Thyroid hormone signaling and deiodinase actions in muscle stem/progenitor cells

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Highlights

1) Muscle stem cells are highly sensitive to thyroid hormone levels;

2) Type 2 and Type 3 deiodinase are expressed and finely tuned in muscle stem cells;

3) Concerted deiodinase activities dynamically regulate local T3 availability in muscle stem cells.

Key Words: Myogenesis, thyroid hormone, deiodinases, muscle stem cells.

Abstract

Thyroid hormone (TH) regulates such crucial biological functions as normal growth, development and metabolism of nearly all vertebrate tissues. In skeletal muscle, TH plays a critical role in regulating the function of satellite cells, the bona fide skeletal muscle stem cells. Deiodinases (D2 and D3) have been found to modulate the expression of various TH target genes in satellite cells. Regulation of the expression and activity of the deiodinases constitutes a cell-autonomous, pre-receptor mechanism that controls crucial steps during the various phases of myogenesis. Here, we review the roles of deiodinases in skeletal muscle stem cells, particularly in muscle homeostasis and upon regeneration. We focus on the role of T3 in stem cell functions and in commitment towards lineage progression. We also discuss how deiodinases might be therapeutically exploited to improve satellite-cell-mediated muscle repair in skeletal muscle disorders or injury.

Muscle progenitor cells and the differentiation pathway

Tissues are constituted by terminally differentiated cells and a few undifferentiated cells that retain the ability to self-renew and differentiate, i.e., tissue stem cells. These cells underlie such physiological processes as the turnover, growth and repair of tissues. Muscle stem cells reside in a niche located between the sarcolemma and basement membrane of mature muscle fibers. They are normally quiescent but, in response to injury, satellite cells can self-activate, proliferate and regenerate new muscle fibers (1-3). Muscle stem cells - also known as satellite cells - are characterized by the expression of the transcription factor Pax7, the transmembrane proteoglycans syndecan-3 and syndecan-4, and the surface antigens CD34 and M-cadherin (4-7). Besides generating new muscle, these cells also reconstitute the muscle stem cell pool (8,9).

In skeletal muscle, after a muscle injury, the resident stem cells undergo a program of lineage progression, which consists of activation, proliferation, fusion and differentiation to form new muscle fibers. Such other stimuli as exercise and chronic disease, can induce a similar cell activation program. After activation, satellite cells migrate toward the site of injury. At the lesion site, a variety of growth factors and cytokines affect satellite cell behavior and promote proliferation to ensure that a sufficient number of myoblasts is available for regeneration. Indeed, injured muscle or infiltrated macrophages produce HGF (Hepatocyte Growth Factor), FGFs (Fibroblast Growth Factors), IGF-1 (Insulin-like Growth Factor 1), LIF (Leukemia Inhibitory Factor), and IL-6 (Interleukin 6)(10,11). FGF6 is released from injured myofibers and plays dual opposite roles depending on its receptor. In fact, while the FGF6–FGFR1 complex induces satellite cell activation and proliferation, proliferating myoblasts express the FGFR4 receptor, and the FGF6–FGFR4 complex induces cell differentiation by inducing myogenin expression (12). Moreover, HGF, released from the basal lamina of injured muscle, is also a chemo-attractant for satellite cells (13). In addition to satellite cells, muscle-resident fibroblasts and macrophages participate in the process of regeneration and are similarly necessary for muscle repair as are muscle stem cells (14). The cell-to-cell communication between these distinct cell types is crucial, and endocrine and paracrine factors ensure the proper functioning of this mechanism (15).

Tissue regeneration is a process characterized by three partially overlapping and sequential phases: inflammation, tissue reconstruction and tissue remodeling. Although satellite cells are quiescent in the healthy muscle, some of them are already committed to the myogenic lineage and co-express Pax7 and Myf5 (5,10). After injury, satellite cells are activated and start the regeneration program. The hepatocyte growth factor, released from the basal lamina or produced by the satellite cells themselves, is essential for the satellite cell activation (16). Upon injury, muscle-resident fibroblasts proliferate and secrete the cellular matrix and a number of factors that promote myoblast

amplification and differentiation (15,17). Activated satellite cells enter the cell cycle, after which they divide symmetrically or asymmetrically. The cells that express only Pax7 but not Myf5 can divide both ways, thereby producing either two $Pax7^{+}/Myf5^{-}$ cells by symmetric division or one $Pax7^{+}/Myf5^{-}$ cell and one $Pax7^{+}/Myf5^{+}$ cell by asymmetric division. On the other hand, cells that already express both Pax7 and Myf5 will produce two $Pax7^{+}/Myf5^{+}$ cells by symmetric division (18). Subsequently, in these cells, Pax7 cooperates with FoxO3 to ensure MyoD expression that is induced within 24 hours, and triggers the differentiation program (19,20). Thyroid hormone plays an important role in this context. In fact, the cross-talk between TH and FoxO3 is particularly tight because this transcription factor is both a direct TH-target gene and a potent inducer of D2 (the T3-producing enzyme), and it creates an auto-sustaining molecular loop that controls its own expression (21,22). In this phase, most myoblasts start to reduce Pax7 expression but continue to express MyoD and myogenin. Therefore, these myoblasts stop proliferating and fuse to form multinucleated myotubes. Differentiating myoblasts express the transcription factors MRF4 and MEF2 that promote terminal differentiation, and p21Cip1 and Rb proteins that induce cell cycle arrest in G0 phase (23,24). Finally, new myofibers originate from terminally differentiated myotubes. Their maturation consists in the synthesis of myofibrils that are required for the contractile function of muscle fibers. In this process, a subset of proliferating myoblasts/satellite cells can turn off MyoD expression, revert to $Pax7^{+}/MyoD^{-}$ and return to the quiescent state. In these cells, Pax7 suppresses MyoD and myogenin expression, while the receptor tyrosine kinase (RTK)–Ras–ERK cascade antagonist Sprouty1, which is usually expressed only in quiescent satellite cells, is re-expressed (25,26).

In this complex scenario, TH acts on several molecular pathways involved in the lineage progression of activated stem cells, in a spatial- and temporal-regulated fashion. This finely and temporally tuned control of the active T3 concentration is obtained by the precise regulation of the deiodinase activities. In this context, the deiodinases in satellite cells are necessary for correct myogenesis and muscle regeneration (21,27). MyoD1, a master gene controlling myogenesis, is a direct T3 target. Retinoic acid is also able to potently induce MyoD1, however its effects is distinct and not additive with T3 in murine C2 myoblasts (28,29). Other proteins relevant in TH metabolism are expressed by muscle stem cells. Of note, transthyretin, a major T4-binding protein is expressed in C2C12 cells and is regulated during cell differentiation (30). Interestingly, the TH membrane transporters, MCT-8, MCT-10, SLCO3A1 and OATP1C1 are expressed in FACS sorted satellite cells and, with the exception of MCT-10, are all induced during differentiation (unpublished data).

Role of type 3 deiodinase

Type 3 deiodinase (D3) converts T3 and T4 into inactive metabolites that do not interact with TH receptors, reverse triiodothyronine (rT3) or 3,3'-diiodothyronine (T2). This enzyme is considered the major physiologic inactivator of TH action. The mouse *Dio3* gene and its human homolog *DIO3* encode a protein of 278 residues that have a molecular weight of about 32 kDa. All vertebrate D3 cDNAs cloned to-date include a Sec-encoding TGA codon, as well as an SECIS element in the 3'-UTR (31). A study conducted with *Dio3* knock-out mice showed that the *Dio3* gene is subject to genomic imprinting and is preferentially expressed by the paternal allele in the mouse fetus (32). D3 is predominantly expressed in fetal tissues and in the human placenta, where it blocks the excessive maternal-to-fetal transfer of T4 and T3. D3 expression has been identified in only a few postnatal tissues i.e., brain, skin, placenta and, at low level, also in liver, intestine and skeletal muscle (33-36). However, several studies have revealed the re-expression of D3 in adult life in various pathophysiological conditions (37), namely, cardiac hypertrophy, chronic inflammation (38) and such hyperproliferative conditions as tissue repair (39,40) and cancer (41-43).

All machinery related to TH-metabolism, namely TH transporters (MCT8 and MCT10), deiodinases (D2 and D3), and receptors (TRα and TRβ), are present in both human and rodent skeletal muscle, where TH plays an important role in regulating muscle fiber metabolism, protein synthesis and catabolism, and thermogenesis (44,45). Expression of D3 and D2 is exquisitely regulated during myogenesis within the muscle stem cells and this dynamic expression impacts on the proliferation and differentiation balance of muscle stem cells. In fact, D3 is expressed only in the proliferative phase of the regeneration process as opposed to D2, which is expressed in the late phase of myogenesis and is essential for proper muscle cell differentiation (21). Dentice et al. (27) demonstrated that D3 is highly expressed in proliferating myoblasts in culture and its expression decreases upon differentiation. D3 is also expressed in proliferating stem cells on myofiber cultures in vitro, although no information is currently available about putative regulators of its expression in this cell context. Satellite cells are characterized by the expression of the transcription factor Pax7; however, Pax7-positive cells represent a very heterogeneous population (46). In fact, there are two types of Pax7-positive cells: the $Pax7^{Hi}$ population, which is considered the more stem cell-like population and that remains in a quiescent state, while the $Pax7^{Lo}$ population is already primed for commitment and contributes rapidly to muscle repair (47). We have shown that D3 is predominantly expressed in the $Pax7^{Lo}$ cells where it intracellularly attenuates the TH signal (27). The significance of this restricted expression is unknown, but it is conceivable that, besides maintaining the TH signal at a low level in order to enable the amplification of stem cells, D3 also prevents premature exposure of activated cells to circulating TH levels which could favor cell

differentiation. Indeed, when proliferating stem cells were treated with TH they underwent massive apoptosis, likely due to the mitotic catastrophe in proliferating cells abnormally exposed to a potent differentiating agent, i.e., TH. A similar effect was observed when D3 was genetically depleted (27). Stem cell death is highly TH-dependent and cell- and time-specific since it does not occur in proliferating fibroblasts or when myoblasts are already primed to differentiate or when D3-depletion occurs in TH-deprived serum (27). In this context, D3-depletion as well as TH treatment induces the expression of FoxO3 and MyoD, which are pro-apoptotic genes in muscle. Similarly, during muscle regeneration in vivo, D3-depletion in stem cells (cD3KO mice, (27)) causes rapid apoptosis of these cells and the entire regeneration process is disrupted. In fact, cD3KO mice were unable to complete regeneration and still 40 days after injury, the injured muscles were characterized by large necrotic areas and fat infiltration. This failure of regeneration was confirmed by the myogenic markers MyoD, myogenin and myosin heavy chain (MHC) that are sharply down-regulated in the absence of D3. This dramatic event is due to the TH excess in the muscle upon D3-depletion. Accordingly, systemic hypothyroidism attenuates apoptosis in cD3KO mice. Taken together, these data demonstrate that D3, by enabling normal myoblast proliferation, is essential for the stem cell activation program and represents a survival mechanism by which muscle stem cells attenuate their exposure to T3 in accordance with their functional state during the regeneration process.

Role of type 2 deiodinase

Quiescent satellite cells become activated and proliferate to produce committed myoblasts. To complete the regeneration process, activated myoblasts must cease proliferating and initiate a differentiation program by fusing in the resident muscle fibers (48). Type 2 deiodinase is the major activator of TH at local level; it converts the pro-hormone T4 into the active hormone T3. This enzyme is encoded by the *DIO2* gene localized on human chromosome 14. In humans as well as in mice, *Dio2* is expressed in skeletal myocytes (49) and its enzymatic activity in mouse and human muscles is comparable (50). It is an endoplasmic reticulum resident protein whose expression is negatively regulated by thyroid hormone both pre-and post-transcriptionally (51) *via* a TH-regulated ubiquitin-mediated inactivation process (52,53). Several studies indicate that *DIO2* expression in skeletal muscle is increased in patients with chronically low T4 levels in the serum (54), in hypothyroidism and in fasting (55).

In mouse muscle, *Dio2* is highly expressed in the newborn (when the number of stem cells is elevated), and in adult stem cells during myoblast differentiation. In myocytes, this enzyme plays a critical role by producing the active T3 that contributes to induction of MyoD and of the

downstream myogenic factors (21). Indeed, *Dio2*-depletion impairs myoblast differentiation thereby giving rise to a phenotype similar to *MyoD1*$^{-/-}$ mice (56,57). This effect is less evident in primary mouse myoblasts with genetically depleted D2 (58), which indicates that D2 action is more relevant in the muscle stem cell context. The entire lineage progression of activated satellite cells is regulated by the D2-produced TH that governs the expression of major genes involved in the control of myogenesis (Graphical abstract). Importantly, D2-depleted cells have increased proliferative potential, which counteracts their failure to differentiate properly. Are these effects reversible? Recent data demonstrate that excessive TH can rescue or induce proper differentiation in D2-depleted cell, so that D2 impairment causes only a transient and reversible block in the differentiation (21).

What promotes D2 expression in muscle cells? In primary human skeletal myocytes, Pioglitazone and insulin increase D2 activity in a dose-dependent manner (59), while kaempferol upregulates D2 approximately ten fold (60). At a transcriptional level, several signals are able to regulate *Dio2* expression. Among them, D2 is induced in myoblast differentiation by FoxO3a (21), which is a forkhead transcription factor involved in several cellular processes, including differentiation, metabolism and survival (61,62). FoxO3 directly binds the *Dio2* promoter and induces its expression. Indeed, Dio2 expression is significantly reduced in FoxO3-null mice (21).

Muscle differentiation is regulated by epigenetic mechanisms that involve different class I and class II histone deacetylases (HDAC) and/or histone demethylases (63). Usually, HDACs repress muscle differentiation by acting on the muscle-specific transcription factors MyoD and Mef2 (64,65). Similar mechanisms operate on *Dio2* expression. Indeed, several histone deacetylase inhibitors induce *Dio2* expression and increase TH signaling in myoblasts (66). In differentiating myoblasts, the histone demethylase LSD1 together with FoxO3 regulate remodeling of chromatin in the *Dio2* promoter region thereby promoting its expression (66). In this context, FoxO3, D2 and MyoD are related in an intricate network in which FoxO3, through D2 induction, increases intracellular T3 production that promotes muscle differentiation thereby inducing MyoD and other differentiation factors. MyoD is the master regulator of the myogenic development and regeneration program (67). Besides MyoD, the expression of the muscle-specific genes myogenin and MHC are required for correct muscle differentiation (21). It is not coincidental that all these genes are positively regulated by TH and are all down-regulated in the D2 null genetic background (45).

In conclusion, type 2 deiodinase is induced in the late phase of muscle differentiation and, by increasing intracellular T3 concentration, it promotes MyoD expression and enables the proper differentiation process to occur. By ensuring adequate levels of TH during the whole process, D2 is required to guarantee a fully differentiated phenotype.

Therapeutic potential of the deiodinases in satellite-cell-mediated muscle repair

Accumulating evidence suggests that the deiodinases play a role in diseases characterized by a loss of muscle mass. A case in point is Duchenne muscular dystrophy (DMD), which is the most common form of muscular dystrophy, affecting 1 in 3,500 male births. DMD Patients suffer from severe, progressive muscle wasting, with clinical symptoms first detected at 2–5 years of age. Currently, treatment options for DMD patients focus primarily on relief of symptoms and prevention of complications, as there is no effective therapy (6). In the dystrophic (mdx) mouse model, skeletal muscle undergoes cycles of degeneration and regeneration, and satellite cells continue to proliferate at a time when counterpart cells in normal healthy muscle enter quiescence. Earlier studies indicated that T4 may be directly involved in the regulation of satellite cell proliferation and myonuclei accumulation (68) and that hypothyroid status affects muscle precursor cells mainly by depressing their ability to differentiate and fuse with existing myofibers (69). Given the rate-limiting role of regeneration in DMD muscles, we hypothesize that manipulation of the intracellular thyroid status of stem cells via manipulation of deiodinase activity can produce beneficial effects in this disease. Preliminary data strongly suggest that deiodinase-mediated control of TH action significantly affects the physiology of muscle stem cells (21,27). Most importantly, these changes are reversible, as it is the intracellular hypothyroidism caused by D2-depletion, which could be reverted by appropriate T3 replacement. The finding that we transiently attenuate MyoD level by reversibly blocking D2 in myoblast precursors could have several clinical implications for patients with muscle disease. For example, myogenic precursor cells could be kept in low T3 conditions when increased cell proliferation and cell expansion is desired. Subsequently, the situation could be reversed by removing the blocking agent or by supplying T3, thereby promoting myotube formation and terminal differentiation. This process could significantly improve the success of myoblast transplantation protocols in which differentiation limits the degree of muscle precursor cell proliferation (70) and could be useful in the treatment of dystrophic patients. In summary, it is conceivable to manipulate deiodinase activity to therapeutically control TH action in muscle stem cells. It is important to emphasize that the deiodinases can be easily modulated in vivo by such molecules as iopanoic acid and rT3. This raises the future possibility of using tissue-specific modulation of TH action to impact on the biology of muscle stem cells in a therapeutical context.

Conclusions

Here we review the data available regarding the functional role of the deiodinases in muscle stem cells. Skeletal muscle is a target of thyroid hormone (TH), as demonstrated by the capacity of THs to affect composition, strength, contraction, glucose metabolism and energy homeostasis in this tissue. An emerging concept in this field is that skeletal muscle stem cells are a major target of TH action. The proliferation and differentiation of skeletal muscle stem cells depends strictly on locally regulated TH action. Thyroid hormone levels are locally controlled by a family of enzymes, the deiodinases. Deiodinases type 2 and 3 are involved in the local modulation of TH in skeletal muscle. D2 plays a pivotal role in the differentiation of activated satellite cells and is highly expressed in differentiating myoblasts in culture as well as in the late phase of the regeneration of skeletal muscle. Conversely, D3 is essential for amplification of satellite cells and is expressed in early phase in myogenesis (21).

In the myoblast differentiation program, D3 and D2 expression increase at different specific times so as to allow proliferation in advance of differentiation. A future exciting challenge in the field of TH and muscle physiology is to exploit the basic knowledge emerging in this area in a therapeutically relevant context. In dystrophic muscles, the functional muscle impairment is caused by exhaustion of the stem cell muscle reservoir that abolishes the ability to repair the chronically damaged muscles. Thus, one may envisage the possibility of manipulating the deiodinases to improve the capacity of muscle stem cells to proliferate and differentiate.

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LEGEND OF THE GRAPHICAL ABSTRACT

The relationship between muscle regeneration, thyroid hormones and deiodinases.
Differential expression of D2 and D3 alters the active thyroid hormone concentration (T3) that regulates critical factors in the lineage progression and differentiation of muscle stem cells. The role and activity of the deiodinases in the quiescent state of muscle stem cells remains to be established. The genes directly regulated by thyroid hormone are shown in red. MHC: myosin heavy chain

GRAPHICAL ABSTRACT