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Molecular biology

Differential expression of zinc transporters accompanies the differentiation of C2C12 myoblasts

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ABSTRACT

Zinc transporters facilitate metal mobilization and compartmentalization, playing a key role in cellular development. Little is known about the mechanisms and pathways of Zn movement between Zn transporters and metalloproteins during myoblast differentiation. We analyzed the differential expression of ZIP and ZnT transporters during C2C12 myoblast differentiation. Zn transporters account for a transient decrease of intracellular Zn upon myogenesis induction followed by a gradual increase of Zn in myotubes. Considering the subcellular localization and function of each of the Zn transporters, our findings indicate that a fine regulation is necessary to maintain correct metal concentrations in the cytosol and subcellular compartments to avoid toxicity, maintain homeostasis, and for loading metalloproteins needed during myogenesis. This study advances our basic understanding of the complex Zn transport network during muscle differentiation.

1. Introduction

Zinc (Zn) is an essential trace nutrient necessary for normal physiological functions in all forms of life [1,2]. Zn is a cofactor in up to 10% of mammalian proteins and it is crucial for proper folding and for the catalytic activity of numerous enzymes [1,3]. With a Zn-deficient diet, mammals develop anemia, growth retardation, hypogonadism, skin abnormalities, diarrhea, chronic inflammatory and compromised immune function [1–6]. Conversely, excess Zn is cytotoxic and causes deficient copper absorption [7–9]. Therefore, cellular Zn homeostasis must be tightly controlled. Two families of Zn transporters, ZnTs (solute-linked carrier 30, SLC30) and ZIPs (Zrt- and Irt-like proteins, SLC39) export and import Zn, respectively, between the cytosol, organelles, and extracellular milieu [1,2,10]. In mammals, ten ZnT (1–10) exporters and fourteen ZIP importers (1–14) have been identified. Nonetheless, the breadth of cellular functions for all these Zn transporters remains to be elucidated. ZnTs and ZIPs are transmembrane proteins predicted to have 6 or 8 transmembrane domains [2]. Zn transport occurs in response to an ionic gradient. Most ZnTs dimerize and export Zn by a diffusion-mediated Zn2+/H+ exchange, while ZIPs import the ion through a channel-like activity [2]. However, some ZIPs import Zn through a bicarbonate/Zn2+ symport mechanism [11]. Certain transporters like ZIP8 and ZIP14 also transport Fe, Mn, and Cd [12] while ZnT10 is the only ZnT known to primarily transport Mn over Zn [13,14].

Members of the ZnT and ZIP families have a prominent role in a wide range of developmental processes. For instance, in chondrocytes, increased levels of cellular Zn have been associated with the presence of ZIP8 in lysosomal vesicles, which may activate MTF1, a metal responsive transcription factor [15]. MTF1 in turn promotes the expression of metalloproteases (MMPs) that degrade different components of the extracellular matrix [16–19]. ZIP12 has been found to induce neuronal differentiation via activation of cAMP response element binding protein (CREB) [20]. ZIP14 transports Fe, Mn and Cd in addition to Zn [21–23]. Interestingly, severely affected phenotypes are observed in Zip14 knock-out animals. For instance, these mice have impaired systemic growth, decreased body sizes, and impaired skeletal development due to impaired CREB activation [24]. Moreover, mice lacking Zip14...
present a proliferation defect in hepatocytes, are hypoglycemic, hyperinsulinemic, and have higher levels of glucose in the liver [21,25]. Importantly, CREB activation is also necessary for proper development and function of the skeletal muscle lineage. It is required for differentiation of embryonic skeletal muscle progenitors, for survival of adult skeletal muscle, for promoting myoblast proliferation in mice that were subject to muscle injury, and also for enhancing muscle regeneration in a dystrophic mouse model [26]. ZIP2 is induced by Zn deprivation in monocytes, and is necessary for keratinocyte differentiation [1,27,28].

Experimental evidence suggests a major role in development for ZIP2, as knock-out mice are sensitive to dietary Zn deficiency during pregnancy [29]. ZIP5 is required for systemic Zn excretion and to promote the development of the sclera and retina [30–32]. ZIP6 over-expression has been associated with the epithelial-mesenchymal transition of different cancer types [33–37]. ZIP6 also contributes to dendritic cell maturation [38]. ZIP1 is proposed to be involved in activation of microglia, embryonic development, and prostate metabolism [2,39–42]. ZIP4 is required for early development, dietary Zn uptake in the intestine, and Zn levels regulate its expression [43,44]. ZIP10-dependent Zn signaling is required for early development of B-cells by suppressing caspase activity in the bone marrow [45]. The above mentioned ZIPs are located to the plasma membrane. Other ZIPs participate in Zn transport for protein maturation, a process that occurs in intracellular compartments. ZIP7, as an example, is located in the endoplasmic reticulum (ER) and the trans Golgi network (TGN), and it has an important role in glycemic control of skeletal muscle [46]. ZIP9 is located at the cell surface and TGN [47,48] and is considered to be an androgen receptor in addition to its role in Zn transport [48]. ZIP13 mobilizes Zn to the TGN and secretory vesicles, controls Zn homeostasis in the ER and is involved in BMP/TGF-β/Smad signaling pathway [49–51].

ZnT1 is the only recognized Zn exporter located to the plasma membrane and is essential during embryonic development, as deletion of the ZnT1 gene in mice is embryonic lethal [52,53]. On the other hand, the majority of the ZnT transporters are localized to secretory structures and the TGN [2]. Their involvement in growth and differentiation has been reported in several cellular systems. For instance, ZnT7 deletion causes growth retardation, decreased systemic Zn, reduced fat accumulation, and impaired glucose tolerance and insulin resistance in male mice [54–56]. ZnT10 is also located in endosomal vesicles, potentially contributing to Zn and Mn export [3]. ZnT5 acts in the early secretory pathway, and knock-out mice for this transporter present with growth delays, muscle weakness and cardiac deficiencies [57]. A ZnT5/6 dimer mobilizes Zn to activate enzymes such as alkaline phosphatases in the early secretory pathway [58]. Other transporters located to the early secretory pathway and TGN are ZnT2, ZnT3 and ZnT8. ZnT2 expression is induced by elevated Zn levels, and has been detected in mammary glands, prostate, pancreas, small intestine, kidney, and retina [31,59,60]. ZnT2 is necessary for secretion of Zn into breast milk in humans [61–64]. ZnT3 is located mainly in synaptic vesicles of glutamatergic neurons located in the hippocampus, but is proposed to also modulate insulin production in pancreatic β cells [65–67]. ZnT8 activity has important implications in diabetes mellitus [68]. ZnT8 knock-out mice fed a high-fat diet became glucose intolerant or diabetic, and islet cells became less responsive to glucose. This is due to a failure to transport Zn into insulin granules, which has been shown to co-crystallize with insulin in pancreatic beta cells [68,69].

Transport of Zn to the nucleus is fundamental for cell growth and development. A large number of transcription factors require Zn for DNA binding and activation of gene expression [70–74]. Zn binding transcription factors can be metallated in the cytosol or subcellular compartments, although Zn acquisition may also occur in the nucleus [75]. Zn has also been found in the nucleolus and chromosomes, and is implicated in DNA replication and stabilization of DNA, RNA, and ribosomes [76]. ZIP11 is the only transporter proposed to be located at the nucleus, although no information on its cellular role is available [77]. Finally, ZnT9 homology to the ZnT family is under debate, as it lacks the distinguishing ZnT transmembrane Zn-binding domains and is mainly located in the cytoplasm [78,79]. ZnT9 acts as nuclear receptor coactivator and was renamed as GAC63 [78].

In spite of all this accumulated research, little is known about Zn requirements, regulation, and distribution in differentiating skeletal muscle cells. Because the majority of Zn in mammalian cells is bound to metalloenzymes and stored in organelles and cellular vesicles, free zinc levels remain quite low [2,80–84]. Within the cell, about 50% of Zn is found in the cytoplasm and in vesicles, 30–40% in the nucleus, and about 10% in the cell membrane [85,86]. Moreover, 60% of the total systemic Zn is sequestered and used in skeletal muscle, making this organ the greatest reservoir of Zn in the body [87]. Whether Zn is necessary for myoblast differentiation is a matter of controversy. Experimental evidence suggests that addition of Zn to differentiation medium inhibits myogenesis, but promotes myoblast proliferation and activation of quiescent myogenic satellite cells [88]. Yet other evidence demonstrated that Zn is required for differentiation, as myoblast differentiation in both C2C12 cells and chicken embryo myoblasts was inhibited in Zn-deficient medium [88,89]. Furthermore, mice fed with a Zn-deficient diet may have reduced muscle regeneration upon injury [4]. C2C12 myoblasts are an excellent model to study myogenesis due to their similarities to progenitor myogenic lines [90]. Upon serum depletion, the cells commit to differentiate and the myoblasts fuse together to become multinucleated myotubes. Myoblasts express specific regulators of the skeletal muscle lineage like MyoD, myogenic factor 5 (Myf5), and myogenin. Upon induction of differentiation, MyoD and Myf5 drive the expression of myogenin, a transcription factor fundamental for the coordination of skeletal muscle development and repair. Once the myoblasts commit to differentiate, myogenin expression continues to increase as myogenesis progresses leading to the expression of muscle-specific proteins such as myosin heavy chains, skeletal actin, muscle-specific creatine kinase and MMPs, among others. Additionally, myotubes express insulin receptors and require insulin binding for growth [90].

We hypothesized that the intracellular levels of Zn in differentiating myoblasts is tightly controlled by a timely and differential expression of ZnT and ZIP transporters that is specific to the skeletal muscle lineage. The C2C12 stable murine cell line was used as a model of myogenesis. We analyzed the kinetics of Zn accumulation at different stages of this process. Dynamic changes of Zn levels were observed over the course of myogenic differentiation. Gene and protein expression analyses suggest that cellular changes in Zn content in myogenesis may be dependent mainly on four ZIP transporters located at the plasma membrane (ZIP3, 8, 5 and 6), and four transporters located in subcellular compartments (ZnT7, 4, 8 and ZIP11). This study set the basis for understanding how Zn is distributed and potentially utilized during skeletal muscle differentiation.

2. Materials and methods

2.1. Cell culture

C2C12 cells are immortalized mouse myoblast used as an in vitro model for myogenesis studies. C2C12 cells proliferate rapidly under high serum conditions, and undergo myogenic differentiation under low serum conditions [91,92]. C2C12 myoblasts were seeded at 1 × 10⁵ cells/cm² in DMEM media containing 10% fetal bovine serum (Gibco) to support proliferation. After 48 h, cells reached confluence, and the media was changed to differentiation media (DMEM, 2% horse serum). At least three independent biological replicates were collected for analysis at different time points of proliferation for 24 h (P) and throughout myogenic differentiation (0, 3, 6, 12, 24, 48, and 72 h).

2.2. Whole cell Zn content analysis

C2C12 cells from the indicated time points were rinsed three times
and immediately re-suspended in 100 μl of PBS. Cells were lysed by three sonication cycles (30 s on by 30 s off) for 5 min at medium intensity using a Bioruptor UCD-200 (Diagenode). Total protein content was determined by Bradford [93]. Then, samples were acid digested in concentrated HNO3 (trace metal grade) for 1 h at 80 °C and overnight at 20 °C. Digestions were concluded by adding 30 μl H2O2 [94–97]. The resulting solution was measured by triplicate using atomic absorption spectroscopy (AAS) equipped with a graphite furnace (PerkinElmer, Analyst 800), which provides an ideal limit of detection (LOD) of Zn from currently available techniques [98]. The estimated LOD, calculated as 3σ of 0.01 μmol L−1, allowed quantification of all samples. The quantification of Zn, at the ppb level, was completed using analytical grade standards diluted in 18 MΩ water. Concentration of Zn measured via AAS was normalized to the initial mass of protein in each sample.

2.3. Zinc imaging

Proliferating and differentiating C2C12 cells were loaded with 2 μM Fluo-Zin3 AM, cell permeant (Invitrogen) in the corresponding culture media for 40 min at 37 °C. Fluo-Zin3 has a high affinity to bind Zn2+. Then, the cells were washed with fresh media and allowed to rest for another 20 min at 37 °C. For live imaging of Zn the media was exchanged for PBS right before microscopic analyses, to avoid fluorescent interference of the culture media. In addition, cells were fixed with 10% formalin-PBS and counterstained with DAPI. Fluorescence images were obtained with a Leica TCS SP5 II AOBS confocal laser-scanning microscope (Leica).

2.4. Gene expression analysis of Zn transporters

RNA was purified from at least three independent biological replicates of proliferating and differentiated primary myoblasts with TRIzol (Invitrogen). cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix (ThermoFisher Scientific). Quantitative RT-PCR was performed with Fast SYBR green master mix (ThermoFisher Scientific) on the ABI StepOne Plus Sequence Detection System (Applied Biosystems) using the primers listed in Supp. Table 1 and normalized to the levels of the housekeeping gene Ef1-a.

2.5. Western blot analysis

C2C12 cells were washed with PBS and solubilized with RIPA buffer (10 mM piperazine-N,N-bis(2-ethanesulfonic acid), pH 7.4, 150 mM NaCl, 2 mM ethylenediamine-tetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, and 10% glycerol) containing complete protease inhibitor cocktail. Protein content was quantified by Bradford [93]. Samples (20 μg) were resolved by SDS-PAGE and electro-transferred to PVDF membranes (Millipore). The proteins of interest were detected with the specific antibodies (Supp. Table 2), followed by species-appropriate HRP-conjugated secondary antibodies and chemiluminescent detection (ECL PLUS; GE Healthcare). GAPDH was used as a loading control.

2.6. Immunocytochemistry

Proliferating and differentiating C2C12 cells were fixed overnight in 10% formalin-PBS at 4 °C. Samples were washed with PBS and incubated overnight with Hybridoma supernatants against myogenic markers (Supp. Table 2) in PBS buffer containing 5% horse serum and 0.2% Triton X-100. Secondary antibody binding and HRP staining were performed with the universal ABC kit (Vector Labs).

3. Results

We first asked whether cellular concentration of Zn varies as a function of the myogenic program. To address this question, we analyzed Zn levels in proliferating and differentiating C2C12 cells (Fig. 1). Samples were collected and whole cell metal content was determined by AAS. Analyses detected 0.93 ± 0.09 μmol Zn/mg of protein in proliferating myoblasts (Fig. 1A). Confocal microscopy of live and fixed proliferating myoblasts using Fluo-Zin3 showed that free Zn2+ is localized in cytosolic vesicles (Figs. 1B; Supp. S2). Whole cell Zn content significantly decreased when the differentiation program was initiated at 0 h (0.32 ± 0.02 μmol Zn/mg of protein; Fig. 1A). Fluorescent labelling of Zn2+ is in agreement with the quantitative analysis by AAS, and showed a large decrease of unbound Zn-containing vesicles (Figs. 1B; Supp. S2). After 12 h of differentiation, a gradual increase in cytosolic Zn was detected, reaching a maximal level in mature myotubes at 72 h (2.3 ± 0.3 μmol Zn/mg of protein; Fig. 1A). Fluo-Zin3 imaging also showed a gradual and sustained increase of cytosolic Zn2+ after 12 h, and until 72 h (Figs. 1B; Supp. S2), suggesting that the cellular Zn content is dynamic over the course of myogenesis. AAS analysis of Zn levels in both, proliferation and differentiation culture media showed no significant differences as indicated in Supp. Table 3, which support the idea that the Zn variations in the cells are independent of
the metal levels in the media.

We then asked which transporters, among all the recognized ZIPs and ZnTs, might be responsible for those changes. Therefore, we investigated whether myogenesis prompts transcriptional changes in Zn transporters located in the cell membrane of differentiating C2C12 myoblasts. Most of the transporters in the cell membrane belong to the ZIP family [1,2,11]. We found that Zip3 and Zip8 were upregulated over the course of myogenesis (Fig. 2). Zip3 showed the greatest increase in expression, starting at 12 h and going up to 20 fold-change by 72 h (Fig. 2A). ZIP3 is proposed to participate in Zn reuptake in other cellular systems [99], which is consistent with its expression at early stages of myogenesis (Fig. 2E). Zip8 mRNA exhibited a 4-fold increase 12–48 h after initiation of differentiation (Fig. 2B), but protein accumulation peaked in mature myotubes (Fig. 2E). Considering the gradual increase in Zn levels after 12 h of differentiation (Fig. 1), ZIP3 and 8 might participate in Zn re-uptake after the initial stages of myogenesis. ZIP8 could also contribute to the establishment of the differentiated phenotype by promoting the expression of MTF1 and trans-activation of MMPs, which are necessary for myotube fusion [15]. Zip5 and Zip6 transcripts were upregulated during the early stages of C2C12 differentiation, showing a peak at 12 h after induction of myogenesis (Fig. 2C,D); in both cases, the protein levels were constant during the first 12 h of differentiation, and decreased after 24 h. Additional genes encoding for Zn importers located at the cell membrane (Zip1, 2, 4, 10, 12, 14) had moderate changes in expression during myogenesis (Supp. Fig. 3A–F), and basal protein levels were sustained over time (Supp. Fig. S3H). Some of these transporters have been implicated in the development and differentiation of several tissues [2]. Nonetheless, it seems from our data that their role in skeletal muscle differentiation might be secondary. Altogether, our data indicate that changes in Zip expression, both transient and sustained over time, accompany changes in Zn content during myogenesis.

ZnT1 is the only proposed Zn exporter located to the plasma membrane [52]. Considering the significant decrease in the cellular levels of Zn during the initial hours of differentiation of C2C12 cells (Figs. 1, Supp. S2), we expected a major change in the expression of ZnT1. However, ZnT1 gene expression and protein abundance remained stable during differentiation of C2C12 cells (Supp. Fig. 3G, H);
suggesting that the early decrease in Zn in differentiating myoblasts may occur via additional transporters localized to distinct secretory elements.

Metal acquisition by enzymes is a post-translational modification critical for folding and catalytic activity, which in some cases occurs in the ER and TGN [100-102]. Therefore, we analyzed the expression patterns of transporters located in these compartments. ZnT7 mRNA expression peaked 12 h after induction of differentiation of C2C12 cells (Fig. 3A). ZnT7 participates in signaling pathways which involve the insulin receptor substrate 2 and AKT, which may have an important role for myogenesis [56]. Expression of other ER and TGN transporters, like ZIP7, remained constant during C2C12 differentiation, suggesting its constitutive expression (Supp. Fig. S4A), which is in agreement with a role for this transporter in glycemic control of skeletal muscles [46]. A modest increase in Zip9 and ZnT10 transcript levels was observed (Supp. Fig. S4B, C); however, Western blot analyses showed a differential increase in abundance of these two proteins at early (ZIP9) or late (ZnT10) stages of differentiation (Supp. Fig. S4I). Importantly, homozgyous mutations of ZnT10 produces muscle-related pathologies like Parkinsonism and dystonia with hypermanganesemia [103,104]. These muscular defects are consistent with abnormalities of mature tissue, supporting a role for ZnT10 in fully developed skeletal muscle cells.

Then, we analyzed the expression of Zn transporters located in compartments of the secretory route: lysosomes, endosomal vesicles, and secretory granules. These transporters potentially contribute to the metallation of secreted Zn-binding proteins. ZnT4 is an exporter associated with the secretory route. Interestingly, ZnT4 mRNA expression was induced during myogenesis (Fig. 3B). However, a decrease in the protein abundance was observed at early time points (Fig. 3E). Considering our quantification of Zn by AAS and confocal microscopy observations of Zn distribution (Figs. 1; Supp. S2), it is plausible that ZnT4 is responsible for Zn export via cytoplasmic vesicles, which is partially supported by Western blot analyses showing a decrease in protein levels early in differentiation (Fig. 3E). However, the mechanisms by which ZnT4 may fulfill this role remain to be elucidated and are beyond the scope of this paper. ZIP13, ZnT6, and ZnT5 are localized to secretory vesicles [2,68]. ZIP13 and ZnT6 showed a modest but significant increase in ZnT10 stages of differentiation (Supp. Fig. S4I). Importantly, homozgyous mutations of ZIP13 produces muscle-related pathologies like Parkinsonism and dystonia with hypermanganesemia [103,104]. These muscular defects are consistent with abnormalities of mature tissue, supporting a role for ZIP13 in fully developed skeletal muscle cells.

Fig. 3. Expression profiles of Zn transporters found in subcellular compartments during C2C12 differentiation. Gene expression profiles of the transporters located in the ER and TGN: ZnT7 (A), ZnT4 (B), ZnT8 (C) and Zip11 (D). Data represent the mean of at least three independent experiments +/- S.E; two-tailed unpaired t-test; *P < 0.01. Representative Western blots of ZnT7, ZnT4, ZnT8 and ZIP11. GAPDH as loading control.
role for these proteins and for nuclear Zn in mature myotubes, which is worth exploring in the future.

4. Conclusions

The expression, localization, and roles of Zn transporters in different tissues and stages of development are largely unknown. In this study we investigated the differential expression of Zn transporters and how intracellular levels of Zn vary during differentiation of C2C12 myoblasts. We found that Zn levels decrease during early stages of myogenesis, only to be restored by 12 h after the initiation of the myogenic program. Zn levels continue to increase until the end of the differentiation period. We also found that the majority of the transporters were expressed as a consequence of the myogenic program and were often detected during the first 12 h upon induction of differentiation. Only ZnT8 was down-regulated in differentiating C2C12 cells. Our data suggest that myogenesis requires specific and optimal Zn levels in different subcellular compartments. Where most of this Zn is needed remains unclear, but our data indicate that a variety of Zn-binding proteins secreted from cellular organelles and granules are most likely necessary during myogenesis. Our findings also suggest that a dynamic network of transport and distribution of Zn occurs as a consequence of initiation of myoblast differentiation.

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Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.jtemb.2018.04.024.

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