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Analysis of UCP1 Expression in Rainbow Trout Gonadal Cell Line RTG-2 Indicates its Marginal Response to Adipogenic Inducers Compared to Mammalian Cell Lines

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Uncoupling protein 1 (UCP1) is a unique mitochondrial membranous protein expressed in brown adipose tissue (BAT) in mammals. While its expression in response to cold temperatures and adipogenic inducers is well-characterized in mammals and human infants, the molecular characterization and expression of UCP1 in fish remain unexplored. To address this gap, we analyzed UCP1 expression in response to adipogenic inducers in a fish cell line, rainbow trout gonadal cells (RTG-2), and compared it with UCP1 expression in three mammalian preadipocytes, 3T3-L1, T37i, and WT1 exposed to the Peroxisome proliferator-activated receptor gamma (PPARγ) agonists, rosiglitazone (Rosi). In mammalian preadipocytes, UCP1 protein was highly expressed by Rosi, with an induction of adipogenesis observed in a time-dependent manner. This suggests that UCP1 plays a significant role in adipogenesis in mammals. However, RTG-2 cells showed no response to adipogenic inducers and exhibited only marginal expressions of UCP1. These results imply that RTG-2 cells may lack crucial responsive mechanisms to adipogenic signals or that the adipogenic response is regulated by other mechanisms. Further studies are needed to confirm these phenomena in fish preadipocytes when an appropriate cell line is established in future research.

Keywords: Fish, Adipogenesis, UCP1 expression, Rosiglitazone

In mammals, adipose tissues are typically classified into two main types: white adipose tissue (WAT) and BAT (Gesta et al., 2007). WAT, in general, serves as a repository for triglycerides, playing a crucial role in energy storage, maintaining glucose homeostasis, regulating inflammatory processes, and participating in endocrine functions (Billon and Dani, 2011). Conversely, the physiological role of BAT has been the subject of extensive investigation. Brown adipocytes within BAT are characterized by their heightened metabolic activity, which is facilitated by a large number of mitochondria and numerous small triglyceride droplets (Cannon and Nedergaard, 2004). They are also known to possess specialized functions primarily centered around the expenditure of energy through non-shivering thermogenesis (NST) (Foster and Frydman, 1978; Himms-Hagen, 1984). This process generates heat energy either through the UCP1-dependent uncoupling of mitochondrial respiration or via UCP1-independent signaling cascades (Cannon and Nedergaard, 2004; Wang et al., 2016). Furthermore, several studies have highlighted BAT's involvement in the endocrine system, as well as its role in maintaining glucose and lipid homeostasis (Stanford et al., 2012; Villarroya et al., 2017).

UCP1, a member of the mitochondrial carrier protein superfamily, is predominantly expressed in the inner mitochondrial membrane of BAT. Its primary function is to uncouple mitochondrial fatty acid oxidation from ATP production, achieving this by mediating a controlled release of the proton gradient generated by the respiratory chain (Ledesma et al., 2002; Ikeda and Yamada, 2020). This energy-dissipating mechanism is essential for NST and cold survival in various mammals, including rodents, hibernators, and human infants (Cannon and Nedergaard, 2004; Tine et al., 2012). The gene expression of UCP1 in BAT is mainly regulated

by the upregulation or stimulation of several crucial components, such as PPARγ, β3Ad, and PGC1 (Seale et al., 2011; Boström et al., 2012; Park et al., 2016). In contrast to mammals, teleost fish has only WAT, not beige adipose tissue (BeAT) or BAT (Salmerón, 2018). However, UCP1 genes have been identified not only in mammals but also in fish (Jarmuszkiewicz et al., 1999; Jastroch et al., 2005), and it was reported that cold-water fish, such as rainbow trout lived below 21℃, contain a higher accumulation of unsaturated fatty acids for cold adaptation (Johnston, 1990; Crespi and New, 2009). To date, it remains unclear whether the classical mechanism of NST involving UCP1 exists in fish adapted to long-term low temperatures.

In this study, our objective was to investigate the potential for brown and/or beige adipocyte differentiation induced by PPARγ agonists in rainbow trout. Although there is limited research on adipogenic differentiation within BAT in fish, a recent report suggested the presence of adipocyte progenitors (APs) capable of adipogenic differentiation within gonadal WAT (Vishvanath and

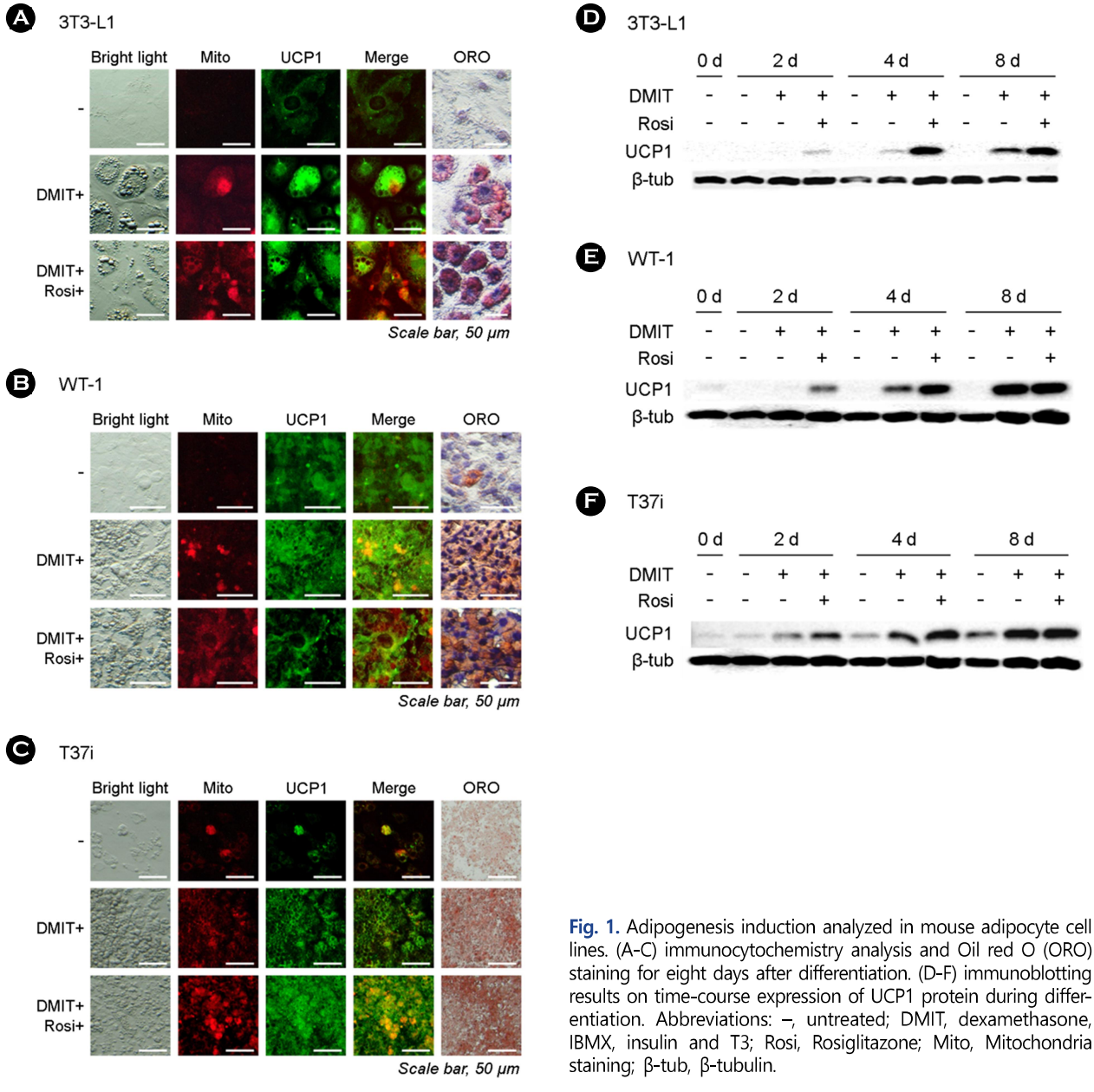

Fig. 1. Adipogenesis induction analyzed in mouse adipocyte cell lines. (A-C) immunocytochemistry analysis and Oil red O (ORO) staining for eight days after differentiation. (D-F) immunoblotting results on time-course expression of UCP1 protein during differentiation. Abbreviations: –, untreated; DMIT, dexamethasone, IBMX, insulin and T3; Rosi, Rosiglitazone; Mito, Mitochondria staining; β-tub, β-tubulin.

Gupta, 2019). Hence, we chose to perform our experiment with a gonadal cell line, RTG-2, derived from rainbow trout. Three types of preadipocytes (3T3-L1, T37i, and WT1), previously studies on brown or beige adipogenesis, and RTG-2 were treated with the PPARγ agonists. This approach allowed us to compare the results between the fish cell line RTG-2 and the mammalian cell lines.

The mouse preadipocyte cell lines, 3T3-L1 (CL-173), and RTG-2 (CCL-55), were procured from ATCC (Manassas, VA, USA). Mouse brown preadipocyte (SCC255) and mouse brown adipocyte (SCC-250) were obtained from Sigma-Aldrich (St. Louis, MO, USA). To induce adipogenesis in the mouse preadipocyte cell lines, they were exposed to DMIT, as a mixture with 1 μM dexamethasone, 500 μM 3-Isobutyl-1-methylxanthine, 0.5 μg/mL insulin and 1 nM 3,3',5-Tniodo-L-thyronine, and/or 5 μM Rosi dissolved in culture media containing 10% FBS, for eight days. The culture media were refreshed every two days. A similar exposure regime was employed for the RTG-2 cells. Specifically, the RTG-2 cells were exposed to EMEM with DMIT and 5 μM Rosi. Additionally, cells were harvested, and total protein was extracted for the analysis of time-course UCP1 expression using immunoblotting. Cell samples were collected at day 0, 2, 4, and 8, and immediately fixed with 10% formalin for 20 min at room temperature for subsequent immunocytochemistry analysis and Oil Red O (ORO; O0625; Sigma-Aldrich) staining. Antibodies against UCP1 and β-tubulin were sourced from Abcam (ab10983; Cambridge, UK) and Cell Signaling Technology (2146S; Beverly, MA, USA), respectively. To visualize mitochondria, 1 μM MitoTracker® Deep Red FM (8778P; Cell Signaling Technology, Beverly, MA, USA) was applied to the cells for 30 min before fixation. For immunocytochemistry analysis, cells were subjected to permeabilization and background epitope blocking by incubation for 15 min in a blocking solution containing 2.5% BSA, 2% Normal Goat Serum, and 0.2% Triton X-100 in PBS. Subsequently, UCP1 as primary antibodies, diluted to 1:500 in blocking solution, were applied to the cells overnight at 4℃. After a 15-minute wash, secondary antibodies were introduced and allowed to incubate for 60 min at room temperature. Nuclei were visualized using DAPI (D9542; Sigma-Aldrich), and coverslips were mounted using Fluorescence Mounting Medium (S3023; Dako, Glostrup, Denmark). For ORO staining, the fixed cells were incubated for 1 h with 0.3% ORO in 60% isopropanol. Cell lysates, in a 2 × SDS sample buffer, were separated on SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). To block nonspecific proteins, membranes were treated with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 30 min. Subsequently, membranes

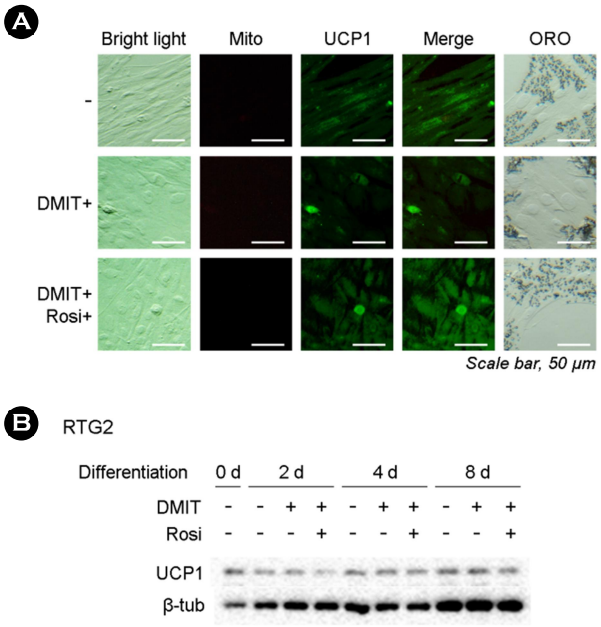

Fig. 2. Adipogenesis induction analyzed in rainbow trout gonadal cell line RTG-2. (A) immunocytochemistry analysis and ORO staining for eight days after differentiation. (B) immunoblotting results on time-course expression of UCP1 protein during differentiation. Abbreviations: –, untreated; DMIT, dexamethasone, IBMX, insulin and triiodothyronine; Rosi, Rosiglitazone; Mito, Mitochondria staining; β-tub, β-tubulin.

were incubated overnight with primary antibodies diluted to 1:1,000 in the blocking solution. After this, the membranes were exposed to a horseradish peroxidase-conjugated secondary antibody for 1 h in the blocking solution. Blots were visualized using the Clarity Western ECL Substrate kit (Bio-Rad, Hercules, CA, USA).

Immunocytochemistry analysis revealed that the three preadipocyte cell lines exhibited an increase in UCP1 expression, which coincided with the results obtained from mitochondrial membrane measurements (Fig. 1A-C). These increases were further validated by ORO staining results. Immunoblotting demonstrated a time-dependent increase in UCP1 expression in all three cell lines (Fig. 1D-F). However, in the case of RTG-2 cells, no significant differences were observed in the potential induction of adipogenesis, as well as UCP1 expression (Fig. 2A). Additionally, there were no discernible morphological differences in RTG-2 cells following exposure to PPARγ agonists. ORO staining did not yield any positive results in RTG-2 cells. When we assessed the expression of UCP1 via immunoblotting, only marginal expressions were observed on each sampling day (Fig. 2B).

In summary, these findings suggest that the fish cell line RTG-2

may lack crucial responsive mechanisms to adipogenic signals, or that the adipogenic response is regulated by alternative mechanisms. Given the high expression of UCP1 protein in mammalian preadipocytes induced by DMIT and/or Rosi, we can assume that our experimental conditions were appropriate to support the role of UCP1 in adipogenesis. However, a limitation of this study is the absence of suitable preadipocyte cell lines in fish and rainbow trout-specific or fish-specific UCP1 antibodies. To investigate the precise expression and function of UCP1, adipose stem cells, rather than gonadal cells, must be extracted from rainbow trout or other fish and differentiated into fat cells. Further research is necessary to confirm these phenomena in fish preadipocytes once an appropriate cell line becomes available in future studies.

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Competing Interests

The authors declare that there is no conflict of interest.

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