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Role of *c-Cbl* Gene in Muscle Energy Metabolism

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Abstract: *It is important to understand the molecular mechanisms regulating energy expenditure in skeletal muscle cells. In particular, mitochondrial function and mitochondrial oxidative capacity has been a major research focus in the fight against obesity. This study was conducted under the hypothesis that the protein product of the c-Cbl gene negatively regulates the energy expenditure of muscle cells by inhibiting the mitochondrial capacity for oxidative function. It investigated the role played by the c-Cbl gene by examining c-Cbl gene knockout C2C12 cells for energy metabolism.*

Keywords: *C-Cbl Gene, Mitochondrial Function, Mitochondrial Capacity, Mitochondrial Dynamics, C-Cbl Knockout C2C12 Cells*

1. INTRODUCTION

It has been shown that *c-Cbl* gene knockout mice exhibit enhanced energy expenditure **[1,2]**. These gene knockout mice are protected against High Fat diet-induced obesity due to an increase in whole-body energy expenditure **[3]**. These studies indicate that *c-Cbl* gene expression may inhibit whole-body energy expenditure. However, these studies do not explain whether this is due to a direct action of *c-Cbl* protein product in skeletal muscle cells or other tissues. It was found that in wild-type mice, the *c-Cbl* gene expression is increased specifically in skeletal muscle cells when a High Fat diet is used, which also induces obesity and insulin resistance. This suggests that skeletal muscle is a major site for the regulatory role of *c-Cbl* in whole-body energy expenditure **[3]**.To follow up on these findings, the present study investigated the cellular mechanism underlying the role of *c-Cbl* in regulating energy metabolism in muscle cells. Understanding the *c-Cbl* associated cellular mechanism controlling energy expenditure may reveal novel drug targets for the treatment of obesity.

Energy metabolism in muscle cells is largely dependent on mitochondria because mitochondria are the apparatus used to generate energy within cells. Different mechanisms of mitochondrial dysfunctions have been suggested as potential causes for the development of obesity and/or insulin resistance, including impairment in mitochondrial capacity, mitochondrial function, and mitochondrial dynamics. It has been shown that mitochondrial dysfunction can cause intra-myocellular lipid accumulation due to decreases in the utilization of fuels, particularly fatty acids **[4,5]**. The intra-myocellular lipid accumulation is associated with insulin resistance in skeletal muscle. It has been reported that obesity is related to the diminished activity of mitochondrial oxidative enzymes in skeletal muscle **[6]**. Thus, it is important to investigate the role of *c-Cbl* in maintenance of mitochondrial capacity, oxidative function, and dynamics of muscle cells to understand how *c-Cbl* may affect the energy expenditure of muscle cells by regulating mitochondrial metabolism.

The hypothesis for the studies was that the protein product of the c-Cbl gene negatively regulates the energy expenditure of muscle cells by inhibiting the mitochondrial capacity or oxidative function. This hypothesis was tested by conducting a series of experiments in cultured C2C12 cells, a well-established murine muscle cell line. The role of c-Cbl gene was investigated by examining the effects of c-Cbl knockout on energy metabolism, mitochondrial function, mitochondrial capacity, and mitochondrial dynamics, as well as the differentiation of C2C12. As a recent study reports that obesity may be related to the impairment in muscle differentiation [7,8,9]. This study compared the effect of c-Cbl knockout between undifferentiated C2C12 (myoblasts) and differentiated C2C12 (myotubes).

2. MATERIALS & METHODS

2.1. Cell Culture

The C2C12 murine cell line was cultured on the growth medium (GM), α MEM supplemented with 10% heat-inactivated FBS and 1% PSG, (all from Invitrogen, Australia) and maintained in a humidified incubator (95% air, 5% CO_2) at 37°C. The C2C12 were seeded and allowed to reach full confluence. Cells were monitored and photographed using the Nikon TS100F inverted microscope and camera (Nikon Instruments Inc., USA) (Figure 1).

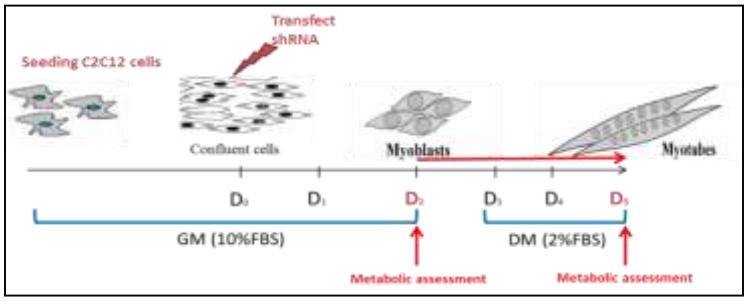

Figure1. *Schematic showing the experimental design of the c-Cbl study. C2C12 cells were seeded the night before transfection. On Day0, muscle cells were transfected with control or c-Cbl targeted shRNAs when the cells reached 70%–80% confluence. After two days (Day2) of transfection, myoblasts were lysed for further metabolic assessment. Myotubes were grown in differentiation medium (DM) and were lysed 5 days (Day5) post-transfection for further metabolic assessment.*

2.2. Transfection of Cells

At 70%–80% cell confluence, *c-Cbl* specific and negative control short hairpin RNAs (shRNAs) plasmids (Invitrogen, Australia) were transfected with Lipofectamine™ 2000 (Invitrogen, #11668-019) following manufacturer protocols on Day0 (Figure 1).

2.3. Differentiation of Myoblasts

To induce myoblasts differentiation, at Day2 the GM in myoblasts was changed to differentiation medium (DM); α MEM supplemented with 2% heat-inactivated FBS and 1% PSG for at least 4 days, as illustrated in Figure 1, to assess the metabolic function of myotubes.

2.4. Western Blotting

Cells were washed with ice-cold PBS and homogenized in ice-cold lysis HES-buffer supplemented with protease and phosphatase inhibitors. Protein levels in the homogenate were measured using the BCA protocol. Equal amounts of proteins (20–40 μg) were loaded and resolved on SDS-PAGE. Levels of protein were determined by the immunoblotting method using specific antibodies.

2.5. Real Time PCR

Total RNA was extracted from the cells using TRIzol® reagent according to the manufacturing protocol. Real-time PCR was carried out using SYBR Green PCR Master Mix method for murine PGC1α, FOXO1, and UCP3 (Genework, Australia). The expression of each gene was analyzed in triplicate and normalized against the expression of housekeeping gene 18S using 2 delta CT methods.

2.6. Measurement of Metabolic Parameters

The XFe24 analyzer measures physiological changes in cellular energy and metabolic pathways. It measures the shift between aerobic respiration through the oxygen consumption rate (OCR) and glycolysis through extracellular acidification rate (ECAR). OCR and ECAR were determined in myoblasts and myotubes. C2C12 myoblasts were seeded into an XFe24 cell culture plate in GM; however, depending on the experiment, the medium may be replaced with DM to promote myotube formation. On the day of the experiment, cell medium is removed and replaced with assay (XF base) medium supplemented with glucose, sodium pyruvate and glutamine (pH 7.4) for 1 hour in a non-CO_2 incubator at 37 ºC to allow the medium temperature and pH to reach equilibrium. The cartridge sensors were calibrated and hydrated by an XFe24 analyzer using XF calibrant as described by the manufacturer. The XFe24 analyzer measured OCR and ECAR simultaneously for baseline and after the addition of Oligomycin (H^+-ATPase inhibitor), FCCP (Carbonyl cyanide 4-(trifluoromethoxy)

phenylhydrazone) (H^+gradient uncoupler), Antimycin A (complex III inhibitor), and Rotenone (complex I inhibitor) (all from Sigma-Aldrich, Australia).

2.7. Statistical Analyses

The data were treated as means ± SE. A two-tailed Student's t-test was used for the comparison of relevant groups. The analysis was performed using GraphPad Prism software (5.0, GraphPad Software Inc, CA, USA). Differences at $p<0.05$ were considered to be statistically significant.

3. Results & Discussion

The seeding density and injection compound concentrations were first optimized. OCR and ECAR were analyzed using 2 mixes followed by 3 measurements cycle. Basal OCR increased in a proportionate manner to the increasing cell number from 5000, 10,000, 25,000, and 50,000. These cells were stimulated with different FCCP concentrations (0.3 µM– 0.6 µM), (0.5 µM– 1.0 µM), and (0.75 µM– 1.5 µM). After the addition of 1.0 µM Oligomycin, FCCP showed the higher peak point at 0.5 µM – 1.0 µM on OCR, and the level of FCCP began to exhibit much lower respiration at 25,000 cells (Figure 2, A, C). For both basal and FCCP stimulated ECAR, 5000 and 10,000 cells did not increase (Figure 2, B, C). Further, basal OCR and ECAR values for different cell numbers were plotted to show the differences in the metabolic profile. Thus, the optimal seeding density is around 25,000 cells per well with 0.5 µM–1.0 µM FCCP concentrations. A seeding density of 30,000 C2C12 cells and a 1.0 µM FCCP concentration were chosen for further experiments.

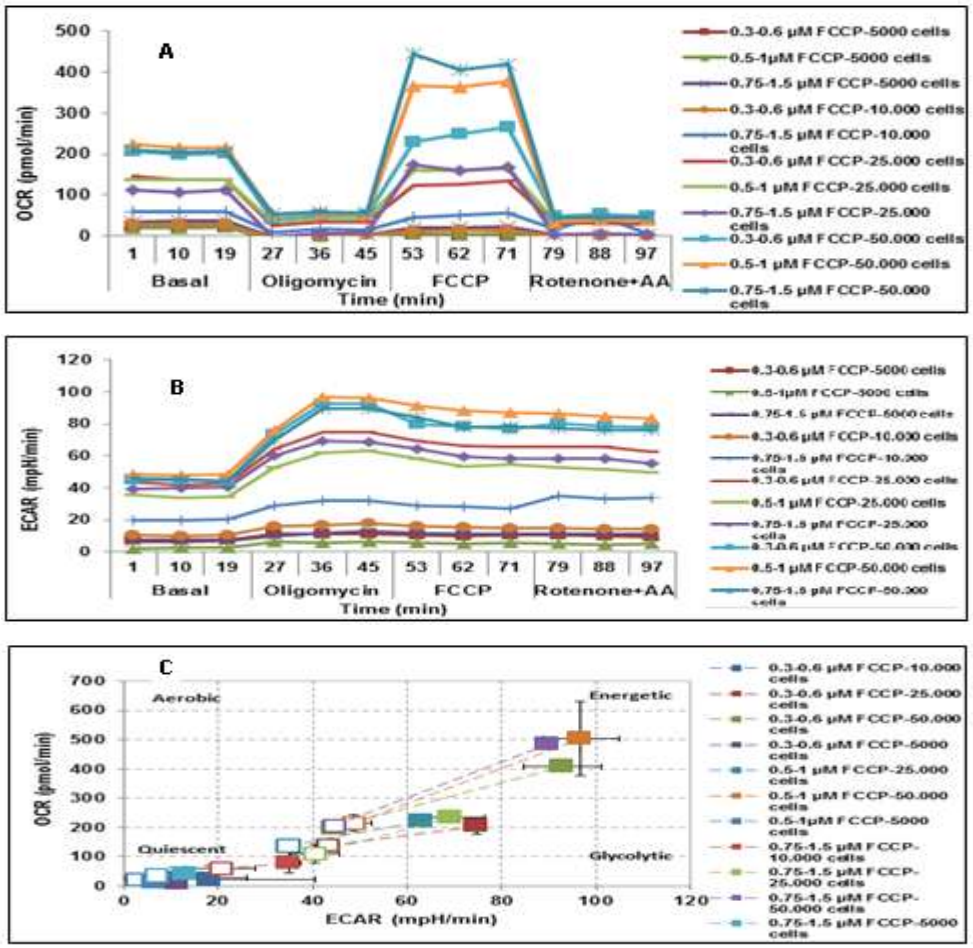

Figure2. *Optimization of the numbers of C2C12 cells and energy substrate concentrations. OCR (A) and ECAR (B) were measured on different muscle cell numbers. 5000, 10,000, 25,000, and 50,000 cells/well were seeded and incubated for 24 hours under basal respiration followed by the addition of Oligomycin (1 µM), FCCP (0.3–0.6 µM, 0.5–1 µM, 0.75–1 µM), Rotenone (1 µM), and Antimycin A (AA, 1 µM). (C) Relationship is shown between OCR and ECAR relative to cell numbers and FCCP concentrations. (Oligo: Oligomycin, FCCP: Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, AA: Antimycin A, Rot: Rotenone).*

The experiments were performed on C2C12 mouse myoblasts and differentiated myotubes over a period of 7 days to examine cell morphology (Figure 3).

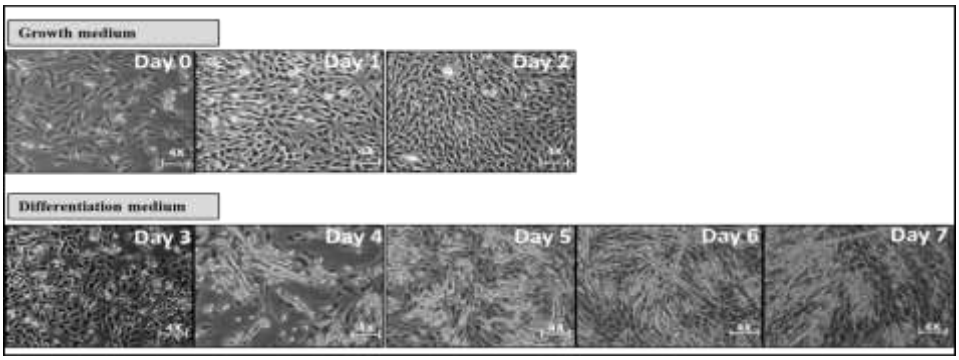

Figure3. *C2C12 myoblasts subjected to a 7-day time course of differentiation. Representative morphological images were taken using a Nikon Eclipse TS100 microscope with a Nikon Digital Sight camera of C2C12 cells. Muscle cells were grown in GM αMEM supplemented with heat-inactivated 10% FBS and 1% PSG from seeding until confluence on day 2, followed by DM αMEM with heat-inactivated 2% FBS and 1% PSG for at least 5 days.*

To assess the development of oxidative respiration in C2C12, the cellular bioenergetics function was measured in proliferating C2C12 myoblasts and differentiated myotubes using the XF24 analyzer (Seahorse, Biosciences). OCR was measured in proliferated myoblasts and differentiated myotubes in response to different substrates. The basal respiratory rate was higher in myotubes when compared to myoblasts ($p<0.01$). FCCP-induced maximal respiration was 1.3 times higher in myotubes than in myoblasts ($p<0.01$). ATP production, which is stimulated with the Oligomycin inhibitor, was increased in myotubes compared to myoblasts. Additionally, the ATP inhibition stimulated by Antimycin A and Rotenone was increased in myotubes (Figure 4, A). ECAR displayed the cellular maximum glycolytic capacity. The basal glycolysis (ECAR) level was reduced significantly in myotubes compared with myoblasts ($p<0.01$, Figure 4, B). The total cell numbers in myotubes were significantly higher compared to myoblasts ($p<0.01$, Figure 4, C).

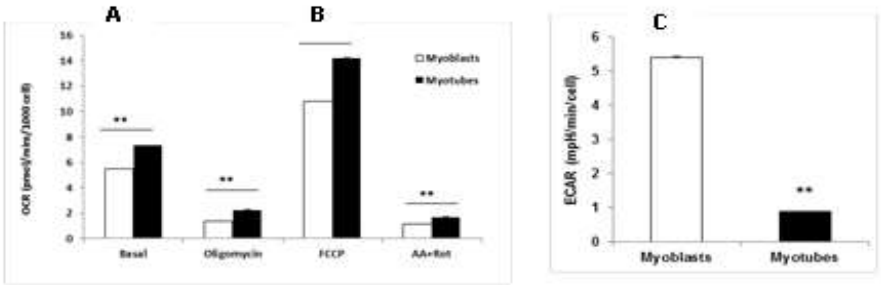

Figure4. *Comparison of mitochondrial respiration and glycolysis between myoblasts and myotubes. The OCR in basal; Oligomycin inhibitors which induced ATP production; FCCP- induced maximal respiration; and Antimycin A (AA), and Rotenone (Rot) inhibitors that inhibit ATP (A) and basal ECAR in myoblasts and myotubes (B) were normalized to the total cell numbers (C). The data shown is means ± SE from four independent experiments per cell type.*

This study investigated the effect of *c-Cbl* on the energy expenditure of muscle cells with the focus on mitochondrial function. Loss of skeletal muscle mass or function may cause obesity **[10]**. It has been shown that *c-Cbl* deficient mice show increased energy expenditure in skeletal muscle cells **[1]**. The *c-Cbl* gene is over expressed in skeletal muscle cells during High Fat diet-induced obesity, suggesting that *c-Cbl* may suppress the ability of muscle to increase the energy expenditure. Thus, it is likely that *c-Cbl* plays an important role in muscle energy metabolism through the regulation of mitochondrial function, mitochondrial capacity, mitochondrial biogenesis, and mitochondrial dynamics.

Muscle cells (myotubes/myofibres) develop from myoblasts. Muscle myotubes are frequently accompanied by a dramatic increase in the levels of mitochondrial function and mitochondrial content **[11]**. It has been reported that myotubes are a highly metabolic cell type that relies on oxidative respiration, while myoblasts rely on glycolysis for their metabolic demands **[12,13]**. To meet the high energetic demands, myotubes have large numbers of mitochondria arrayed in complex networks **[13]**. Consistent with that, it was found that the basal and the maximal respiration rate were 1.3 times higher in myotubes when compared to myoblasts, suggesting that myoblasts were relying on glycolysis for ATP production rather than oxidative respiration.

4. Conclusion

The results demonstrate that the increased energy expenditure in *c-Cbl* knockout muscle cells is likely due to the increased mitochondrial function and oxidative capacity. However, the precise role of *c-Cbl* in mitochondrial dynamics is still not completely clear.

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