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Feasibility of Tubulin as a Control for Gene Expression following Transfection in Mouse Monocyte/Macrophage-like Cells

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Feasibility of Tubulin as a Control for Gene Expression following Transfection in Mouse Monocyte/Macrophage-like Cells

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Abstract

Transfection, which is the ability to modify host cells' genetic content, has broad application in studying normal cellular processes, molecular mechanism of disease and gene therapy. There are several transfection techniques, and all require either a control or a reference gene. Commonly used controls for transfection experiments are housekeeping genes, which maintain expression for a given cell/tissue, experimental conditions, and treatment. However, recent research has uncovered that expression levels of housekeeping genes may vary depending on the gene, cell type and experimental conditions. A growing body of evidence demonstrates that housekeeping genes are inadequate internal standards for measuring gene expression levels as they are affected by many factors, including experimental treatment and conditions. Current literature is lacking about adequacy of tubulins, specifically β-tubulin isotypes as controls for transfection experiments. This research aims to fill this gap by testing whether tubulin is a suitable control gene for transfection in RAW264.7 cells. It is hypothesized that tubulin expression changes following transfection because tubulins play a role in genetic material uptake. RAW264.7 cells were transfected by electroporation with either non-targeting siRNA (NT-siRNA) or cyclophilin A siRNA (CyPA siRNA). Levels of β-tubulin isotypes mRNA was quantified by RT-qPCR for cells collect either 24 hours or 48 hours after transfection. Increased expression of βI (+50%, $p<0.01$) was observed 24 hours after electroporation with NT-siRNA. βI(-50%, $p<0.001$), βII(-50%, $p<0.01$) and βIII (-92%, $p<0.01$), βIV (-55%, $p<0.001$) expression decreased 24-hours post-electroporation (0.5±0.2, $p<0.00124$hours post-electroporation with CyPA. All four β tubulin isotypes were deemed unusable reference gene following transfection with electroporation involving CyPA

targeting siRNA, specifically βIII tubulin would not be suitable control for transfection with electroporation due to these causing significant changes in expression levels, 24h post-transfection.

Introduction

Transfection is a process by which foreign nucleic acids are delivered into a eukaryotic cell to modify the host cell's genetic makeup (Kim and Eberwine 2010). The main purpose of transfection is to study the function of genes or gene products, by enhancing or inhibiting specific gene expression in cells, and to produce recombinant proteins in mammalian cells (Wurm 2004). Enhancement or inhibition of gene expression in cells is performed by modifying genetic material of the host cell, to learn about the effect of presence or absence of the gene (Wurm 2004). This ability to modify host cells' genetic content enables the broad application of this process in studying normal cellular processes, molecular mechanism of disease and gene therapeutic effect (Chong et al, 2021). Development of transfection techniques has led to advancements in transfection-based research studies. There are several different transfection techniques, which are selected based on cell type and desired result of transfection. The ideal method should have high transfection efficiency, low cell toxicity, minimal effects on normal physiology, and be easy to use and reproducible (Kim and Eberwine 2010).

Transfection methods are broadly categorized in three classes: biological, chemical, and physical mediated (Kim and Eberwine 2010). According to Kim and Eberwine, biological transfection refers to virus-mediated transfection which is also known as transduction (Kim and Eberwine 2010). It is the most commonly used method in clinical research. This method involves introduction of viral vector containing gene of interest into cells of host organism. Virus then integrates its DNA into the host DNA. Integrated DNA replicates as host genome does.

Considering the non-clinical nature of this research, viral transfections will not be studied. This research will focus on one physical and one chemical transfection method.

Chemical transfection methods are widely used in contemporary research (Schenborn and Goiffon 2000). The underlying principle of chemical methods of transfection is as follows: positively charged chemicals make nucleic acid-chemical complexes with negatively charged nucleic acids; these positively charged nucleic acid-chemical complexes are attracted to the negatively charged cell membrane (Kim and Eberwine 2010). While the exact mechanism of how these nucleic acid/chemical complexes pass through the cell membrane is unknown, it is believed that endocytosis and phagocytosis are involved in the process (Kim and Eberwine 2010). Transfected DNA must be delivered to the nucleus to be expressed, however the translocation mechanism to the nucleus is not known (Kim and Eberwine 2010). Some examples of cationic-dependent transfection include lipofectin and lipofectamine (Kim and Eberwine 2010). Lipofectamine RNAiMAX is a proprietary RNAi specific cationic lipid formulation, which works on the described principle, was designed for the delivery of siRNA and miRNA into cells. On the other hand, physical transfection methods involve delivering the nucleic acids into cells, which requires skill and can cause cell death (Kim and Eberwine 2010). Electroporation is a common physical transfection technique (Inoue and Krumlauf 2001). The exact mechanism is unknown, but it is supposed that a short electrical pulse disturbs cell membranes and makes holes in the membrane through which nucleic acids can pass (Inoue and Krumlauf 2001). Electroporation was used as the physical transfection technique

To determine whether transfection occurred, using any of the above techniques, gene expression is usually quantified using real-time quantitative polymerase chain reaction (RT-qPCR). An RT-qPCR works on the same principle as a qPCR which is amplification of the desired genetic

material, and in case of the RT-qPCR the process can be monitored “real-time”. The desired genetic product is tagged with a fluorescent dye prior to the amplification (Schmittgen et al, 2000). The resultant amplified gene expression is measured by a fluorometer that detects the fluorescence of the amplified genetic products (Schmittgen et al, 2000). RT-qPCR is popular mainly due to its ability to efficiently amplify small quantities of RNA in a relatively short period of time (Turabelidze et al. 2010). Despite the advantages of the RT-qPCR technique, it can have a few drawbacks such as adequate primer specificity and presence of inhibitors in samples (Turabelidze et al. 2010). To overcome such issues, the results of a RT-qPCR are usually normalized using controls for internal standards. Commonly used controls are housekeeping genes because the expression of housekeeping genes is expected to not change across different treatment groups (Li and Shen 2013).

Housekeeping genes are constitutive genes that are required for the maintenance of basal cellular functions that are essential for existence of a cell (Silver et al. 2006). Therefore, they are expressed in all cells regardless of cell type, origin and conditions. (Silver et al. 2006). Some examples of common housekeeping genes include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin, β-actin, β-tubulin, hypoxanthine phosphoribosyl transferase (HPRT) and phospholipase-A2 (PLA2) (Li and Shen 2013). These genes are also conserved across species (Li and Shen 2013). This makes housekeeping genes very useful as controls, internal standards, or reference genes for quantification in a range of experiments such as qPCR and western blots.

However, there is a growing body of evidence that demonstrates that some of the commonly used housekeeping genes serve as inadequate internal standards for measuring gene expression levels as they are actually affected by a large number of factors, including drug and experimental treatment and conditions (Ferguson et al, 2005). For example, one of the most frequently used

housekeeping genes, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) has been found to be unstable depending on the type of tissue, metabolic process or under certain experimental conditions (Panina et al. 2018). GAPDH is not stable in age-induced apoptosis in neurons, in insulin stimulated adipocytes and hepatoma cell lines, and in human omental and subcutaneous adipose tissue from obesity and type 2 diabetes patients (Gong et al. 2016). Some recent studies also reported that microRNA inhibition or over-expression in vitro might also regulate β-actin and GAPDH expression (Sikand et al. 2012), suggesting β-actin and GAPDH should not be used as an internal control for normalization of microRNA targeted mRNA expression (Li and Shen 2013). This observation is further confirmed by other independent research groups reporting that β-actin is not a suitable reference gene in qPCR analysis due to its extensive variability in expression in mouse lymphocytes (Albershardt et al. 2012). For instance, a study performed by Schmittgen and Zakrajsek (2000) determined that while βII microglobulin and 18S rRNA were suitable internal control genes in quantitative serum-stimulation studies, β-actin and GAPDH were not. In addition to this, studies performed by Dr. Contreras revealed that the levels of β tubulin proteins were altered by transfectants (unpublished data). Recent literature has focused on testing the feasibility of tubulins as a housekeeping gene for western blots, however there is a lack of the knowledge of feasibility of tubulins as transfection controls and standards. This necessitates an investigation of effect of transfection on tubulins and whether tubulins can be used as internal control genes.

Tubulins play many roles in the cell system. Highly conserved α- and β-tubulin heterodimers assemble into dynamic microtubules and perform multiple important cellular functions such as structural support, pathway for transport and force generation in cell division (Binarová and Tuszynski 2019). For example, one of the β-tubulin isotypes, namely, βII has been observed to have an increased expression in several types of tumors cells due to its role in cell division (Yeh

and Ludueña 2004). Tubulins are also involved in transport of substances via endocytosis, phagocytosis and transfection mediated processes; these microtubule-mediated pathways have been shown to involve, specifically βIII (Hasegawa et al. 2001). The act of transfection, therefore, could cause an increased expression of βIII. Other studies have found that tubulins play a significant role in the immune response generated by the cell (Ilan-Ber and Ilan 2019, Alves-Silva et al. 2017). The process of transfection, i.e., electroporation along with the presence of any siRNA could cause the cell to generate an immune response. This in turn could cause an increased expression of tubulins Therefore, it is hypothesized that tubulin expression levels will change following transfection.

The aim of this project, therefore, was to determine if tubulin is an appropriate gene expression control for siRNA transfection experiments by evaluating individual β-tubulin gene expression. For this study, RAW264.7 cells were used. The RAW 264.7 cells are monocyte/macrophage-like cells, originating from Abelson leukemia virus transformed cell line derived from BALB/c mice (Taciak et al. 2018). According to the American Type Culture Collection (ATCC), main supplier of this cell line, RAW264.7 cells are suitable transfection hosts. RAW264.7 cell line is easy to propagate, has a high efficiency for DNA transfection and sensitivity to RNA interference (Hartley 2008). RAW264.7 cells were transfected using electroporation with either non-targeting siRNA (NT-siRNA) or cyclophilin A siRNA (CyPA siRNA), which targets the housekeeping gene cyclophilin A in human cell lines, not mice cells. Quantification of tubulin gene expression post transfection was performed.

Methods

1. *Cell Culture*

RAW 264.7 cells were seeded at 3.3×10^5 cells/well (nucleofection) in 24-well culture plates with DMEM media (10% FBS). Control cells were cells that did not receive transfectant/electroporation nor nucleic acids and were maintained in normal growth media. Mock transfection groups received all reagents except the siRNA and were treated with the different transfection method (Mock Trx). Transfection groups of different experiments were RAW264.7 cells transfected with nucleofector and received either 0.012 nM cyclophilin A (CyPA) siRNA or non-targeting siRNA (NT siRNA). All cell plates were incubated at 37˚C, 5% CO2 for either 24 hours or 48 hours, after which the cells were collected.

2. *Transfection methods*

Nucleofection & Electroporation

Cell samples were prepared for electroporation by reconstituting cell pellets ($2 \times 10^6 cells$) in 100 µL of nucleofector solution (Amaxa Nucleofector Kit, Lonza Kit V). Control group did not receive siRNA or nucleofector solution. Mock Trx group received nucleofector solution but did not receive any siRNA. The 100 µL of nucleofector solution diluted cell groups received 100 pmol of either NT siRNA or 100 pmol of CyPA siRNA. Following manufacturer's protocol, cells were transfected by using Amaxa Nucleofector program no. D032. Post-electroporation cells (final siRNA is 55.6 nM) were resuspended in 500 µL of prewarmed media and 100 µL transferred to one well of a 24-well plate and incubated in a total volume 300 µL of media for either 24 hours or 48 hours at 37˚C, 5% CO2, after which the cells were collected for RNA isolation

3. *RNA extraction, purification & quantification*

Total cellular RNA was isolated from the cells using the RNA Microprep isolation kit (Zymo Research). Following manufacturer's instructions, DNA was removed from RNA samples using DNase I digestion treatment as recommended. RNA was eluted from the Zymo spin IC columns with 15 µl of pre-warmed, RNase-free water. The concentration of RNA extracted from the cells was quantified using the Nanodrop. RNA was stored at −80°C.

4. *Reverse Transcription: qPCR*

cDNA was synthesized from RNA by using reverse transcription reagents (Applied Biosystems) following manufacturer's instructions ("High-Capacity cDNA synthesis" protocol). cDNA synthesis was performed using a thermal cycler by incubating at 25°C for 10 minutes, then at 37° for 120 minutes followed by 85°C for 5 minutes. cDNA was stored at -20°C until further use.

5. *Real time qPCR*

Real-time PCR was performed with complementary DNA (cDNA) obtained from RNA samples using TaqMan Assays (Table1), Universal Master Mix (BIORAD), and the StepOnePlus Real Time PCR System (Applied Biosystems). Data were normalized using GAPDH cDNA as an invariant transcript and the control cells as the calibrator to calculate relative quantification (RQ) using the delta-delta Ct method (ΔΔ Ct).

6. *Statistical Analyses*

Statistical Analyses were performed using GraphPad Prism (GraphPad Softwars, La Jolla, CA, USA). Data was expressed as the mean ± SD. Differences in the means of relative quantification for each treatment group were determined by an analysis of variance (ANOVA) incorporating repeated measures. For comparison of different treatment groups, ANOVA was performed using additional post-hoc analyses. Tukey's comparison was used to determine any differences among

the individual treatment groups. To correct for multiple comparisons of β tubulins, statistical significance was determined using Bonferroni correction, where p=0.05 was divided by the number of β-tubulin isotypes (n = 4). Therefore, a p <0.0125 is considered significant.

Table 1. TaqMan Assays used in RT qPCR

Tubulin	Gene	TaqMan Assay
βI	Tubb5	Mm00495806
βII	Tubb2A	Mm00809562
βIII	Tubb3	Mm00727586
βIV	Tubb4b/2c	Mm00847804

Results

To determine the effects of transfection using electroporation, the expression of β tubulin isotypes was quantified using RT-qPCR.

β-tubulin I post-transfection

Expression levels of β tubulin isotype I did not change 24 hours, after mock transfection(Figure 1A). However, βI expression levels in RAW264.7 cells electroporated with NT-siRNA significantly increased by 50% (1.55±0.51, p<0.01) whereas in cells electroporated with CyPA siRNA, expression significantly decreased by 50% (0.51±0.21, p<0.001) when compared to the untransfected (1.02±0.27)(Figure 1A). βI expression level in cells electroporated with NT-siRNA significantly increased in comparison to cells that were transfected but did not receive any SiRNA as well as cells that received CyPA siRNA (p<0.001) (Figure 1A). No change in βI tubulin gene as expression levels was observed 48-hours after electroporation (Figure 1B).

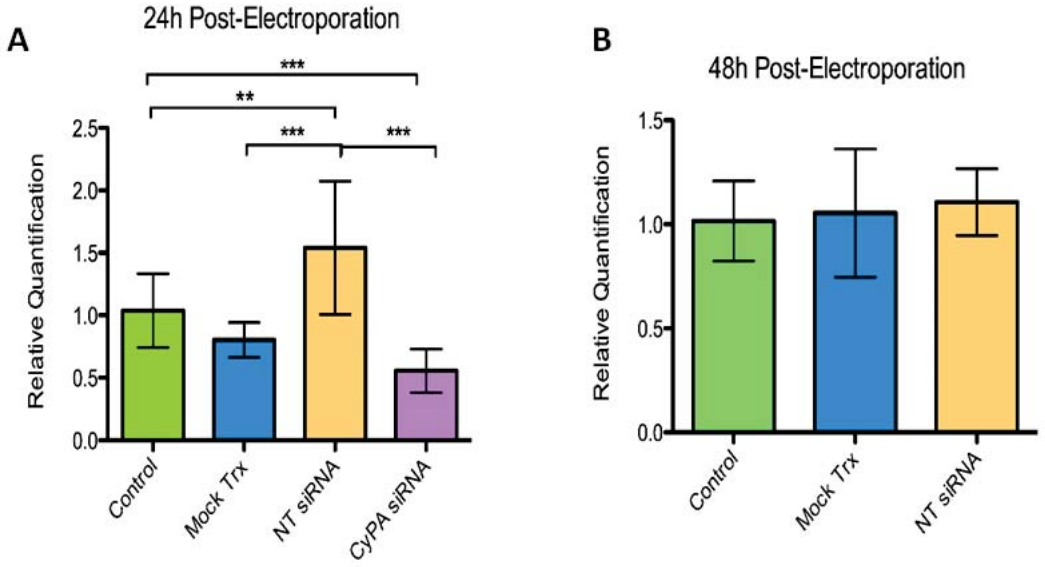

Figure 1. Relative expression of β1 (Tubb5) in +/- transfected RAW 264.7 cells. Data are mean RQ values +/- SD. Each bar is mean of n = 4-10 samples. (A) RAW 264.7 cells were transfected using electroporation and expression levels measured 24-hours post-transfection. (B) RAW 264.7 cells were transfected using electroporation and expression levels measured 48-hours post-transfection. Statistical test performed by ANOVA with Tukey's comparison. **p-value<0.01, ***p-value< 0.001.

β-tubulin II post-transfection

Compared to untransfected control expression levels of β tubulin isotype II did not change, after 24 hours, in cells that were mock transfected (Figure 2A). βII expression levels in RAW264.7 cells electroporated with NT siRNA did not change, however expression levels did appear to somewhat increase. Expression in cells electroporated with CyPA siRNA decreased by 50% (0.51±0.2, $p<0.01$) when compared to all other groups (Figure 2A). No change in βII tubulin gene expression levels was observed 48-hours after electroporation (Figure 2B).

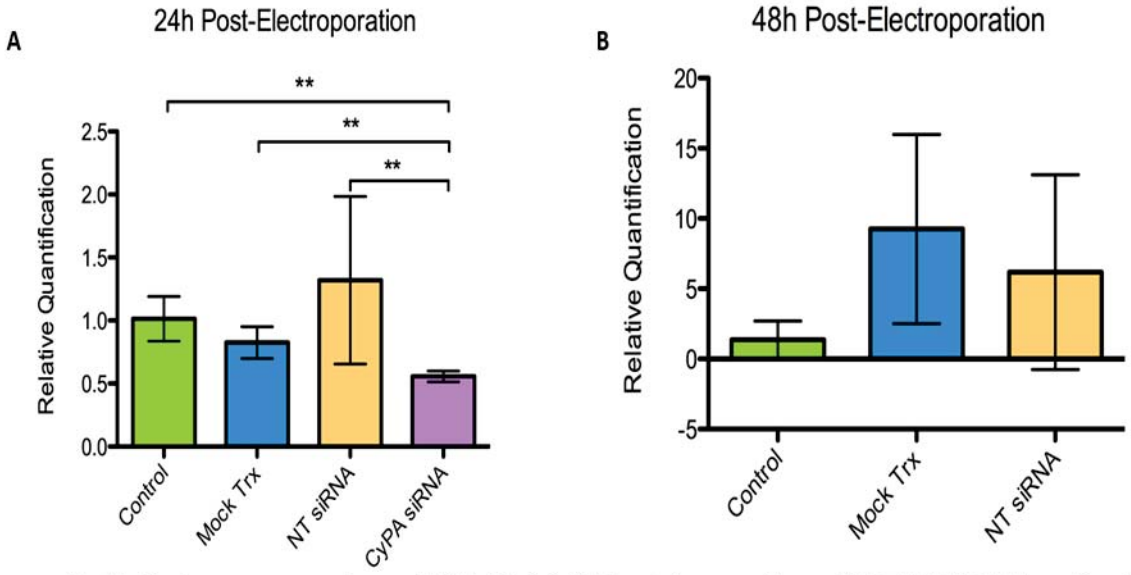

Figure 2. Relative expression of β2 (Tubb2) in +/- transfected RAW 264.7 cells. Data are mean RQ values +/- SD. Each bar is mean of n= 4-10 samples. (A) RAW 264.7 cells were transfected using electroporation and expression levels measured 24-hours post-transfection. (B) RAW 264.7 cells were transfected using electroporation and expression levels measured 48-hours post-transfection. Statistical test performed by ANOVA with Tukey's comparison. **p-value<0.01, ***p-value< 0.001.

β-tubulin III post-transfection

Expression levels of β tubulin isotype III did not, after 24 hours, in cells that were mock transfected (Figure 3A). Similarly, expression in cells receiving NT siRNA also did not change. However, βIII expression in RAW264.7 cells electroporated with CyPA siRNA significantly decreased by 92% (0.5±0.02, $p<0.001$) after 24 hours when compared to cells that were electroporated but did not receive any siRNA (Figure 3A). βIII gene expression was not detected 48 hours post-electroporation regardless of whether the cells received siRNA (Figure 3B).

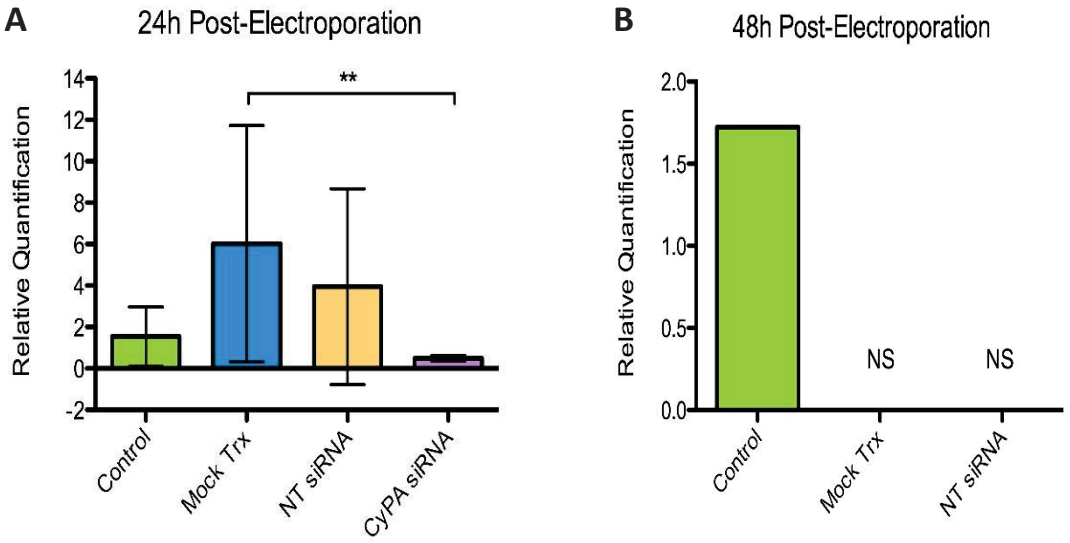

Figure 3. Relative expression of β3 (Tubb3) in +/- transfected RAW 264.7 cells. Data are mean RQ values +/- SD. Each bar is mean of n= 4-10 samples. (A) RAW 264.7 cells were transfected using electroporation and expression levels measured 24-hours post-transfection. (B) RAW 264.7 cells were transfected using electroporation and expression levels measured 48-hours post-transfection. Statistical test performed by ANOVA with Tukey's comparison. **p-value<0.01, ***p-value< 0.001.

β-tubulin IV post-transfection

Expression levels of β tubulin isotype IV did not, after 24 hours, in cells that were mock transfected (Figure 4A). βIV expression in RAW264.7 cells electroporated with NT-siRNA significantly increased by 54% (1.2±0.25) in comparison to cells that were mock transfected (0.78±18, $p<0.01$) (Figure 4A). Expression levels of CyPA siRNA transfected cells significantly decreased by 55%, 24-hours post-electroporation (0.5±0.2, $p<0.001$) when compared to the untransfected control as well as all other cell groups (Figure 4A). No change in βIV tubulin gene expression levels was observed 48-hours post-electroporation (Figure 4B).

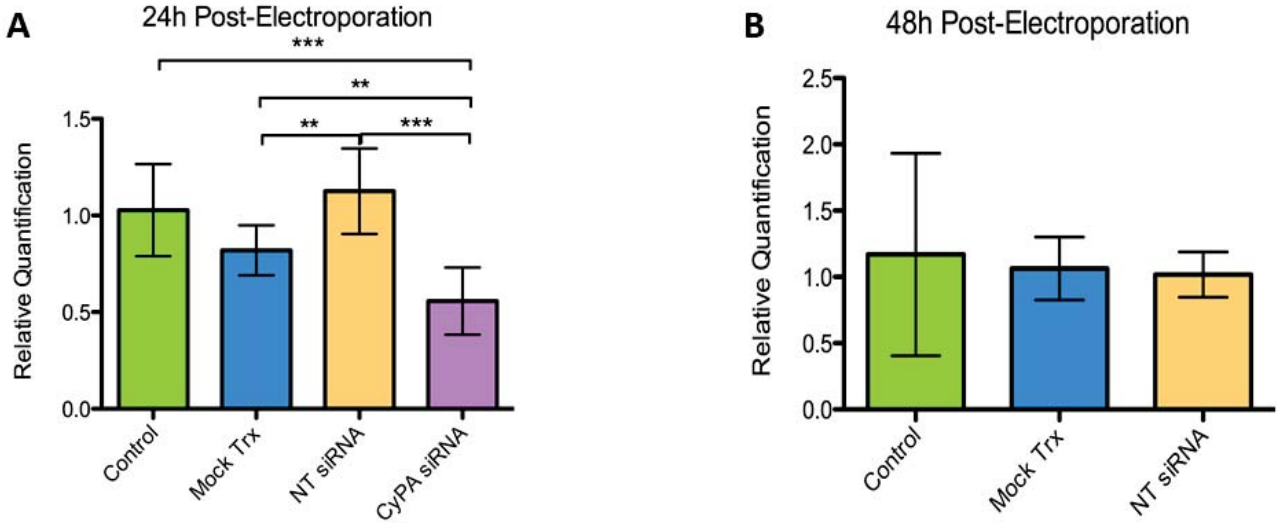

Figure 4. Relative expression of β4 (Tubb4) in +/- transfected RAW 264.7 cells. Data are mean RQ values +/- SD. Each bar is mean of n= 4-10 samples. (A) RAW 264.7 cells were transfected using electroporation and expression levels measured 24-hours post-transfection. (B) RAW 264.7 cells were transfected using electroporation and expression levels measured 48-hours post-transfection. Statistical test performed by ANOVA with Tukey's comparison. **p-value<0.01, ***p-value< 0.001.

Discussion

The purpose of this study was to determine if tubulin is an appropriate control for gene expresiion following siRNA transfection experiments. Previous literature has focused on inadequacy of tubulins as controls for western blot analyses (Li and Shen 2013). However, a similar analysis of individual tubulins has not been performed using RT-qPCR after transfection. In this study, we examine the adequacy of β-tubulin as control/reference genes for RT-qPCR analyses following different transfection methods.

A summary of all the observed significant increases and decreases is presented in Table 2. Cells transfected with non-targeting siRNA (NT-siRNA), using electroporation had no significant difference in the expression of βI, βII, βIV tubulins (Table 2). This suggests βI, βII, βIV tubulins may be adequate as controls for gene expression following transfection with NT-siRNA using

electroporation. However, expression of βIII also significantly decreased 48 hours after transfection with electroporation in both, treatment group without and treatment group with NT-siRNA (Table 2). In these treatment groups, βIII tubulin expression was too low for detection within forty PCR amplification cycles. It is supposed that the short electrical pulse to the cell during electroporation causes a disturbance in the cell membranes and makes holes in the cell membrane (Inoue and Krumlauf, 2001). This disruption of the cell membrane could generate a signal for apoptosis of the cell (Zhang et al, 2018). Apoptosis is a non-inflammatory cell death and ruptured membrane is a hallmark indicator of apoptotic cell death (Zhang et al. 2018). Additionally, programmed or regulated cell death has been found to be interconnected with several immune response pathways (Riera, 2021). In such a circumstance, it is reasonable to suppose that production of some β-tubulins might be reduced, since apoptosis is a non-inflammatory process whereas production of other β-tubulins could increase, since microtubules play a potential role in immune response, which is discussed later. Another observation which supports this postulation is that cell death was, in fact, observed 24-hours post electroporation (due to presence of debris in cell plate and reduced confluency).

Table 2. Summary of changes observed in β tubulin isotype expression due to different transfection techniques. All comparisons made with untransfected control group. * represents significant change in expression, '-' represents no change in expression.

Transfection Type	**siRNA type**	**βI**	**βII**	**βIII**	**βIV**
Electroporation	NT	↑*	-	↓ after 48h	-
	CyPA	↓*	↓*	↓*	↓*
	Mock	-	-	↓ after 48h	-

This suggests that βIII tubulin isotype would not be a suitable control for transfection experiment performed with using either electroporation. It has been demonstrated that βIII-tubulin is part of a complex, pro-survival, molecular pathway activated by hypoxia and poor nutrient supply (Mariani

et al. 2015). An interesting investigation, for future research, would be to quantify expression 48-hours after transfection under similar conditions to determine whether βIII expression remains high.

Transfection of RAW264.7 cells with CyPA siRNA by electroporation resulted in significant decrease in expression of βI, βII, βIII tubulin and significant increase in expression of βIV-tubulin (Table 2). The cyclophilin A silencing RNA used in this study, targets the cyclophilin A housekeeping gene, which belongs to immunophilin protein family in human cells (Song et al. 2004). Therefore, this siRNA should act as a non-targeting RNA for RAW264.7 cells. This implies that it should not cause an increase or decrease in expression of housekeeping genes in mouse cells. However, this is contradictory to the observed increased expression of βIV and decreased expression of βI, βII, βIII and suggests the act of transfecting cells with certain kinds of nucleic acids can lead to alterations in individual tubulins.

Variations in β-tubulin expression after transfection could result from the involvement of microtubules in immune response. The microtubule cytoskeleton regulates several cellular processes related to the immune system (Binarová & Tuszynski, 2019). Recent studies on microtubule structure and function contributed to the understanding of their potential role as players in the innate and adaptive immune systems, which involves inflammation (Ilan-Ber and Ilan 2019). For instance, an intricate intracellular transport mediated by microtubules is responsible for the proper localization of vesicular receptors of innate immunity and its adaptor proteins (Alves-Silva et al. 2017). A significant increase in expression of any β-tubulin isotype could, therefore, suggest its role in microtubule assembly required for immune response. Particularly so in RAW 264.7 cells, since these cells are a macrophage cell line derived from a tumor in a male mouse induced with the Abelson murine leukemia virus (ATCC). Macrophages

are effector cells of the innate immune system that phagocytose bacteria and secrete both pro-inflammatory and antimicrobial mediators (Hirayama, 2017). According to a study by Khandani et al., activation of macrophages coincides with an increased population of microtubules (Khandani, 2007). From this, it is reasonable to suppose that transfection could activate the macrophage resulting in an immune response and therefore, increased expression of β tubulins. Similarly, a significant increase in βIV expression after transfection with CyPA siRNA using electroporation could also indicate involvement of βIV in the immune response generated, suggesting that the sequence of some exogenous RNAs may be detected as a pathogen by the RAW264.7 cells.

Difference in expression of β-tubulin isotypes is possibly because of different evolution of the isotypes. According to a study by Bhattacharya and Cabral (2004), an alignment of mouse β-tubulin sequences reveals numerous amino acid mismatches which in turn suggests that β-tubulin falls into two distinct evolutionary branches consisting of βI, βII, and βIV on the one hand, and βIII and βV on the other. This could explain the difference in βIII expression variation on transfection. It also explains the similarity in expression variation (significant decrease) for βI and βII, which was observed upon transfection with cyclophilin A with electroporation.

In this study tubulins, specifically β-tubulin isotypes, have variations in expression dependent on the given experimental conditions. This study finds that all four β-tubulin isotypes examined would not be appropriate as control for gene expression following transfection with cyclophilin siRNA using electroporation in mouse monocyte/macrophage-like cells. From the data and analyses, it is also reasonable to suppose that β-tubulin would not be an adequate control for gene expression following transfection with other exogenous RNA. In addition to this, βIII tubulin is unsuitable as a control for transfection using physical methods such as electroporation. The reason behind the

expression variation with experimental conditions is unclear and require further investigations. It is also unclear how changes to single tubulins affects overall tubulin expression or protein levels. Future research could include testing the feasibility of tubulin as a control for RT-qPCR followed by transfection experiments in different cell lines. More transfection techniques could also be used to test the adequacy of tubulins as controls. The scope of this research could be broadened by testing the adequacy of other housekeeping genes such as α-tubulins, cyclophilin A, GAPDH for different cell lines as well as transfection techniques.

As a continuation of this study, our current lab work is focusing on determining the feasibility of tubulins as a control for gene expression following transfection in THP-1 cell line by evaluation of individual β tubulins. THP-1 is a human leukemia monocytic cell line, which is extensively used to study monocyte/macrophage functions, mechanisms, signaling pathways, and nutrient and drug transport (Chanput et al. 2014). Feasibility of tubulins as a control for gene expression after transfection for the THP-1 cell line will be performed similar to RAW264.7 analyses. For RAW264.7 cells, adequacy of tubulins as controls will be analyzed following transfection with either CyPA siRNA or NT siRNA using Lipofectamine 2000. Adequacy will be assessed 24 & 48-hours post-transfection. In addition to electroporation and lipofectamine 2000, a third polymer-based transfection method, namely Viromer Blue, will also be used to test the adequacy of tubulins as controls for gene expression. Electroporation, Lipofectamine 2000 and Viromer Blue are widely used transfection methods. It is essential for any valid experiment to have adequate controls. Continued work in this field, therefore, will aid in determining whether β-tubulins isotypes are feasible control for gene expression following different transfection methods.

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