCT8) and 10 (MCT10), organic anion transporting protein 1C1 (OATP1C1), and the L-type amino acid transporters LAT1 and LAT2 (Bernal et al., 2015; Heuer and Visser, 2009, 2013). MCT8, the most intensively studied TH transporter, is encoded by the Slc16a2 gene and exhibits the highest specificity toward the prohormone 3,3’,5,5’-tetraiodothyronine (thyroxine; T4) and the active form 3,3’,5-triiodothyronine (T3) (Friesema et al., 2003, 2005; Visser et al., 2011). Inactivating mutations in MCT8 were identified to underlie the clinical picture of the Allan-Herndon-Dudley syndrome (AHDS), a severe form of psychomotor retardation (Dumitrescu et al., 2004; Friesema et al., 2004; Schwartz et al., 2005). As a hallmark of this disease, patients present characteristic alterations in the serum TH profile with highly elevated T3 and decreased T4 concentrations along with signs of a peripheral thyrotoxicity such as hypermetabolism, low body weight, and muscle wasting. Moreover, affected patients suffer from pronounced neuromuscular abnormalities and uncontrolled motor movements (Dumitrescu et al., 2004; Schwartz et al., 2005).

In order to gain insights into the pathogenic mechanisms of AHDS, Mct8 knockout (KO) mice were generated (Dumitrescu et al., 2006; Trajkovic et al., 2007; Wirth et al., 2009). Though these Mct8 KO mice fully replicate the abnormal serum TH profile and the peripheral hyperthyroidism of AHDS patients, they do not show any neurological impairment or behavioral abnormalities (Ceballos et al., 2009; Trajkovic et al., 2007). Indeed, we recently demonstrated that, in mice, only the concomitant inactivation of two TH transporters, MCT8 and OATP1C1 (encoded by the Slco1c1 gene), results in a strongly diminished transport of TH across the blood–brain barrier (BBB) and, consequently, in highly reduced TH concentrations within the CNS (Mayerl et al., 2014). Since, in rodents, OATP1C1 is strongly expressed in brain endothelial cells but absent in endothelial cells of the primate CNS (Ito et al., 2011; Roberts et al., 2008), it has been postulated that OATP1C1 can compensate for the loss of MCT8 at the BBB in mice but not in humans.

Despite their central state of TH deprivation, Mct8/Oatp1c1 double-knockout (M/O DKO) mice show a similar state of peripheral hyperthyroidism as Mct8 KO mice. Moreover, unlike the single-mutant animals, only M/O DKO mice exhibit pronounced behavioral alterations including an ataxic gait and reduced locomotor performance making it a suitable model for AHDS (Mayerl et al., 2014).

Skeletal muscle is a major target of TH signaling, and changes in TH homeostasis are often linked to myopathic symptoms (Lee et al., 2014; Salvatore et al., 2014). Among those factors that are transcriptionally regulated by TH are...
合同性确定的蛋白质，如肌动蛋白-重链（MHC）类型的1型（MHC1），MHCIIa，MHCIIb，和MHCIIx，以及转录因子MYOD和MYOGENIN，这些在肌肉细胞损伤中被激活，并涉及肌细胞的激活。后者代表一个高度协调的进程，它被触发于肌肉损伤，从而激活静息肌细胞（卫星细胞[SCs]）的分化，产生新的肌原纤维，并最终成熟为新的肌纤维。因此，我们的研究是利用门控功能的TH转运蛋白，从而验证了SC功能和再生能力在Mct8/Oatp1c1缺陷小鼠中的活性。我们证实了所有TH转运蛋白在激活SCs后在肌肉组织中增强，尽管分别在Mct8/Oatp1c1缺陷小鼠中（Figure 1B），而T3和D2活性显示出3倍和6倍的显著上升，分别解释了TH浓度下降。通过二聚体化基因甲基化分析，这一缺陷在Mct8和Oatp1c1缺陷小鼠中完全复制的（Di Cosmo et al., 2013），而mRNA水平，一个标记活性SCs，没有显示出显著的水平。D3活性在Mct8/Oatp1c1双缺陷小鼠中显著升高，表明TH状态的骨骼肌超装可能与缺乏MCT8和OATP1C1有关。此外，我们发现肌纤维类型比例在Mct8 KO和M/O DKO小鼠中有所改变，具有肌肉纤维表达的快速MHC类型中的差异，表明了在TH保持机理中没有导致SCs的激活，这可能暗示了不同类型的肌肉小鼠（Chang et al., 2016）。这促使我们通过分别识别慢速MHC的抗体来进一步澄清MCT8和OATP1C1在肌肉组织中的分布。

**RESULTS**

**Thyroidal State of Skeletal Muscles**

Mct8缺陷在雄性小鼠中导致TH浓度的升高，而在雌性小鼠中较轻。在Mct8/Oatp1c1缺陷雄性小鼠中，TH浓度未影响静息TH浓度（Dumitrescu et al., 2006; Mayerl et al., 2012; Trajkovic et al., 2007）。为了确定性别的TH状态，我们利用我们的之前发布的Affymetrix基因表达分析，利用两种肌纤维类型的抗体来区分慢速MHC（MHCIIa，IIb，和IIx），在Mct8 KO小鼠中，M/H DKO小鼠中，Mct8 KO和Mct8/Oatp1c1双缺陷小鼠中，表达了的慢速MHC，而Mct8 KO小鼠中，M/H DKO小鼠中，Mct8 KO和Mct8/Oatp1c1双缺陷小鼠中，表达的快速MHC，分别代表了不同的TH激素转运蛋白（Bernal et al., 2015）。为了获得广泛的表达模式，我们使用了分别识别MHCI（慢速MHC）和MHCII（快速MHC）的抗体，其中MHCI在静息SCs中表达最高，而MHCII主要在慢速肌纤维中表达，并且在静息SCs中表达的百分比有所增加。这表明了TH浓度的改变并未导致SCs的激活，这可能暗示了不同类型的肌肉小鼠（Chang et al., 2016）。
Figure 1. Thyroid State of Skeletal Muscle in TH Transporter-Deficient Mice

(A) Measuring serum TH concentrations in 4-month-old female mice (n = 8) revealed highly elevated serum T3 and decreased serum T4 values in Mct8 deficiency, thereby replicating the characteristic TH serum profile of male Mct8 KO animals.

(B) TA muscle T3 content was almost 4-fold elevated in Mct8 KO and M/O DKO mice, while muscle T4 levels were reduced in M/O DKO mice only (n = 4 Mct8 KO; n = 5 WT, Oatp1c1 KO, M/O DKO mice).

(C) D2 and D3 enzymatic activities were measured in TA muscle homogenates. M/O DKO mice showed a pronounced rise in D2 and D3 activities (n = 6).

(D) qPCR analyses were performed using TA muscle homogenates from 2- to 3-month-old female mice to assess transcript levels of TH-regulated genes. Group means + SEM are shown. n = 3 Mct8 KO; M/O DKO; n = 5 WT, Oatp1c1 KO mice. *p < 0.05; **p < 0.01; ***p < 0.001. Two-way ANOVA and Bonferroni-Holm post hoc testing.
acid transporter Lat1 (Slc7a5), which appears to be present in all myogenic cell types, while Lat2 (Slc7a8) and Oatp1c1 mRNA expression were rather low in all cell types analyzed.

Unfortunately, our array analysis did not provide any information about the expression profile of genes present in activated SCs. We therefore isolated primary extensor digitorum longus (EDL) myofibers with their adjacent SCs from 4-month-old female WT mice and analyzed expression of MCT8 and OATP1C1 in quiescent (0 hr), activated (42 hr), and differentiated (72 hr) SCs by immunocytochemistry (Figure 3B). The specificities of the immunofluorescence signals were confirmed by concomitant processing and staining of EDL myofibers from M/O DKO mice that were devoid of any specific labeling (data not shown). Immediately after the isolation of WT fibers, faint MCT8 staining was observed in PAX7-positive, still quiescent SCs, whereas OATP1C1-specific staining was not detectable (Figure 3B). Intriguingly, with increasing time in culture leading to activation, proliferation, and differentiation of SCs, expression of both TH transporters became visible in PAX7-positive cells, indicating that MCT8 and OATP1C1 expression is strongly upregulated in activated SCs.
Figure 3. TH Transporter Expression in SCs

(A) Fold changes based on log2-transformed RNA values from quiescent SCs, cultured myoblasts, myocytes, and myotubes were used to generate global ratio heat maps. While *Lat1* is highly expressed in all cell types, *Mct8* and *Mct10* exhibit strongest expression in SCs. *Oatp1c1* and *Lat2* mRNA levels are low compared with *Mct8*.

(B) Primary EDL myofibers isolated from female WT mice and cultured for 0, 42, or 72 hr. Immunofluorescence studies revealed MCT8 expression (in green, arrow) in PAX7-positive (red) SCs at all analyzed time points. In comparison, OATP1C1 (in green, arrow) could not be detected at 0 hr but was clearly visible after 42 and 72 hr in PAX7-positive (in red) SCs. Nuclei stained in blue. Representative images of n = 3. Scale bar: 5 μm.
THs are known to orchestrate the differentiation process within the myogenic program (Dentice et al., 2013). Therefore, we wondered whether the absence of MCT8 and/or OATP1C1 might interfere with the activation and differentiation of SCs. Hence, EDL myofibers were isolated from WT and TH transporter-deficient, 4-month-old female mice and analyzed directly or after 72 hr in culture. Staining for the SC marker PAX7 and the differentiation marker MYOD demonstrated no aberrant activation of SCs under resting conditions in TH transporter-deficient mice (Figure 4A). Though the overall percentage of PAX7-positive cells per cluster was comparable between the genotypes after 72 hr in culture (Figure S1A), differences in their differentiation state became apparent: the percentage of non-differentiated SCs expressing only PAX7, but not MYOD, per cluster was almost doubled in EDL cultures of Mct8/Oatp1c1 DKO mice (Figure 4B), whereas the percentage of committed SCs (PAX7/MYOD double-positive) was reduced by 47% (Figure 4C), suggesting a delay in differentiation of SCs. In addition, the overall percentage of MYOD-expressing cells per cluster was significantly decreased in MCT8 and OATP1C1 deficiency (Figure 4D), but not the percentage of only MYOD-positive myoblasts (Figure S1B). Obviously, a combined MCT8 and OATP1C1 inactivation compromises directly and/or indirectly SC differentiation in vitro.

As an additional in vitro approach to clarify the muscle-specific function of MCT8 and OATP1C1 and to avoid possible compensatory effects, we prepared EDL fiber cultures from WT and Oatp1c1 KO mice that show normal serum TH values and a normal muscle TH content. These fiber cultures were treated for 72 hr with Silychristin (25 μM) as this compound has been recently shown to specifically inhibit Mct8 (Johannes et al., 2016). Immunostaining of fiber cultures with PAX7 and MYOD indeed revealed the highest number of cells expressing only PAX7 in Oatp1c1 KO mice treated with Silychristin (Figure 4E), whereas the percentage of PAX7-positive cells per cluster was not altered (Figure S1C). Likewise, the percentage of PAX7/MYOD-positive, differentiated SCs was slightly reduced in Silychristin-treated cultures (Figure 4F), though neither the percentage of MYOD-expressing cells nor the percentage of MYOD only-positive nuclei were significantly affected by the inhibitor treatment (Figures 4G and S1D). Altogether, these in vitro data suggest an impaired differentiation of SCs entering the myogenic program in the absence of MCT8 and OATP1C1.

Muscle Regeneration and SC Differentiation Are Impaired In Vivo in TH Transporter-Deficient Mice

We wondered whether impairments in the myogenic program also occur in vivo after injury when both TH transporters are absent. Therefore, we injured the TA muscle of adult female mice by injection of cardiotoxin (CTX).

First, we investigated myofiber size in resting conditions but did not observe significant differences between the genotypes by analyzing the minimal fiber feret (diameter) as a measure of fiber size (Figures 5A and 5B). Intriguingly, 5 days after CTX injection (5 days post injury [dpi]), the minimal fiber feret was reduced in all TH transporter-deficient animals, with the most pronounced and statistically significant reduction in M/O DKO mice (14.4 μm compared with 20.5 μm in WT animals). This impaired regeneration was still evident at 10 dpi when fiber size was still clearly reduced in all TH transporter KO mice, with lowest values in M/O DKO animals (19.7 μm compared with 25.2 μm in WT controls). Interestingly, we did not observe significant differences in myofiber size at 21 dpi, suggesting rather a delay in regeneration than a general inhibition in the absence of MCT8 and OATP1C1.

Since regeneration of skeletal muscle depends on functional SCs (Lepper et al., 2011; Murphy et al., 2011; von Maltzahn et al., 2013), we speculated that the delayed regeneration of M/O DKO mice is due to impaired SC function. Therefore, we enumerated the number of SCs expressing PAX7 and SCs that also express the differentiation marker MYOD at different time points after injury as well as under resting conditions (Figure 6A). Surprisingly, already under resting conditions we found a 2.1-fold increase in the number of PAX7-positive SCs in M/O DKO mice, suggesting alterations in apoptosis and/or proliferation of SCs in the absence of both TH transporters (Figure 6B). Quantification of proliferating Ki67-positive cells, however, revealed similar numbers under basal conditions in all TA sections, arguing against pronounced alterations in SC proliferation in Mct8/Oatp1c1 deficiency (Figure 6C). Likewise, counting of PAX7/TUNEL double-positive cells did not show any significant differences between the genotypes as the number of apoptotic PAX7 cells were, as expected, in general very low under basal conditions (Figure 6D). Thus, we exclude differences in proliferation or apoptosis as the cause for the elevated numbers of SCs in MCT8- and OATP1C1-deficient muscles.

Quantification of PAX7-positive cells at 5 dpi demonstrated decreased numbers in Mct8-deficient muscles, while Oatp1c1 and M/O DKO muscles did not show significant differences compared with control mice (Figure 6E), in the latter case possibly due to the higher number of SCs under resting conditions. Importantly, we observed significantly reduced numbers of PAX7/MYOD-positive SCs 5 dpi in Mct8 KO and M/O DKO muscles, suggesting impairments in either activation or differentiation. This prompted us to quantify PAX7- and MYOD-positive cells at later time points (10 dpi and 21 dpi). Notably, we found reduced numbers of PAX7/MYOD double-positive cells at 10 dpi in

1964 Stem Cell Reports | Vol. 10 | 1959–1974 | June 5, 2018
Figure 4. SC Activation and Differentiation Is Delayed in Myofiber Cultures of M/O DKO Mice

(A) WT, Mct8-, and/or Oatp1c1-deficient primary EDL myofibers (n = 3) were either fixed directly (0 hr) or cultured for 72 hr and incubated with antibodies against PAX7 (in red) and the activation and differentiation marker MYOD (green) as well as with Hoechst33528 to label cell nuclei (in blue). MYOD immunoreactivity was only detected after 72 hr of culture as expected. 

(B) Marker-positive cell nuclei were quantified and revealed a higher percentage of PAX7 only immunopositive nuclei in M/O deficiency at 72 hr. 

(C–D) Both the percentage of PAX7/MYOD double-positive (C) and total MYOD-positive (D) nuclei per cluster were reduced in cultures derived from M/O DKO mice.

(E–G) EDL myofibers from WT (n = 4) and Oatp1c1 KO mice (n = 6) were cultured for 72 hr with the MCT8 inhibitor Silychristin or DMSO. Analysis of PAX7- and MYOD-immunopositive cells demonstrated a tendency toward a higher number of PAX7+ only cells (E) and a reduced number of PAX7/MYOD-immunopositive cells (F), but not toward total MYOD-positive cell numbers (G) in Silychristin-treated Oatp1c1-deficient myofibers. These findings support a delayed SC activation and differentiation if OATP1C1 and MCT8 are absent or inhibited. Group means + SEM are shown.

*p < 0.05; **p < 0.01; ***p < 0.001. Two-way ANOVA and Bonferroni-Holm post hoc testing. Scale bar: 10 μm.
M/O DKO mice, while numbers of PAX7-positive cells were not significantly altered at 10 dpi or 21 dpi (Figures 6E and 6F), suggesting inhibition of the differentiation process.

One may speculate that the abnormal serum profile and, consequently, the global changes in muscle TH content contribute to the delayed regenerative capacity in Mct8-deficient animals. To exclude any systemic effects due to the global loss of Mct8 and/or Oatp1c1, we generated mice with a specific inducible deletion of both TH transporters in SCs (Figure S2A).

For that purpose, we obtained conditional Mct8 mouse mutants from the KOMP (Knockout Mouse Project) repository that carry loxP sites flanking exon 3 of the murine Mct8 gene (Figure S2A). Cre recombinase-mediated deletion of exon 3 was predicted to result in a frameshift mutation from exon 4 to 5 and thus loss of function of the Mct8 gene product. Mct8 fl/fl or Mct8 fl/y mice were mated with Oatp1c1 fl/fl (Mayerl et al., 2012) mice and mice carrying the Pax7 CreERT2 transgene (Murphy et al., 2011). CRE recombinase activity was induced prior to all experiments by tamoxifen. The successful removal of both Mct8 exon 3 and Oatp1c1 exon 3 was confirmed on DNA level (Figure S2B). To further prove that our cell-specific KO strategy was successful, we isolated EDL muscle fibers from M/O-SC KO animals and analyzed MCT8 and OATP1C1 expression by immunostaining after 72 hr of culture. TH transporter-specific fluorescent signals were absent in 70% of PAX7-positive cell clusters (Figures 7A, 7B, and S2C), suggesting that Cre recombinase-mediated inactivation of Mct8 and Oatp1c1 was not complete but was efficient enough for further analysis. As expected, M/O-SC KO mice were phenotypically indistinguishable from control animals and exhibited normal serum TH parameters (Figure S2D).
Figure 6. Delayed SC Activation and Differentiation in M/O DKO Mice upon Injury

(A) The TA muscle of female mice was injured with CTX at the age of 2.5–4 months and mice were sacrificed 5, 10, or 21 dpi. TA sections were stained for the SC marker PAX7 (in red), the early differentiation factor MYOD (in green), and Hoechst 33258 (in blue). Representative images for WT and M/O DKO mice are depicted.

(legend continued on next page)
We injured the TA muscle of young adult female M/O-SC KO mice and analyzed muscle regeneration at 10 dpi. Importantly, we did not observe differences in SC numbers under resting conditions between M/O-SC KO and control mice (Figure S2E). After injury, we found a significant decrease in the number of PAX7/MYOD double-positive SCs (Figures 7C and 7D) and myofiber size (Figures 7E and 7F) resembling the phenotype of M/O DKO mice. This prompted us to conclude that MCT8 and OATP1C1 fulfill SC-specific functions during regeneration.

DISCUSSION

Skeletal muscle is a well-established TH target tissue as TH regulates the expression of numerous muscle proteins critical for proper muscle development and contractility (Ambrosio et al., 2017; Milanesi et al., 2016, 2017; Soukup and Smerdu, 2015). Thus, highly elevated serum T3 levels, as detected in AHD5 patients, are expected to heavily affect skeletal muscle homeostasis and regeneration. Elevated T3 levels may even cause or contribute to the overt muscle wasting and locomotor deficits of these patients. Indeed, increased serum concentrations of lactic acid and ammonia as markers of skeletal muscle catabolism are indicative for a thyrotoxic state of skeletal muscle tissues in those patients (Herzovich et al., 2007). Likewise, Mct8 KO mice as well as M/O DKO mice, which both replicate the abnormal circulating TH concentrations of the patients (Dumitrescu et al., 2006; Mayerl et al., 2014; Trajkovic et al., 2007) (Figure 1A), show an almost 4-fold increase in TA muscle T3 content (Figure 1B). This rise in T3 is accompanied by an increased expression of the T3-regulated target gene hairless. Furthermore, expression analysis of different MHC isoforms and analysis of the fiber type distribution in different skeletal muscles (Figures 1D and 2) indicate a shift from slow-twitch to fast-twitch fibers as expected from a hyperthyroid muscle tissue (Figure 2) (Soukup and Smerdu, 2015). These observations are in line with previous data describing increased skeletal muscle T3 content and TH action in Mct8 KO mice (Di Cosmo et al., 2013).

One might even suggest that MCT8 does not play a major role in muscle TH transport as the circulating T3 levels appear to be fully sensed by MCT8-deficient skeletal muscle cells. Of note, Mct10, a close relative of Mct8 and a potent T3 transporter (Friesema et al., 2008), shows a similar skeletal muscle expression pattern as Mct8 according to our cell-specific RNA profiling (Figure 3A). Thus, as also suggested by Di Cosmo et al. (2013), MCT10 might at least partially compensate for the absence of MCT8 in the murine muscle. Such a scenario could be tested by assessing the thyroidal skeletal muscle phenotype of Mct10/Mct8 DKO mice, particularly as studies of double-mutant animals have already shown a concerted action of MCT10 and MCT8 in mediating TH transport in liver, kidneys, and the thyroid (Müller et al., 2014). However, it also should be kept in mind that muscle tissue contains various cell types with distinct TH transporter repertoire. Thus, the cell-specific TH status may vary greatly between different skeletal muscle cell types in mice that lack Mct8 globally.

That this is indeed the case becomes already evident with the determination of T4 concentrations and deiodinase activities in skeletal muscle homogenates. Despite the low T4 levels in the circulation, skeletal muscle T4 content was found to be surprisingly normal in Mct8 KO mice. The observed increased mRNA levels and activities of the TH-activating enzyme D2 would be indicative of a hypothyroid state, although the total tissue T3 content and our qPCR data argue for a thyrotoxic situation in TA muscle (Figures 1B–1D). A different situation was found in M/O DKO mice that displayed reduced T4 muscle content in combination with highly elevated D2 and increased activities of the TH-inactivating enzyme D3 (Figure 1C). A simultaneous rise in D2 and D3 activities is contradictory to general expectations as D2 is negatively and D3 is positively regulated by TH (Bianco et al., 2002). Rather, our observations point to muscle-intrinsic, cell-specific changes in TH homeostasis in Mct8 KO or M/O DKO muscles, with some cells being in a hyperthyroid and others in a hypothyroid state. As another explanation, M/O DKO mice present a more than 2-fold increase in the number of SCs at baseline, which are the main cellular source of D3 expression in skeletal muscle (Dentice et al., 2014). Hence, the high D3 activities in those animals might simply reflect the increased number of D3-expressing cells in vivo.
Figure 7. Compromised Muscle Regeneration upon Conditional Inactivation of *Mct8* and *Oatp1c1* in SCs. EDL Myofibers Were Isolated from Tamoxifen-Treated Control and *M/O*-SC KO Littermates and Cultured for 72 hr
(A and B) Immunofluorescence staining for MCT8 (in green) (A) and OATP1C1 (in green) (B) demonstrated signals in clusters of PAX7-positive cells (red) in control samples, whereas signal intensities were strongly reduced in *M/O*-SC KO animals (nuclei in blue). Arrows depict cells positive for PAX7 and MCT8 or PAX7 and OATP1C1, respectively.
(C) TA muscles of 3-month-old control and *M/O*-SC KO mice were injured with CTX and analyzed by immunostainings 10 dpi (PAX7 in red; MYOD in green, nuclei in blue).
(D) Quantification showed a significantly reduced number of PAX7/MYOD double-positive SCs in *M/O*-SC KO mice.
(E) Immunofluorescence staining for LAMININ (in red) and cell nuclei (in blue) indicated similar fiber diameters in TA muscles in the uninjured state, while *M/O*-SC KO animals showed a reduced myofiber size at 10 dpi.
(F) Minimal fiber feret determination in uninjured TA muscle revealed similar numbers. However, injured TA muscles of *M/O*-SC KO mice revealed a reduced myofiber diameter 10 dpi. Group means ± SEM are shown. n = 4.
*p < 0.05, unpaired 2-tailed Student’s t test. Scale bars: 5 μm (A and B) and 50 μm (C and E).
In a first approach to unravel the muscle-specific functions of MCT8 and OATP1C1 we determined the cell-type-specific expression patterns of these two transporters. It has already been reported that Mct8 transcripts are present in skeletal muscle tissue (DiCosmo et al., 2013), and our cell-type-specific profiling as well as immunohistochemical data are in line with this earlier report. Intriguingly, we could detect strongest MCT8 expression in quiescent and activated SCs, whereas OATP1C1 protein expression was only transiently observed in activated SCs (Figure 3). This temporally regulated expression pattern indeed suggested a specific function of both transporters in SCs that are required for regeneration of skeletal muscle (Lepper et al., 2011; Murphy et al., 2011; von Maltzahn et al., 2013).

TH tightly regulates muscle regeneration as unambiguously demonstrated in various studies (Dentice et al., 2010, 2013, 2014; Lee et al., 2014; Salvatore et al., 2014). According to the current model, high expression of the inactivating enzyme D3 ensures strongly reduced intracellular TH levels in SCs in order to keep them in a proliferative state (Figure S3). Low intracellular TH concentrations seem to be a prerequisite for SC proliferation and survival. Indeed, Dentice et al. (2014) elegantly demonstrated that D3 acts a “survival factor” in these cells as inactivation of this enzyme specifically in SCs leads to caspase3-dependent apoptosis of activated stem cells. Absence of MCT8 and OATP1C1 in SCs possibly results in impaired TH uptake into these cells, thereby evoking low intracellular TH concentrations, which in turn keeps the SCs for a prolonged period in a proliferating state. Such a scenario would also explain the elevated number of PAX7-positive cells as well as the elevated D3 activities found in M/O-deficient muscle under resting conditions in vivo (Figure 6B).

Upon injury, SCs become activated, enter the cell cycle, and either divide symmetrically, thereby maintaining the pool of SCs, or asymmetrically to give rise to myogenic progenitor cells (Bentzinger et al., 2013a). Once activated, PAX7-positive SCs express the transcription factor MYOD, a master regulator of myogenesis that in turn regulates expression of downstream myogenic factors important for proper myogenesis (Bentzinger et al., 2012; von Maltzahn et al., 2012). Interestingly, upregulation of MyoD expression is controlled by local TH signaling via numerous pathways of which T3-stimulated Foxo3 expression and, consecutively, Foxo3-induced D2 expression represents one important mechanism (Dentice et al., 2010). Elevated D2 activities in turn locally generate “supraphysiological” T3 concentrations that are needed for robust induction of MyoD expression and thereby trigger the differentiation process (Figure S3). Indeed, inactivation of D2 in mice has been shown to cause impaired differentiation of myoblasts similar to the phenotype described for MyoD/Myf5-deficient mice (Rudnicki et al., 1993). Thus, adequate and precise regulation of intracellular TH concentrations with low T3 levels in proliferating cells and a high-T3 condition in differentiating cells appears to be mandatory for proper myogenesis.

The strictly regulated TH action during myogenesis is obviously disturbed if Mct8 and Oatp1c1 are missing. Myofibers prepared from M/O DKO mice and cultured for 72 hr contained far fewer differentiated PAX7/ MYOD double-positive and MYOD-positive cells, indicating that the differentiation process of PAX7-positive SCs is compromised (Figures 4C and 4D). Accordingly, an almost 50% reduction in Pax7/MYOD-positive cells was detected in muscles derived from M/O DKO mice when analyzed at 10 dpi. A significant drop in Pax7/MYOD cells after injury was even found in M/O-SC mice in which Mct8 and Oatp1c1 were exclusively deleted in Pax7-positive SCs (Figure 7C). Overall, these findings firmly demonstrate a critical cell-intrinsic role of MCT8 and OATP1C1 in SCs allowing proper timing of the differentiation process only in the presence of both transporters (Figure S3).

Are Mct8/Oatp1c1-deficient muscles able to fully regenerate in response to injury? Our in vivo studies indicate that the regeneration process is only transiently blocked if Mct8 and Oatp1c1 are absent. Myofiber size was temporarily reduced in TH transporter-deficient animals 5 and 10 dpi, but ultimately muscles fully regenerated to a normal size at 21 dpi with similar numbers of SCs (Figure 4). Possibly, elevated muscle T3 content together with the presence of other TH transporters such as MCT10 can partially compensate for a diminished cellular T3 transport caused by the absence of MCT8. In addition, and similarly to the situation in the brain (Bernal et al., 2015), the rise in muscle D2 activities in Mct8-deficient animals might provide another compensatory mechanism to counteract the compromised cellular T3 uptake. However, such a mechanism can only provide full compensation if sufficient amounts of T4 are available for local T3 production. Consequently, a combined deletion of Mct8 and Oatp1c1 that putatively compromises both T4 and T3 uptake into SCs affects proper SC differentiation to a much greater extent than inactivation of Mct8 alone.

Overall, our data provide solid evidence for a unique gate-keeper function of MCT8 and OATP1C1 in SC activation and underscore the importance of a finely tuned TH signaling within the myogenic program. Unraveling the function of those TH transporters during regeneration of skeletal muscles might open additional therapeutic strategies for AHDS patients.
EXPERIMENTAL PROCEDURES

Animals
Mct8 KO mice were obtained from Deltagen and have been described previously (Trajkovic et al., 2007). Oatp1c1 KO mice were generated from Oatp1c1 b/b animals as reported before (Mayerl et al., 2012). Breeding pairs were set up as described previously in order to obtain WT, Oatp1c1 KO, Mct8 KO, and Mct8/Oatp1c1 KO mice on a C57BL/6 background (Mayerl et al., 2014). Mct8 KO and Oatp1c1 KO mice were genotyped as described previously (Mayerl et al., 2012; Trajkovic et al., 2007).

Conditional Mct8 mutant mice (C57BL/6 background) were obtained from the KOMP repository (Sle16a2m(cre)KOMP2061). Further information is provided in Supplemental Information.

Mct8 b/b/l mice were mated with Oatp1c1 b/b/l mice and transgenic animals expressing a tamoxifen-inducible Cre recombinase driven by the Pax7 promoter (Murphy et al., 2011). Detection of the Pax7 CreERT2/+ transgene was performed as stated elsewhere (Murphy et al., 2011). Mct8 b/b/l, Oatp1c1 b/b/l, Pax7-CreERT2 mice were treated with tamoxifen to delete Mct8 and Oatp1c1 specifically in SCs (M/O-SC KO) as described previously (von Maltzahn et al., 2013). Mct8 b/b/l, Oatp1c1 b/b/l mice negative for the CreERT2 transgene and treated with tamoxifen served as controls. Genomic DNA obtained from tail biopsies, brain, and gastrocnemius muscle were subjected to PCR.

Mice were kept at constant temperature (22 °C) on a 12 hr light, 12 hr dark cycle and were provided with standard laboratory chow and water ad libitum. Animals were sacrificed by cervical dislocation at 2–4 months of age. Serum TH values were measured by radioimmunoassay as published elsewhere (Friedrichsen et al., 2003). For determination of muscle TH content and qPCR analysis, TA muscles were rapidly frozen in liquid nitrogen. Muscle T4 and T3 content was measured following rough homogenization and extraction of the tissues as described in detail by Reyns et al. (2002). Muscle tissues used for deiodinase activity assays were processed as described before (Friedrichsen et al., 2003).

For regeneration experiments, TA muscles of female mice at the age of 2.5–4 months were injured by intramuscular injection of 200 ± 50 μL of CTX (10 μM in 0.9% NaCl) under isoflurane anesthesia and isolated 5, 10, or 21 dpi. Muscles were cross sectioned and watered in liquid nitrogen. Muscle T4 and isolated 5, 10, or 21 dpi. Muscles were cross sectioned and frozen in liquid nitrogen.

Affimetric Microarray Data Acquisition
For cell-specific TH transporter expression analysis, we took advantage of microarray data that have been published by Price et al. (2014). In this study, quiescent SCs were isolated from heterozygous Pax7-zsgreen mice after a 30 min collagenase/dispase digestion and sorted for zsgreen expression (Price et al., 2014). The Pax7-zsgreen mice express the zsgreen reporter under control of the endogenous Pax7 promoter. In addition, Price et al. (2014) sorted SCs from adult mice using alpha7 integrin in order to obtain activated SCs that were consecutively cultured under proliferating conditions (F10 medium with 20% FBS [Gibco] and 2.5 ng/mL fibroblast growth factor [Gibco]) for obtaining myoblasts, or under differentiating conditions (DMEM with 5% horse serum [HS; Gibco]) in order to obtain myocytes (after 2 days in culture) and myotubes (after 5 days in culture), respectively. Unfortunately, activated SCs were not analyzed in this study.

Tissue Cultures
Single EDL myofibers were obtained from 2.5- to 4-month-old mice and cultured for 0 hr, 42 hr, or 72 hr (Bentzinger et al., 2013b). In brief, the EDL muscle was carefully removed, only handling the tendons, and digested with 2.5 mL of collagenase I (Sigma; final concentration, 0.2%) in DMEM for 45 min at 37°C under constant agitation. The muscle was then triturated using Pasteur pipettes coated with HS until single fibers came off. Single myofibers were transferred into culture medium (20% FBS in DMEM, 1% chicken embryo extract [Seralab]) in a 24-well plate coated with HS.

For inhibiting MCT8, Sillychristin (25 μM, Sigma) was applied to the culture medium of WT and Oatp1c1 KO-derived fiber cultures, whereas control fibers received equal amounts of the solvent (DMSO).

Immunofluorescence Studies
EDL myofibers were fixed with 2% paraformaldehyde (PFA) for 10 min, treated with 0.1 M glycine in PBS for 10 min, blocked, and permeabilized with PBS containing 10% HS and 0.2% Triton X-100. Subsequently, fibers were incubated with primary antibodies in PBS overnight at 4°C. Following washing with 0.2% Tween in PBS, fibers were incubated with fluorescence-labeled secondary antibodies raised in goat (1:1,000; Alexa Fluor 488 or 555; Invitrogen) and with Hoechst 33258 (1:10,000) to label cell nuclei. Thereafter, myofibers were washed, transferred on Superfrost slides, mounted with Mountant, and analyzed using an Olympus AX70 microscope.

Muscle crossections (12 μm) were fixed with 2% PFA for 10 min, permeabilized with 0.1% Triton X-100/0.1 M glycine, blocked with MOM reagent (1:40 in PBS, Vector laboratories), and incubated with the respective primary antibody in PBS/5% goat serum overnight at 4°C. Subsequently, sections were incubated with fluorescein Alexa Fluor 488- or 555-labeled secondary antibodies (1:1,000) and Hoechst 33258 (1:10,000).

The following antibodies were used: rabbit anti-Ki67 (1:500; Sigma-Aldrich), rabbit anti-LAMININ (1:1,000; L9393, Sigma-Aldrich), rabbit anti-MCT8 (1:500; HPA003353, Sigma-Aldrich), mouse anti-fast MHC (1:500; M4276, Sigma-Aldrich), mouse anti-slow MHC (1:500; M8421, Sigma-Aldrich), rabbit anti-MOYD (1:250; sc-304, Santa Cruz Biotechnology), rabbit anti-OATP1C1 (1:100; Mayerl et al., 2012), and mouse anti-PAX7 (undiluted; Developmental Studies Hybridoma Bank). TUNEL staining was performed using the In Situ Cell Death Detection Kit (catalog no. 11684817910, Roche) following the manufacturer's instructions.

qPCR
Detailed information on qPCR studies is provided in Supplemental Information.

Quantification
In EDL fiber cultures, numbers of total MYOD-positive, MYOD-only-positive, total PAX7-positive, PAX7-only-positive, and PAX7/MYOD double-positive cells were counted per cell cluster.
72 hr after fiber isolation. Myofiber numbers of whole plantaris and soleus cross-sections were determined by staining 3–6 consecutive sections for LAMININ and the respective MHC antibodies. TA muscle fiber diameter was assessed by measuring the minimal fiber feret (defined as the minimum distance of parallel tangents at opposing borders of the myofiber). For that purpose, whole cross-sections of LAMININ stained muscles were analyzed with an AxioObserver (Zeiss) utilizing the mosaic function provided by the Zen software (Zeiss) to automatically stitch the images. Quantification of fiber types was conducted manually by counting the number of slow and fast MHC-positive fibers and dividing them by the number of total fibers in order to get the percentages of slow and fast MHC-positive fibers per muscle respectively. Following CTX injury, only those muscle fibers were included in the analysis that displayed centrally located nuclei. Numbers of MYOD-positive, PAX7-positive, and PAX7/MYOD double-positive cells were counted per area.

Tamoxifen treatment efficiency was calculated by counting the number of PAX7-positive cell clusters either showing or lacking TH transporter immunofluorescent signals along 10 M/O-SC KO EDL-derived myofibers 72 hr after isolation.

To quantify cell culture results, six to eight independent images per sample and condition have been investigated. For proper comparison between different samples and conditions, only cross-sections of the muscle’s mid-belly regions were used in these analyses, immunostainings were always performed in parallel, images were acquired with the same settings, and all measurements were performed in a blinded manner.

Statistics
Values represent mean ± SEM from at least three animals per genotype and time point. For multigroup comparisons, two-way ANOVA was performed (2 × 2 factorial ANOVA; factor A, WT versus Oatp1c1 KO; factor B, WT versus Mct8 KO) using Daniels XL Toolbox add-in for Microsoft Excel followed by Bonferroni-Holm post hoc testing. Statistical significance between control and M/O-SC KO mice was assessed by unpaired Student’s t test (Microsoft Excel). A p value less than 0.05 was considered significant.

Study Approval
All animal procedures were in accordance with the European Union (EU) directive 2010/63/EU and approved by the Animal Welfare Committee of the Thüringer Landesamt für Lebensmittel-sicherheit und Verbraucherschutz (03-011/14 and 03-48/16; TLV; Bad Langensalza, Germany) and the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (84-02-04.2017.A219; LANUV; Recklinghausen, Germany), respectively.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.03.021.

AUTHOR CONTRIBUTIONS
S.M., J.v.M., and H.H. designed experiments, analyzed data, interpreted results, and wrote the manuscript. S.M., M.S., D.D., S.S.H., and J.v.M. performed in vivo and in vitro experiments on myogenesis and muscle regeneration. C.K. provided valuable reagents and advice on microscopy. A.B., V.M.D., S.L., and T.J.V. determined TH levels, tissue TH content, and deiodinase activities. M.S. and D.D. contributed equally, and H.H. and J.v.M. contributed equally.

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