ATF3-MEDIATED REGULATION OF GENES DIFFERENTIALLY EXPRESSED DURING PHYSICAL ACTIVITY

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Abstract

Previous studies from our laboratory have identified nine genes (ATF3, NR4A2, NFIL3, NR4A3, MAFF, SIK, SLC2A3, MYC, SOCS3) that are differentially expressed between elite athletes and sedentary individuals and suggested their potential interconnection in pathways by using the STRING analysis. As a preliminary step towards revealing the broader gene interaction networks, the current study investigated the ATF3-mediated regulation of SOCS3 expression in the HEK293 cell line. Tamoxifen was applied as a drug that is known to alter the expression level of ATF3. Western blot analysis was conducted to assess protein expression under different conditions. The results demonstrated a dose-dependent increase in ATF3 expression at 2.5 µM and 25.5 µM tamoxifen, suggesting that tamoxifen induces cellular stress, consistent with previous studies. SOCS3 expression was unexpectedly higher in the vehicle control compared to tamoxifen-treated conditions, indicating that tamoxifen's effect on SOCS3 may be independent of ATF3. Additionally, tamoxifen significantly reduced SOCS3 levels, implying a potential role in regulating cytokine signaling pathways. Further research is necessary to confirm the dose-dependent variation between tamoxifen-induced ATF3 gene upregulation and alterations in SOCS3 expression as well as the other remaining genes from the potential pathway network. It will provide a golden opportunity to understand the broader effects of the genes responsible for muscle adaptation in response to physical activity.

Introduction

Over the years, it has been known that skeletal muscle tissue responds to different kinds of stress including physical activity through adaptation mechanisms involving cell signalling pathways (Fowler et al, 2024). Phosphatidylinositol-3-kinase AKT/mTOR signaling pathway (PI3K/AKT/mTOR) is activated under the influence of many growth factors and regulates the processes of cell division and apoptosis. During exercise, mTOR regulates skeletal muscle growth by phosphorylating various protein enzymes, as well as transcription factors for promotion of new protein production (Ashcroft et al., 2024). However, the level of adaptation to physical activity is not only determined by the activity cell signalling pathways, but also by the changes in expression of skeletal muscle genes as well (Furrer & Handschin, 2024).

Since people show different gene expression levels based on the intensity, duration, and type of exercising they perform, our previous studies aimed to investigate the genes that are differentially expressed between elite triathletes and sedentary individuals. They used Gene Expression Omnibus (GEO) datasets and blood sample analyses and identified most common nine genes related to physical activity (ATF3, NR4A2, NFIL3, NR4A3, MAFF, SIK, SLC2A3, MYC, SOCS3). The full name of the genes and their function in skeletal muscle are listed in the table below:

No	Gene	Gene Symbol	Function
1	Activating Transcription Factor 3	ATF3	Maintains the quiescence of skeletal muscle stem cells (Zhang et al, 2023)

2 3	Nuclear Receptor Subfamily 4, Group A	NR4A2 and NR4A3	Transcription factors involved in metabolic regulation (glucose), inflammation, and cellular stress responses (Prince et al, 2017)
4	Nuclear Factor Interleukin 3 Regulated	NFIL3	Binds to ATF sites, regulates the expression of genes involved in immune responses, such as natural killer (NK) cells and T cells (Kim et al, 2019)
5	MAF BZIP Transcription Factor F	MAFF	Oxidative stress response, detoxification, and metabolism (Wang et al, 2020)
6	Salt-Inducible Kinase	SIK	Energy metabolism, immune response, neurophysiology, and has been implicated in muscle insulin sensitivity (Jagannath et al, 2023)
7	Solute Carrier Family 2 Member 3	SLC2A3	Also known as GLUT3, involved in glucose transport (NLM, 2024)
8	MYC Proto-Oncogene	MYC	Regulates the expression of genes involved in metabolism and protein synthesis, which are essential for muscle growth and adaptation (Jones et al, 2024)
9	Suppressor of Cytokine Signaling 3	SOCS3	Involved in controlling inflammatory responses, which are crucial during muscle injury and regeneration processes (Luo et al, 2024)

Table 1. Summary of Nine Genes Modulating Homeostasis and Adaptation during Exercising. This Table Includes the Full Gene Names, Abbreviations of the Genes, and Their Main Function in Human Body

The researchers from our previous studies also performed the STRING pathway analysis, a powerful method of analyzing gene interactions (Szklarczyk et al, 2019), to examine how these

nine genes are possibly connected to each other (Figure 1).

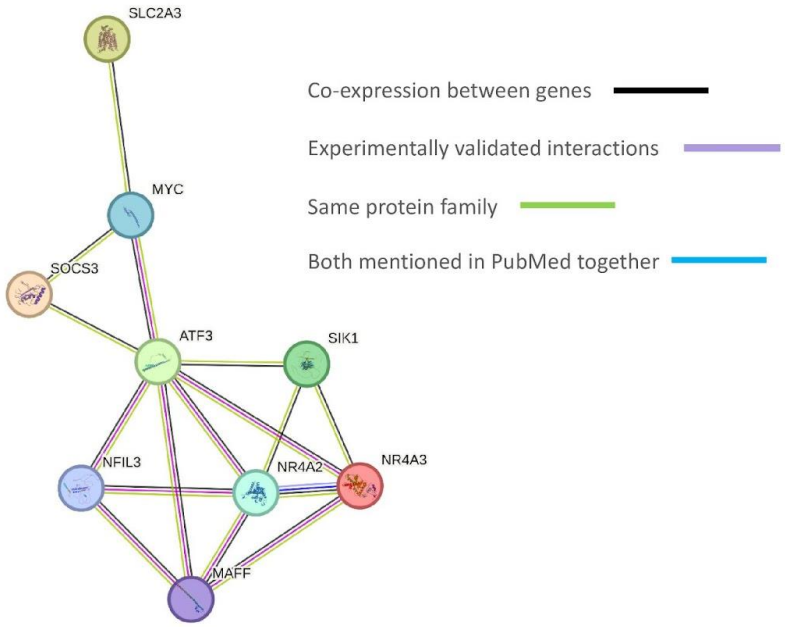

Figure 1. Potential Network of Connections among Nine Genes Revealed by the STRING Analysis (The Work from the Previous Study)

In Figure 1, genes are depicted as circles and each line shows different relationships among them. Black lines indicate co-expression between the genes, while purple lines demonstrate interactions that were proven through experiments. Green lines represent genes that are often mentioned together in scientific articles, mostly in PubMed abstracts, while blue lines show that genes share the identical protein family. However, due to time constraints, this study focuses on two key genes, which are ATF3 and SOCS3.

To begin with, according to Figure 1, ATF3 was the most connected gene, acting as the "hub" gene of the pathway because it was directly co-expressed with seven other genes, except for SLC2A3. Also, the direct relationship between these two genes was discovered in the previous

research further justifying their selection for this analysis. Luo et al. (2024) investigated the role of ATF3 in regulating SOCS3 expression. Cistrome, Jaspar, and other database analyses and in vitro experiments revealed that ATF3 acts as a transcriptional activator of SOCS3, where it binds to its promoter and enhances its protein expression (Luo et al., 2024).

ATF3 gene encodes for a stress-responsive transcription factor that belongs to ATF/cAMP response element-binding (CREB) family and binds to promoters with the consensus sequence TGACGTCA (Hu et al., 2024). Moreover, ATF3 is categorized under the basic leucine zipper (bZIP) family, implying an alternate role of the transcription regulatory factor in the cell.

It means that it can attach to other regulatory proteins (c-Jun, p53, C/EBP) through the bZIP domain rather than directly binding to DNA and impact apoptosis, stress response, and immune regulation in a manner that is not dependent on transcription. (Chen et al., 1994). One of the reasons why ATF3 was chosen for this study is because it was marked out as a “hub” in a possible pathway of interconnections among nine exercise-related genes as noted in the STRING analysis in our previous studies. Furthermore, Hu et al. (2023) also stated it had the “hub” role within the cellular adaptive-response network. This suggests that computational predictions are not just random, but actually highlight the functional importance of the gene within the network. According to Baumann and their colleagues (2023), the ATF3 gene is essential during exercise and sports because it helps skeletal muscles adjust and recover from the stress caused by physical activity. Some studies suggest that it particularly helps through regulation of inflammation response and muscle regeneration. In a study managed by Fernández-Verdejo et al. (2017), scientists found out that ATF3 reduces expression of pro-inflammatory chemokines and cytokines and increases expression of genes related to metabolism through modulation of

oxidative stress in skeletal muscle after exercise. Zhang et al. (2023) showed that ATF3 plays a part in turning on the gene for histone 2B, which is really important for changing how chromatin is arranged. This change helps stop muscle stem cells from turning into mature muscle cells too soon, which means they don't run out too early. Beyond just helping with muscle repair, ATF3 also seems to support muscle strength — when its levels go up, motor neurons not only survive better but also grow more effectively. This, consequently, improved skeletal muscle strength in amyotrophic lateral sclerosis muscle models by increased number of neuronal connections with skeletal muscle cells. All of these features make ATF3 a key regulator in various physiological conditions and an appropriate gene for the study.

SOCS3 gene encodes for a negative feedback inhibitor of cytokine signaling pathways that belongs to the Suppressor of the Cytokine Signaling family (Babon & Nicola, 2012). The SOCS3 protein consists of a C-terminal SOCS box for proteasomal degradation of key signaling proteins and N-terminal kinase inhibitory region (KIR) that blocks the activity of JAK. These structures assist SOCS3 in inhibiting cytokine signaling pathways, specifically Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway (Dai et al., 2021). The JAK/STAT signalling cascade perceives signaling molecules such as interleukins through a surface receptor that specifically bind them, transmit a signal into the cell and trigger a response, making it a universal pathway involved in the processes of inflammation, cell division, tumor growth and intercellular interactions (Hu et al., 2021). Once the appropriate response is obtained, SOCS3 switches off the JAK-STAT pathway via binding to JAK kinase that is attached to cytokine receptors and inhibiting further phosphorylation and dimerization of STAT3 proteins. In turn, it prevents STAT3 from binding to DNA and stops gene expression changes (Babon & Nicola, 2012). So, this causes avoidance of excessive activation and, subsequently,

chronic inflammation and autoimmune diseases (Chaves de Souza et al., 2013; Mahony et al., 2016). The regulation of cytokine signalling as well as the other functions of SOCS3 contribute to skeletal muscle adaptation. Swiderski et al. (2016) emphasized that SOCS3 affects inflammation by ensuring the specificity and duration of inflammatory responses. The other effects of SOCS3 include adequate steroid hormone production and glucose metabolism where the SOCS3-knockout mice in steroidogenic factor 1 cells showed decreased performance in resistance training (Pedroso et al., 2017). Also, SOCS3 impacts insulin signaling as the mice with the skeletal muscle-specific deletion of the gene demonstrated reduced obesity-induced insulin resistance (Jorgensen et al., 2013). Regardless of its role in modulating the immune system and glucose metabolism processes, higher levels of SOCS3 were found to be associated with reduced regenerative potential of muscle stem cells observed in aging (McKay et al., 2013). Collectively, these studies demonstrate that SOCS3 is a critical mediator linking inflammatory regulation, exercise performance, aging-related muscle dysfunction, and obesity-induced metabolic disturbances - the processes that impact muscle adaptation in response to training. To summarize, their necessity in muscle adaptation processes confirms ATF3 and SOCS3 as key candidates for studying muscle gene expression.

The Human Embryonic Kidney 293 cell line (HEK293) was utilized for the *in vitro* experiment. Since the cell line was derived from human embryonic kidney cells in the 1970s, their nature does not resemble the nature of human skeletal muscle cells (Graham et al., 1977). Nonetheless, HEK293 is a popular cell line used in the molecular biology research field (Malm et al., 2020). The transformation involving injection of sheared adenovirus 5 DNA into their host genome turned them into a valuable tool for studying gene expression due to their high viability and high

proliferative capacity of 24-36 hours (Geisse. 2009). All of these features establish them as a perfect model for studying gene expression changes in our experiment (Pulix et al., 2021).

In this regard, we utilized a cell-based assay that permits us to observe the changes both inside and outside of the cells stimulated by different compounds including pharmaceutical drugs (Yang et al., 2019). The reason why it was selected over biochemical assays because, in comparison with biochemical assays that are based on the measurement of the molecules and their corresponding reactions in the system, in vitro assays show a deeper understanding of the physiological context of the cellular response to the stimuli and the details of the mechanisms involved (Berg et al., 2014). We found that tamoxifen would be appropriate for our research since it is known not only as a selective estrogen receptor modulator (SERM) that that is commonly used in the treatment and prevention of estrogen receptor (ER) positive breast cancer, but also for its ability to induce stress (Yao et al., 2020; Denk et al., 2015). It works by binding to the ER, forming a complex, which relocates to the nucleus and subsequently attracts the co-suppressors as NCoR and SMRT. This results in the inhibition of the gene expression involved in cell proliferation (Ali et al., 2016). In addition, tamoxifen causes stress through the disruption of cholesterol homeostasis that leads to changes in ATF3 expression in neuronal cells (Denk et al., 2015). Given that ATF3 responds to different stress signals, tamoxifen is a perfect candidate for investigating the ATF3-mediated regulation of the eight other genes detected in STRING analysis (Figure 1).

Overall, the data obtained by computer modeling from our previous research and the literature review provide a superficial understanding of how the nine genes interact with each other and how expression of these genes alternate in response to physical activity respectively. However, there is still a limited understanding of the precise interactions and regulatory mechanisms of

these genes within the skeletal muscle tissue conditions. In addition, we still lack detailed studies on how the differential expression of these genes contributes to muscle adaptation. Therefore, it is significant to analyze how these genes interact with each other and regulate one another's expression in skeletal muscle cells *in vitro*.

The Premise of the Hypothesis

Previous studies have shown that there is a gene regulatory network between nine genes related to physical activity, where they influence each other in a coordinated manner. Analysis of the effects of the expression changes of the hub gene ATF3 on the other eight genes, particularly on SOCS3 gene expression *in vitro* provides insight into their complex regulatory network where they regulate each other.

Research Question

How does the ATF3 regulate the expression levels of the other eight genes including SOCS3, NR4A2, NFIL3, NR4A3, MAFF, SIK, SLC2A3, MYC?

Objective

To investigate how ATF3 regulates the expression levels of the other eight genes.

Hypothesis

ATF3 regulates the expression levels of the other eight genes.

Specific Aims

1. To obtain ethical approval from Nazarbayev University Biological and Chemical Safety Committee (NU BCSC).

2. To grow Human Embryonic Kidney 293 cells (HEK293) by optimizing viability, culture conditions, and growth characteristics.
3. To induce activity of ATF3 gene by applying drug treatment.
4. To examine the effect of ATF3 activity on the expression levels of the other eight genes by using Western blotting.

Methodology

We used tamoxifen to induce changes in ATF3 gene expression. Western blotting allowed us to identify changes in SOCS3 gene expression via ATF3-mediated regulation at the protein level (Refer to Figure 2 to review the details of the experimental workflow).

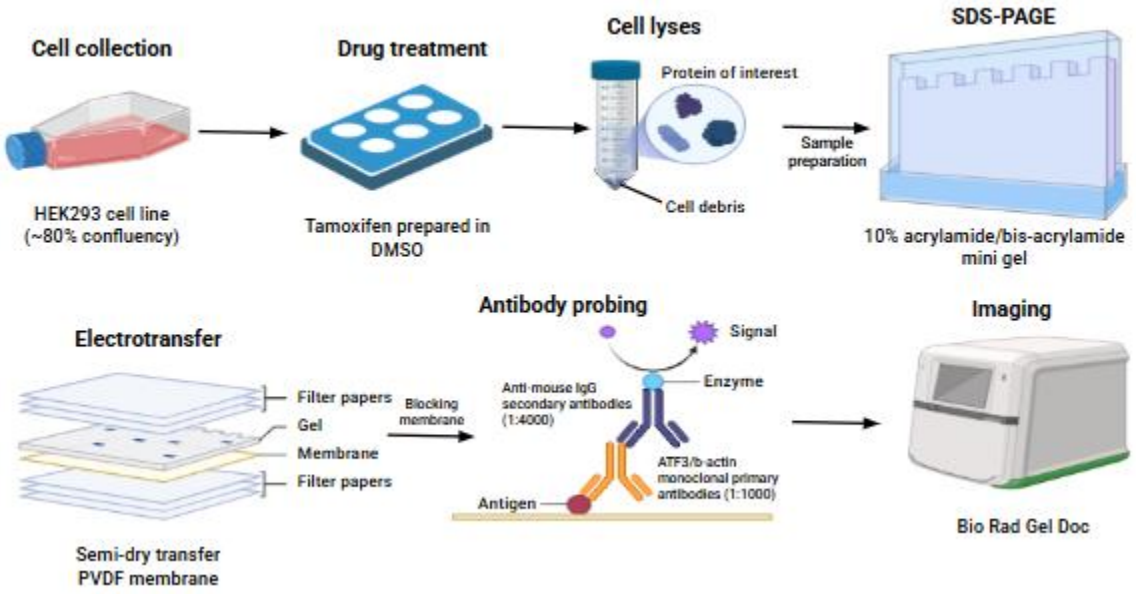

Figure 2. Schematic Representation of the Methodology

Material	**Company/Supplier**	**Catalog Number**
HEK293 cell line	ATCC	CRL-3216

DMEM - high glucose	Sigma-Aldrich	D5796
Fetal Bovine Serum	Sigma-Aldrich	F2442-500ML
Penicillin-Streptomycin	Sigma-Aldrich	P4333
Trypsin-EDTA solution	Sigma-Aldrich	T4049-100ML
Phosphate-buffered saline	ThermoFisher	003002
Tamoxifen	ThermoFisher	J63509.03
Dimethyl sulfoxide	Sigma-Aldrich	276855
Tris	Sigma-Aldrich	10708976001
Sodium chloride	Sigma-Aldrich	S9888
Triton X-100	QazMura	QM5264-500ML
Sodium deoxycholate	Sigma-Aldrich	BCCB0916
Sodium dodecyl sulfate	QazMura	QM4700-500G
Ethylenediaminetetraacetic acid	AppliChem	A1103,1000
Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X)	ThermoFisher	78445
TGX FastCast Acrylamide Kit, 10%	Bio-Rad	1610173
Ammonium persulfate	Sigma-Aldrich	A3678-25G
TEMED	Bio-Rad	1610801
2x Laemmli sample buffer	Bio-Rad	1610737
2-Mercaptoethanol	Sigma-Aldrich	M6250
PVDF Transfer Membrane 0.45um	ThermoFisher	88518
Non-fat dry milk powder	VWR	ROCKB501-0500
Precision Plus Protein Dual Color Standards	Bio-Rad	1610374
ATF3 Monoclonal Antibody	ThermoFisher	5A9B9

SOCS3 Monoclonal Antibody (SO1)	ThermoFisher	MA1-19373
beta Actin Antibody (2A3)	Santa Cruz Biotechnology	sc-47778
Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP	ThermoFisher	31430
EZ-ECL Detection Kit	Sartorius	20-500-120

Table 2. List of Materials and Their Specifications

Cell Culture

We cultured human embryonic kidney 293 (HEK293) cell line in Dulbecco's Modified Eagle Medium (DMEM) which contained 4500 mg/L glucose, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. The cells were incubated in a 37°C incubator supplemented with 5% CO_2. Following the incubation, passaging was performed every 2-3 days when they reached 80-90% confluence: 0.25% trypsin-EDTA solution was used to allow cell detachment and neutralized with the identical amount of DMEM. The cells were then seeded at the desired density.

Drug Treatment

When HEK293 cells reached their confluence level of 80-90%, we conducted drug treatment to investigate the effect of tamoxifen on the cells.

Once HEK293 cells were seeded into six-well plates, they adhered overnight in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C, in a 5% CO_2 incubator. The next day, we prepared a stock solution of tamoxifen dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10mM. The cells were treated with 25.5 µM tamoxifen for 24h based on the IC50 value reported in the MCF-7 cell line since it represented the lowest effective

concentration in the literature (Kalabay et al., 2022). To assess dose-dependent effects, cells were also treated with tenfold lower and higher concentrations (2.5 µM and 25.5 µM respectively). Control wells included: a) vehicle control (DMSO at the same final concentration as in drug-treated wells with the highest drug concentration) and b) untreated control (cells maintained in media without drug or DMSO). Each condition was performed in three biological replicates to ensure reproducibility, consistency, and robustness of the results.

Morphological Analysis

Following 24 hours of tamoxifen treatment, cellular morphology was assessed using an inverted phase-contrast microscope (Carl Zeiss Microscopy GmbH, Germany) to observe potential changes indicative of cytotoxic effects. Images were captured using ZEN 2.6 (blue edition) software at 10x magnification. Morphological changes such as cell shape, clustering, density, loss of adherence and death were observed and compared with control groups.

Cell collection and Lysis

After 24 hours of tamoxifen treatment, HEK293 cells were collected for protein extraction. Culture media was aspirated, and cells were washed with ice-cold PBS. Adherent cells were lysed directly in the plate using a RIPA lysis buffer prepared according to manufacturer's protocol (Biomol GmbH) and supplemented with protease inhibitor cocktail to prevent protein degradations. Cells were incubated on ice for 20 minutes with occasional gentle agitation. Lysates were collected by scrapping, transferred to microcentrifuge tubes, and centrifuged at 16,000x g for 15 min at 4°C. The supernatant containing total protein was transferred (30 µL) to a new tube and mixed with equal volume of 2x Laemmli sample buffer containing 5% 2-mercaptoethanol followed by boiling at 95°C for 5 min to denature proteins.

Western Blot

Equal amounts of protein (20 µl) were resolved on 10% SDS-PAGE gels, prepared using TGX FastCast Acrylamide Kit (Bio-Rad) according to the manufacturer's recommendations (Bio-Rad, 2011), and electrophoresed at 110V for one hour. Proteins were transferred onto PVDF membrane (ThermoFisher) using a semi-dry method using standard protocol of 25V for 30 min (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in PBS-T (Phosphate-buffered saline with 0.1% Tween-20) for one hour at room temperature to prevent nonspecific binding. Membranes were incubated overnight at 4°C with primary antibodies: ATF3 Monoclonal Antibody (1:1000), SOCS3 Monoclonal Antibody (SO1) (1:1000), and beta Actin Antibody (2A3) (1:1000) as a loading control. The next day membranes were washed three times with PBS-T, and incubated with HRP-conjugated secondary antibody (1:4000) at room temperature for 1 hour. Detection was performed using an ECL chemiluminescence substrate (Sartorius) and visualised using Bio-Rad ChemiDoc.

Statistical Analysis

Western blot band intensities were quantified using (Bio-Rad) and analyzed through ImageJ (National Institutes of Health, Bethesda, MD, USA) and protein expression levels were normalised to β-Actin.Data analysis was performed using Microsoft Excel software (Microsoft Corporation, Redmond, Washington, USA), where results were expressed as mean ± standard deviation (SD) and graphs were produced. The results were represented as a fold change relative to a control condition. A p-value < 0.05 was considered statistically significant.

Results

Morphological Analysis

Cells were observed under an inverted microscopy to evaluate the morphological changes. According to Figure 3, cells treated with 2.5 and 25.5 µM displayed slight changes in morphology compared to untreated cells including cell shrinkage, increased cell aggregation, and loss of adherence. At 255 µM concentration of tamoxifen, a significant elevation in cell death was indicated, characterized by cell detachment from the culture surface and rounding of the cells. Finally, cells treated with the vehicle control exhibited more pronounced morphological changes compared to those treated with 2.5 µM and 25.5 µM tamoxifen.

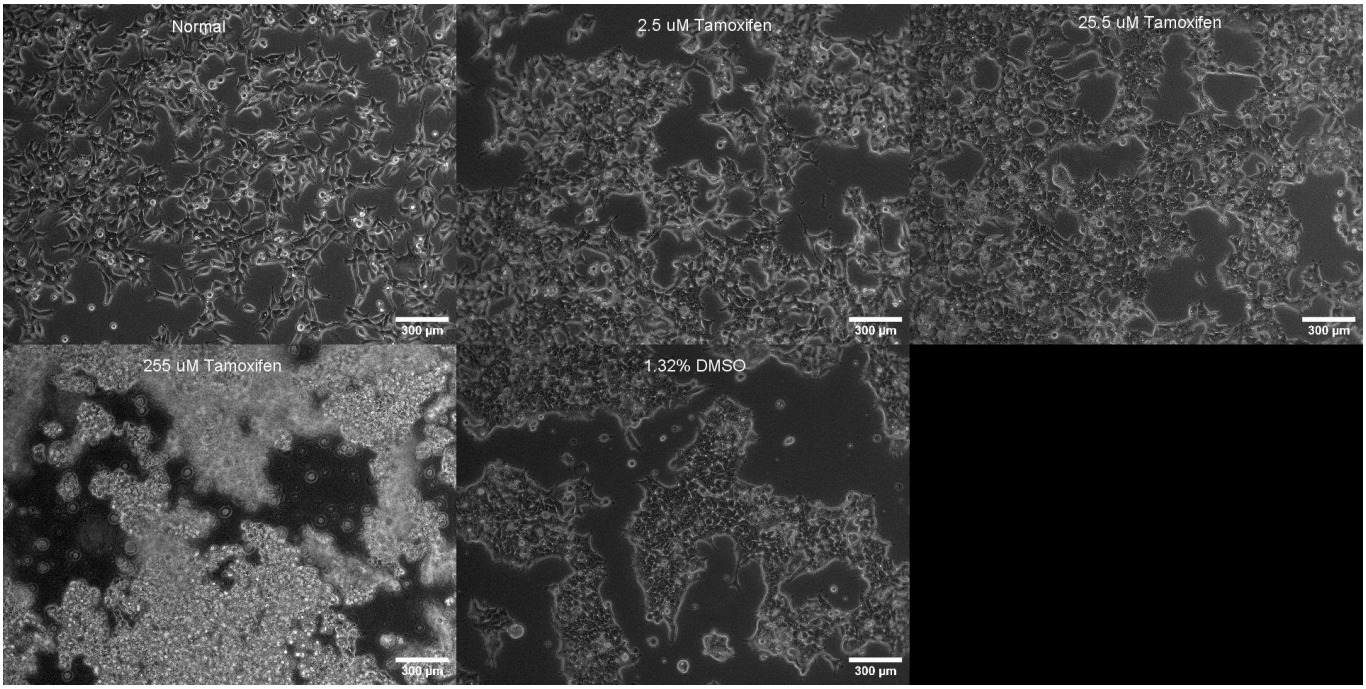

Figure 3. Morphological Changes in HEK293 Cells Following Tamoxifen and Vehicle Treatment

Dose-Dependent Induction of ATF3 and SOCS3 by Tamoxifen

Western blot analysis revealed a dose-dependent effect of tamoxifen on ATF3 and SOCS3 protein levels in HEK293 cells. Distinct bands were observed in all treated samples except at 255 µM, where no bands were detected. B-actin, a housekeeping protein, was used as a loading to correct variation and was almost consistent across samples (Figure 4a). At a lower dose of 2.5 µM, ATF3 expression was slightly higher than the control. A moderate dose of 25.5 µM led to a significant increase in ATF3 expression compared to the control. The same trend could be seen for SOCS3, where the expression was significantly elevated compared to a lower dose. However, at both concentrations of tamoxifen, SOCS3 expression level was lower than in the vehicle control. At the highest dose of 255 µM, a significant reduction of both ATF3 and SOCS3 expressions were evident (Figure 4b).

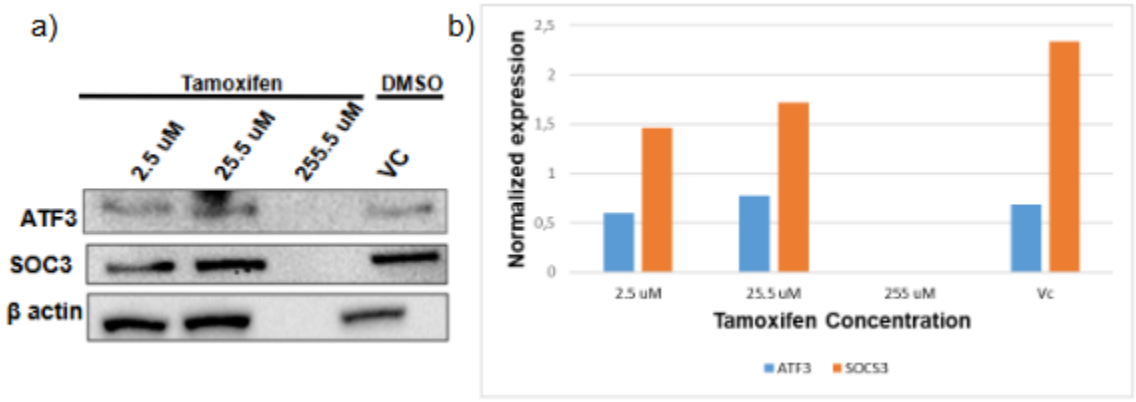

Figure 4. Tamoxifen-Induced Changes in ATF3 and SOCS3 Expression. (a) Western blot analysis of drug treatment. (b) Bar graph on the effect of Tamoxifen Concentration on Normalized ATF3 and SOCS3 Expression

To assess the reproducibility of tamoxifen-induced effects on ATF3 and SOCS3, Western blot analysis was performed in two independent replicates (Figure 5). According to Figure 5, no significant in ATF3 expression was observed between tamoxifen treated groups and the vehicle control ($P > 0.05$). Meanwhile, tamoxifen treatment significantly reduced SOCS3 expression

compared to the vehicle control (for 2.5 µM, P = 0.0145; for 25.5 µM, P = 0.0424). Some inconsistency in β-actin protein bands are observed (Figure 5a).

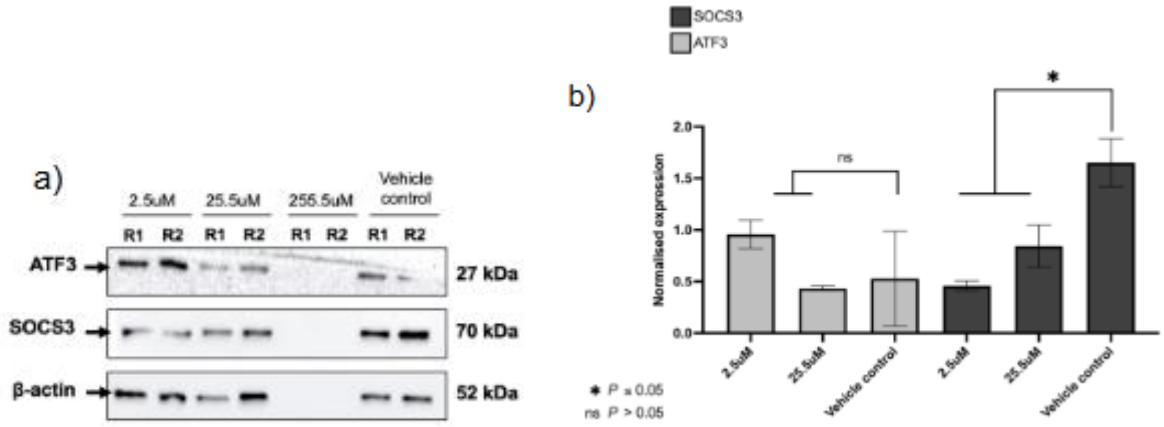

Figure 5. Tamoxifen-Induced Changes in ATF3 and SOCS3 Expression with Two Biological Replicates. (a) Western blot analysis of drug treatment. (b) Bar graph on the effect of Tamoxifen Concentration on Normalized ATF3 and SOCS3 Expression

Discussion

We studied the effects of ATF3-mediated regulation on SOCS3 protein expression in HEK293 cells using Western blotting to observe protein level changes as well as focusing on morphological changes. The results from both the morphological analysis and Western blot indicate a dose-dependent response, with noticeable alterations in both cell structure and gene expression. At lower concentrations (2.5 µM and 25.5 µM), cells showed minor changes in morphology, including slight shrinkage, increased clustering, and partial loss of adherence. These changes correspond with the Western blot findings, where ATF3 and SOCS3 expression levels were elevated.

To begin with, it is suggested that tamoxifen treatment influences ATF3 expression. Especially, Western blot analyses revealed a dose-dependent increase in ATF3 expression at 2.5 µM and 25.5 µM tamoxifen, with a significant upregulation at 25.5 µM, which aligns with the previous studies demonstrating that tamoxifen can induce ATF3 expression. ATF3, a stress-induced transcription factor, can be upregulated in response to stress caused by various factors including chemotherapeutic drugs (St Germain et al., 2010). One of such drugs is tamoxifen as it can disrupt the cholesterol synthesis within neuronal cells and lead to cellular stress that can, in turn, upregulate the expression of ATF3 gene (Denk et al., 2015). Even though the HEK293 cells are not cells of neural origin, they can nevertheless also exhibit such a mechanism of action. For example, a study of Wu et al. (2013) revealed that cholesterol modulation affects ion channel activity and protein expression in HEK293 cells, suggesting the presence of active cholesterol synthesis and regulation pathways. Given the similarities, it is reasonable to consider that tamoxifen could exert similar effects on cholesterol metabolism and associated stress mechanisms in HEK293 cells. Additionally, since tamoxifen functions as a SERM, it might influence ATF3 expression through mechanisms involving estrogen receptor (ER) signaling (Bailey et al., 2012). However, since HEK293 cell line is non-cancerous and known for their low ER expression, this implies the potential involvement of alternative non-classical signaling pathways or off-target actions of tamoxifen, warranting further investigation (Yin et al., 2014; Zhao et al., 2009).

Interestingly, our study observed differential expression patterns of ATF3 and SOCS3 under varying concentrations of tamoxifen. Based on previous studies, ATF3 has been shown to enhance SOCS3 expression as ATF3 acts as an activator that binds to SOCS3 promoter region (Luo et al., 2024), so the increase in SOCS3 protein expression at 25.5 µM tamoxifen aligns with

this expectation. Nonetheless, SOCS3 expression was unexpectedly higher in the vehicle control compared to both 2.5 µM and 25.5 µM tamoxifen-treated conditions. A possible explanation could be that the tamoxifen effect on SOCS3 might be independent of ATF3. A study on tamoxifen's role in breast cancer showed that SOCS3, important for the epithelial-mesenchymal transition (EMT) pathway, was downregulated in response to tamoxifen (Mirzaei et al., 2022). Further research is needed to elucidate these pathways and determine whether factors such as alternative signaling cascades besides ATF3 contribute to SOCS3 modulation.

To confirm reproducibility of the results, Western blot experiments were repeated in two independent replicates. Although initial observations represented ATF3 upregulation, no significant changes in ATF3 protein levels between tamoxifen-treated groups and the vehicle control were observed, which may occur due to technical issues rather than biological effects. The vehicle control band was not clearly visible, likely due to membrane cutting, affecting normalization and accuracy of quantification. For SOCS3, tamoxifen significantly reduced the level of gene expression compared to vehicle control which is consistent with previous results of the one-replicate experiment. It could justify that an alternative pathway where tamoxifen regulates SOCS3 expression might exist and affect its expression (Mirzaei et al., 2022). Further validation with qPCR and increase of replicates' number is needed to eliminate discrepancies in the data that could occur due to technical issues and differences in cellular response.

Finally, it is worth mentioning the results obtained from the cells treated with the highest tamoxifen concentration of 255 µM. We observed that the cells exhibited severe morphological changes at 255 µM tamoxifen: significant detachment from the surface, rounding, and a noticeable increase in cell death. The results from the Western blot further support the cytotoxic effect for HEK293 cell line at this dose due to no band detection for ATF3, SOCS3, and even

housekeeping gene β-actin. It means that 255 μM tamoxifen shows the adverse effects on the structure and function of HEK293 cells that caused them to undergo apoptosis or necrosis. Consequently, this led to the death of the cells or an overall reduction in viable cells capable of protein expression as well as protein degradation. This finding is consistent with a previous study demonstrating tamoxifen's cytotoxic effects at high doses (Brandt et al., 2004). Another study also verified the results and elaborated on the cytotoxic effects of high doses of tamoxifen on non-cancerous cells. Majumdar et al. (2001) reported that the drug suppressed cell proliferation and triggered apoptotic changes in human cervical carcinoma (HeLa) cells and murine erythroleukemic (MEL) cells. Also, the cells showed the signs of nuclear condensation and cell shrinkage (Majumdar et al., 2001), and the last characteristic was observed in our morphological analysis. Thus, we could confirm that tamoxifen's influence on gene expression appears to be concentration-dependent, where lower doses potentially activate stress response pathways and higher doses lead to cytotoxicity and loss of cellular integrity.

Limitations and Future Directions

Some limitations appeared during the project that compromised the consistency and reproducibility of the data. One of such limitations is the limited scope of gene analysis due to time constraints. The study focused only on ATF3 and SOCS3 and did not analyze the remaining exercise-related genes of the potential network. The use of HEK293 cell line can be considered as another limitation as they are not of muscular origin. So, the results may not directly translate to muscle-specific responses. Finally, we implemented Western blot analysis and did not perform other additional methods, hence, obtained only semi-quantitative data on protein expression. Future experiments are required to further investigate how ATF3-mediated regulation impacts the other genes' expression (NR4A2, NFIL3, NR4A3, MAFF, SIK, SLC2A3, MYC) in skeletal

muscle cell lines. Moreover, complementary assays such as qPCR will help to validate and expand the results on mRNA level.

Conclusion

To conclude, ATF3 expression was upregulated in response to tamoxifen treatment, with the highest expression observed at 25.5 µM, indicating a dose-dependent effect. Western blot analysis successfully confirmed gene expression changes, highlighting ATF3 as a tamoxifen-responsive gene in HEK293 cells.

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