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Characterization of *NeuN* expression in the mouse neuronal NSC-34 cell line using RT-qPCR, immunological staining and siRNA-mediated gene suppression

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ABSTRACT

Background: Acute spinal trauma is followed by a secondary injury that causes additional damage to the tissue. The mouse neuronal hybrid cell line NSC-34 is planned for studies regarding this process, wherefore the cell line needed to be established in the laboratory and a proof-of-concept study needed to be performed. A suitable target gene for this study was Neuronal Nucleus (*NeuN)*, a neuronal marker expressed in nearly all neuronal cells although not yet studied in NSC-34.

Aim: The aim of this project was to characterize the expression of *NeuN* in differentiated and undifferentiated NSC-34 cells and silence gene expression by using siRNA.

Methods: RT-qPCR was used to measure *NeuN* expression during passages 5 to 15 and a comparison was performed between one early and one late passage. Lipofectamine® RNAiMAX was used for siRNA-treatment in different concentrations and several different medium compositions were tested as differentiation media.

Results: *NeuN* was expressed in passages 5 to 15, with decreased expression levels in passage 13 (ΔCt 15.36 ± 0.16) compared to passage 5 (ΔCt 15.09 ± 0.16), $p < 0.05$. The expression levels did not change after differentiation. siRNA-treatment yielded knockdown when using high concentrations of the reagent ($p < 0.05$).

Conclusion: *NeuN* was expressed in a stable, low level throughout passages 5 to 15 with a slightly decreased expression during later passages and no change after differentiation. The siRNA-treatment suppressed gene expression, although further optimization is needed to increase the suppression.

KEYWORDS

Rbfox-3, Neuronal Nucleus, motor neuron, differentiation, secondary injury.

INTRODUCTION

Acute trauma to the spinal cord causes damage to, and loss of, neurons and neural tracts and is followed by a degenerative reaction cascade that is referred to as the secondary injury. The primary injury is the initial damage, e.g. a mechanical break, a laceration or a compression of the tissue. The disruption of the tissue causes a release of intracellular components and chemical mediators such as glutamate (Freire 2012) and uric acid (Shi, Evans, and Rock 2003). These molecules initiate local and potentially harmful reaction cascades, such as inducing an excessive inflammatory response in the region, inducing apoptosis, causing oxidative stress or causing cell death by excitotoxicity of surviving neurons (Freire 2012). This is the secondary injury, and it can promote further cell loss and widen the affected area with a potential effect on the quality of life of the patient after the injury as a result.

The mechanisms and potential prevention or limitation of the secondary damage have been the focus of many studies investigating, among other things, the effect of pharmacological drugs (Petrović et al. 2019), antibody-mediated protein inhibition (Gonzalez et al. 2003) and lentivirus-mediated gene knockdown (Wang et al. 2019) on different parts of the secondary injury process. However, few investigations have been done on the potential use of siRNA-mediated gene suppression to limit the inflammatory response. siRNA-mediated gene knockdown is a technique that uses a short sequence (20-25 base pairs) of double-stranded RNA to incite the cells own endogenous defence mechanism against harmful RNAs to target a specific RNA sequence. The siRNA is designed complementary to the mRNA of the target gene and is introduced into the cell via transfection, were it is recognized as an exogenous RNA molecule by the RNA-induced silencing complex (RISC). It is then separated into single strands and incorporated into the protein complex, which can then use the siRNA as a template to bind any complementary strands of RNA. The complex then cleaves the bound

RNA and the RNA fragments are further degraded by other cellular processes, effectively preventing the sequence from producing a functioning protein.

The use of siRNA is a potent method of gene knockdown and a viable option as active component in drugs for the treatment of patients as shown by a previous successful case: a drug using siRNA as the active component has recently been approved as treatment for the neurodegenerative disease hereditary transthyretin-mediated amyloidosis (Hoy 2018). It is therefore warranted to investigate the technique's potential to alleviate the effects of the secondary injury after spinal trauma. In the case of treatment of spinal trauma, there are many potential targets for siRNA. The event cascade that is the secondary injury is large and complex, and suppression of any of the involved cytokines or enzymes could potentially lead to a decrease in the severity of the secondary damage.

Prior to performing animal studies, it would be advantageous to study the effects of siRNA-treatment on cell cultures as a primary screening of possible genes of interest, as well as using cell cultures as a pilot platform to establish viable treatment concentrations. The hybrid cell line of mouse spinal cord neuron and neuroblastoma cells called NSC-34 has been shown to exhibit several characteristics of motor neurons, such as acetylcholine synthesis, generation of action potentials and inducing twitching when cocultured with myotubules (Cashman et al. 1992). The NSC-34 cell line has been successfully used as a model for spinal cord motor neurons in the past, for example when studying amyotrophic lateral sclerosis (Noda et al. 2019; Pinto et al. 2017). NSC-34 was therefore chosen as the platform on which to study the effect of siRNA-mediated knockdown on secondary injury-related genes.

Before using the NSC-34 cell line to study the effects of siRNA-mediated gene knockdown it needed to be established in the current laboratory, and it was decided that a proof-of-concept study has to be performed. The gene chosen for this study was *Rbfox-3* (also known

as *NeuN*) which encodes the protein Neuronal Nucleus (NeuN), a commonly used neuronal marker expressed in nearly all neuronal cells. NeuN is mostly located in the nucleus of the cell although it can also, to a lesser extent, be visualized in the cytoplasm (Mullen, Buck, and Smith 1992). The protein, and to a smaller extent the gene, is highly conserved across species. The NeuN protein is a member of a family of splicing factors responsible for the alternative splicing of several different genes. The different subgroups in the protein family have different expression patterns and recognize different RNA sequences. *NeuN* is exclusively expressed in the central nervous system and is known to bind to DNA and to activate alternative splicing of some pre-mRNA, although exactly which genes it regulates is not yet fully explored (Duan et al. 2016). Since no studies have yet examined the expression of *NeuN* in the NSC-34 cell line, a characterization of the expression would give novel information. The NSC-34 cell line does show many characteristics of motor neurons, however, since they are modified cells the expression pattern in regards to *NeuN* could differ from native motor neurons. In this project, the characterization was done on both the mRNA and protein level using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunological staining, and the effect of *NeuN*-targeting siRNA was examined.

Ideally, the studied cell line should be as similar as possible to the cells of interest *in vivo*. Cell lines derived from tumour cells tend to grow in a more immature form, closer in form to undifferentiated stem cells. This can be mitigated by the fact that it is often possible to treat the cultivated cells in such a way as to induce differentiation in the direction of the matured cell. Differentiated NSC-34 show more characteristics of motor neurons, and would be preferable as study material over undifferentiated cells. Previous studies with NSC-34 use morphology as the means to classify differentiated versus undifferentiated cells, with neurite length often used as criteria for differentiation (Madji Hounoum et al. 2016). A number of different medium compositions have been used to induce differentiation (Madji Hounoum et

al. 2016; Matusica et al. 2008), although common amongst all of them is a decreased serum concentration. Using a less nutritious medium to slow down proliferation and expose the cells to a small degree of stress is a common strategy for differentiation. The normal growth medium for NSC-34 is Dulbecco's Modified Eagle's Medium (DMEM), often supplemented with additional glucose. This is, as is common for most cell cultures, supplemented with antibiotics and antimycotics as well as serum, usually fetal bovine serum (FBS). This provides a nutritious environment resistant to infection and with growth factors to induce proliferation. To allow differentiation, the FBS concentration is reduced to decrease proliferation and to reduce nutrient concentrations. The base medium can also be exchanged for a less nutritious one, such as Minimum Essential Medium Alpha Modification (α-MEM), to further decrease proliferation rate. While the significant reduction in serum concentration and nutrition is intentional, it is important to not reduce the levels of nutrients to such a degree that it adversely affects the cells' health. When the levels of non-essential amino acids (NEAA) are too low, the cells can synthesize them through the Krebs cycle by using glucose from the surrounding medium. To reduce the possible effects of the byproducts of this process, as well as to reserve the reduced nutrients in the medium for other processes, the differentiation medium is often supplemented with NEAA. Another common addition is retinoic acid (RA). The addition of RA has been shown to increase the rate of differentiation in some media, as well as to markedly decrease the proliferation rate of the cells (Madji Hounoum et al. 2016). In an attempt to induce differentiation of the NSC-34 cells, a number of different medium compositions based on those found in the literature were tried during this study.

The aim of this project was to characterize the expression of *NeuN* in the mouse neuronal NSC-34 cell line over a number of passages via RT-qPCR and immunological staining. The secondary aim was to perform preliminary trials of gene knockdown with siRNA and investigate the possibility of differentiating the cells.

MATERIALS AND METHODS

Cell cultures

This study was performed on the mouse spinal cord neuron and neuroblastoma hybrid cell line NSC-34, procured from Cedarlane Laboratories, which were kept in 75 cm^2 culture flasks at 37^oC and 5 % CO_2. The growth medium consisted of DMEM with high glucose (Sigma-Aldrich) with 10 % v/v FBS (Sigma-Aldrich), 60 µg/mL penicillin and 50 µg/mL streptomycin (1 % P/S, Statens Veterinärmedicinska Anstalt). The growth medium was renewed every 3-4 days and the cells were subcultured at >80 % confluence. To subculture, the growth medium was removed via aspiration and the cells washed with phosphate buffered saline (PBS). After removing the PBS, the cells were treated with 750 µL trypsin for approximately 5 min at 37^oC and 5 % CO_2. After visually confirming the cells' detachment from the flask's surface with an inverted microscope (Nikon TMS), 9.25 mL growth medium was added to the flask. The cell solution was then homogenized via aspiration and transferred to a 15 mL centrifuge tube. From there, an appropriate amount of cell solution (usually 2 mL) was transferred to a new culture flask containing enough growth medium to bring up the volume up to a total of 10 mL. The amount of transferred cell solution was dependent on when the new flask would need to reach >80% confluence.

Freezing and thawing

Cells from early passages were frozen to preserve them for later studies. This was done by collecting the cell solution after trypsinization and centrifuging it for 5 min at 300 g. The supernatant was removed, except for approximately 0.5 mL, and the pellet was resuspended in the remaining liquid. This was followed by the addition of 5 mL freezing medium (30 % v/v

FBS and 10 % v/v dimethyl sulfoxide, DMSO, in growth medium). The cells were then frozen in aliquots of 1 mL at -70^{o}C for 24 h, after which they were transferred to storage in liquid nitrogen.

Cells were thawed when needed. The freezing vial was removed from liquid nitrogen storage and thawed quickly by hand. The content of the vial was split to two tubes containing 9.5 mL growth medium and the solutions were, after mixing, transferred to two culture flasks. After incubating the flasks for 4-6 h at 37^{o}C and 5 % CO_2, the medium was exchanged for fresh growth medium and the flasks were then treated as normal culture flasks.

Cell counting and seeding

Cells for the various experiments were collected and seeded in 24-well plates when a culture had reached >80 % confluence, in concurrence with the culture flask being trypsinized. Before seeding, the cell concentration was determined using a NucleoCounter® NC-100™ System (ChemoMetec). To do this, 200 µL cell solution was transferred to a microcentrifuge tube to which 200 µL Reagent A100 followed by 200 µL Reagent B was added. A NucleoCassette™ was then used to aspirate some of the mixture, and the cassette was analyzed in a NucleoCounter® NC-100™. A dilution to the desired concentration for seeding according to the current experiment was made with growth medium, and 1 mL cell solution was added per well. Plates were incubated at 37^{o}C and 5 % CO_2 until the desired degree of confluence had been reached. The medium was refreshed every 2-3 days.

RT-qPCR

RNA extraction

Cells for RT-qPCR were seeded at 80 000 cells/mL as described above. Plates were incubated for 3 days, after which all growth medium was removed and RNA was extracted using TRIzol® Reagent (Thermo Fisher Scientific) according to the manufacturer's description. The amount of TRIzol® Reagent per well used was 300 µL. The solution was homogenized via aspiration and transferred to a microcentrifuge tube, were it was left to incubate in room temperature for 5 min. Chloroform was then added to each tube in volumes of 60 µL, and the tubes were vortexed and then incubated in room temperature for 2 min. The tubes were then centrifuged for 15 min at 12 000 g and 4^{o}C and the aqueous phase transferred to a new tube, to which 150 µL isopropanol was added. The tubes were vortexed again and incubated in room temperature for 10 min. The tubes were then centrifuged for 10 min at 12 000 g and 4^{o}C. The supernatant was removed and 300 µL 70% ethanol was added and the tubes were vortexed. If necessary, tubes were stored at 4^{o}C at this stage until the protocol was continued. The tubes were then centrifuged for 5 min at 7 500 g and 4^{o}C. The supernatant was removed and the pellets left to airdry for 5-10 min, and then resuspended in 20 µL RNAse free water. The RNA concentration was measured using a NanoDrop® ND-1000 (Saveen Werner). For the initial RT-qPCR of the different cell passages, samples were then diluted to the lowest concentration within the same passage. For RT-qPCR-reactions comparing expression levels between groups, all samples were instead diluted to a concentration of 55.6 ng/µL if possible.

cDNA synthesis

cDNA was synthesized from the extracted RNA samples by using the High-Capacity RNA-to-DNA™ Kit (Thermo Fisher Scientific) according to the manufacturer's description. A

master mix containing 10 µL RT Buffer Mix (2x) and 1 µL RT Enzyme Mix (20x) per reaction was prepared, and 11 µL master mix was added per well to a 96-well reaction plate. The diluted RNA samples were then added to the plate, 9 µL per sample, and the plate was sealed with adhesive film, vortexed and centrifuged briefly, and then incubated in a GeneAmp® PCR System 9700 (Thermo Fisher Scientific). The reaction parameters were 30 min at 16^{o}C, 30 min at 42^{o}C, 5 min at 85^{o}C and 4^{o}C on hold. The completed cDNA was stored in the reaction plate at 4^{o}C until the RT-qPCR-reaction was prepared.

RT-qPCR reaction

TaqMan® Fast Advanced Master Mix (2x) together with a TaqMan® Gene Expression Assay (20x) containing a probe and primers targeting *NeuN* (Mm01248771_m1), and a Custom TaqMan® Gene Expression Assay (20x) containing a probe and primers targeting *ActB* (Mm02619580_g1), all from Thermo Fisher Scientific, were used for the PCR-reaction according to the manufacturer's description. A master mix containing 10 µL TaqMan® Fast Advanced Master Mix, 1 µL gene expression assay targeting *NeuN*, 1 µL gene expression assay targeting *ActB* and 4 µL nuclease-free water per reaction was prepared, and 16 µL master mix was added per well to an optical 96-well reaction plate. The cDNA-samples were then added to the plate, 4 µL per sample, and the plate was sealed with adhesive film, vortexed and centrifuged briefly, and then incubated in a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). The reaction parameters were an initial 20 s at 95^{o}C, followed by 40 cycles of 3 s at 95^{o}C and 30 s at 60^{o}C.

Immunocytochemistry

Cells for immunocytochemistry (ICC) were seeded at 40 000 cells/mL as described above. The cells were incubated for 3 days, at which point all growth medium was removed and the cells were washed with PBS. They were then fixed in 4 % paraformaldehyde in phosphate buffer for 10 min, followed by another wash with PBS. Blocking was then done with 10 % v/v goat serum in dilution buffer (1 % v/v bovine serum albumin and 0.15 % v/v Triton in PBS) for 30 min. The cells were then incubated with rabbit polyclonal anti-NeuN (ABN78, Millipore) antibody diluted to 2µg/mL (1:500) in dilution buffer at 4^{o}C overnight. After removal of the primary antibody solution, the cells were rinsed once and then washed three times for 5 min in washing buffer (1 % v/v Triton in PBS). The cells were then incubated with biotinylated goat anti-rabbit IgG antibodies (BA-1000, Vector Laboratories) diluted to 6 µg/mL (1:250) in dilution buffer for 30 min in darkness. After another rinse and three washings for 5 min with washing buffer, the cells were incubated with DyLight® 488 streptavidin (sa-5488, Vector Laboratories) at 20 µg/mL for 30 min in darkness. The cells were then again rinsed and washed three times for 5 min in washing buffer, followed by a rinse with PBS and were then kept in PBS, in darkness at 4^{o}C, until imaging. Stained cells were imaged in the wells with a fluorescent microscope (Leica DMi8).

Gene silencing using siRNA

Cells for siRNA-treatment were seeded at 40 000 cells/mL for the first test and 20 000 cells/mL for the second test as described above. The cells were grown for 3 days before treatment. siRNA transfection was done with Lipofectamine® RNAiMAX (Thermo Fisher Scientific) as per the manufacturer's instructions. A stock solution of 100 µM siRNA was diluted in Opti-MEM® medium (Thermo Fisher Scientific) to 2 µM. Lipofectamine® reagent

was also diluted in Opti-MEM®, to 2 %, 4 %, 6 % or 12 % v/v. Equal amounts of diluted siRNA and diluted Lipofectamine® reagent were mixed and incubated in room temperature for 5 min, after which 50 µL mixed solution was added to each well. The resulting final concentrations in the wells were 50 nM siRNA and 0.05 %, 0.1 %, 0.15 % or 0.3 % v/v Lipofectamine® reagent. Wells treated with 50 µL PBS were used as negative control during the first trial, while the negative controls for later trials were wells treated with 50 µL of Lipofectamine® reagent diluted in Opti-MEM® to equal the concentration in the test group with the highest concentration during that run. The plate was then incubated in normal growth conditions for 24 h, after which the gene expression was examined with RT-qPCR following the same procedure as described above. The total amount of RNA used for cDNA synthesis was kept at 500 ng.

Differentiation

Cells for differentiation were seeded at concentrations ranging from 1000 cells/mL to 100 000 cells/mL, either seeded as described above in growth medium or seeded immediately in the differentiation medium used for that test run. Different lengths of time before switching from growth medium to differentiation medium were tried, ranging from 3 days post seeding to, as mentioned, being immediately seeded in the differentiation medium. A number of different medium compositions were tried as differentiation media, and all compositions were supplemented with 1% P/S and 1% MEM-NEAA supplement (Thermo Fisher Scientific). Either DMEM with high glucose or α-MEM (GE Life Sciences) was used as the medium base. RA was used in some compositions at a concentration of 1 µM. The concentration of FBS in the differentiation media ranged from 0 % to 3 %. The medium was refreshed every 2-3 days. The final medium composition used for differentiation was 1 % FBS, 1 % P/S, 1 %

MEM-NEAA and 1 μM RA in α-MEM. The plates were incubated for up to 9 days post seeding. The cells were imaged with phase contrast using a Leica DMi8 microscope.

Statistics

All RT-qPCR data was processed using the comparative ΔΔCt method in Microsoft Office Excel. Mann-Whitney U tests (2-tailed) were used for statistical analyses regarding passages and differentiation. Kruskal-Wallis tests (1-tailed) with pair-wise comparison and Bonferroni correction were used to analyze data from the siRNA-treatments. Statistical tests were computed using SPSS version 26, with significance level set to $\alpha = 0.05$.

Ethical considerations

This project was performed solely with cultures of a commercial cell line, and thus required no ethical approval. One of this research group's aims in using the NSC-34 cell line is to decrease their number of research projects that require animals.

RESULTS

NeuN expression

Expression of *NeuN* was detected in all passages used (passages 5 to 15). Mean values and standard deviation are presented in table 1 for RT-qPCR with different amounts of input RNA for each passage.

Table 1. Expression levels of *NeuN*, normalized against the expression level of *ActB*. Mean values and standard deviations are presented (n=6). The total amount of input RNA used in the cDNA synthesis varied between passages.

Passage	**ΔCt Mean**	**Total input RNA (µg)**
5	15.16 ± 0,17	3.57
6	15.56 ± 0,09	0.50
7	14.77 ± 0,14	0.50
8	14.37 ± 0,19	1.76
9	15.42 ± 0,23	3.14
10	14.31 ± 0,18	3.47
11	13.69 ± 0,11	4.02
12	13.78 ± 0,23	3.16
13	13.56 ± 0,22	3.96
14	13.65 ± 0,21	3.74
15	13.93 ± 0,13	3.41

RT-qPCR done with equal amounts of input RNA (500 ng) for one early and one late passage (passages 5 and 13) yielded a significant difference ($p < 0.05$, two-tailed Mann-Whitney U test) in *NeuN* expression (see figure 1), with passage 13 (P13) having a higher mean ΔCt value (15.36 ± 0.16) than passage 5 (P5, 15.09 ± 0.16).

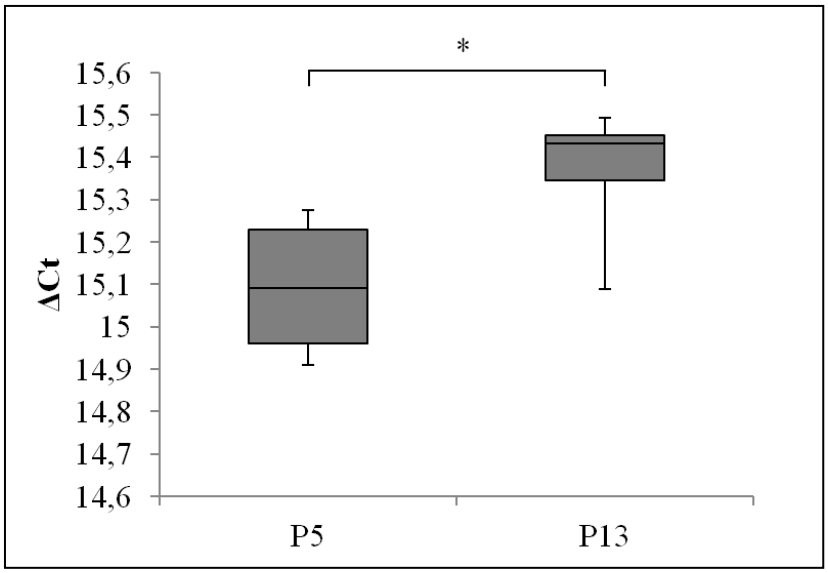

Figure 1. Expression levels of *NeuN* for passages 5 (P5) and 13 (P13) normalized against the expression level of *ActB*. For P5: n=6. For P13: n=5. ”*” indicates $p < 0.05$, two-tailed Mann-Whitney U test.

Immunocytochemistry

Cells were positively stained for NeuN in all investigated passages and no major difference in pattern or intensity was noted. The signal intensity was most intense in the nuclei, although it was also visible in the cytoplasm (see figure 2).

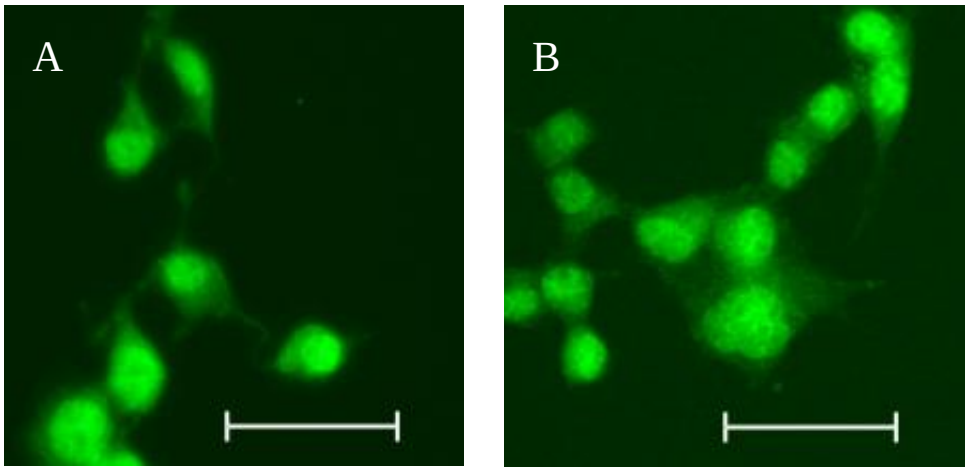

Figure 2. Immunofluorescent labelling of NeuN in the NSC-34 cell line for passages 5 (A) and 13 (B). Scale bar: 50 µm.

siRNA-treatment

The *NeuN* expression levels of NSC-34 cells seeded at 40 000 cells/mL and treated with 0.05 %, 0.10 % or 0.15 % Lipofectamine® were 58 %, 54 % and 47 % respectively (see figure 3) when compared to a PBS-treated control. Only the highest concentration (0.15 %) of Lipofectamine® resulted in a significantly lowered expression ($p < 0.05$, one-tailed Kruskal-Wallis test with Bonferroni correction). The differences between treatment groups were not significant; however a trend indicated lower expression levels with increasing Lipofectamine® reagent concentration.

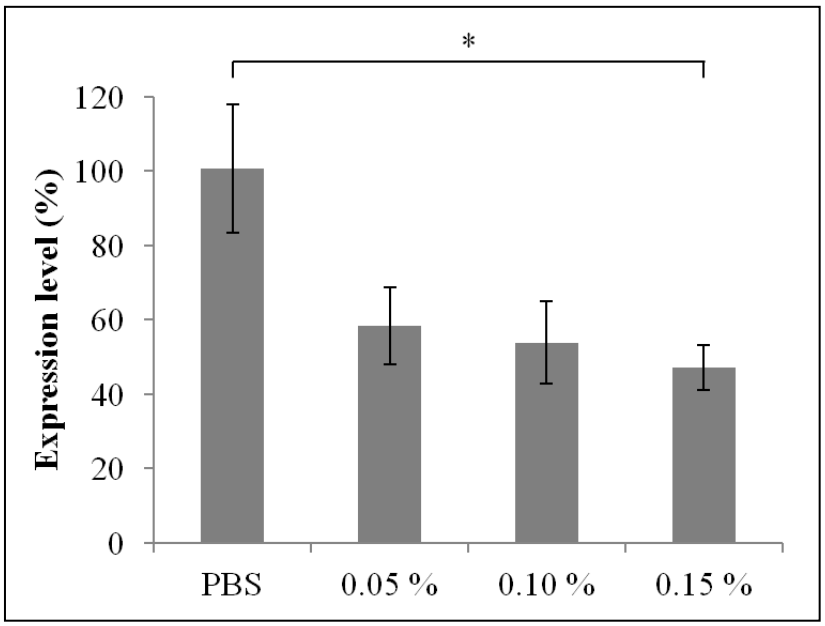

Figure 3. Relative expression levels of *NeuN* normalized against *ActB* in siRNA-treated NSC-34 seeded at 40 000 cells/mL, compared to a PBS-treated siRNA-free control, in three groups treated using different concentrations of Lipofectamine® reagent. n = 3. "*" indicates $p < 0.05$, one-tailed Kruskal-Wallis test with Bonferroni correction.

When the NSC-34 cells were seeded at 20 000 cells/mL and compared to a Lipofectamine® reagent-treated control, treatment using 0.15 % and 0.30 % Lipofectamine® resulted in expression levels of 74 % and 71 % respectively (see figure 4). Only 0.3 %

Lipofectamine® resulted in a significantly lowered expression ($p < 0.05$, one-tailed Kruskal-Wallis test with Bonferroni correction). The relative decrease in expression appeared smaller than when compared to a PBS-treated control.

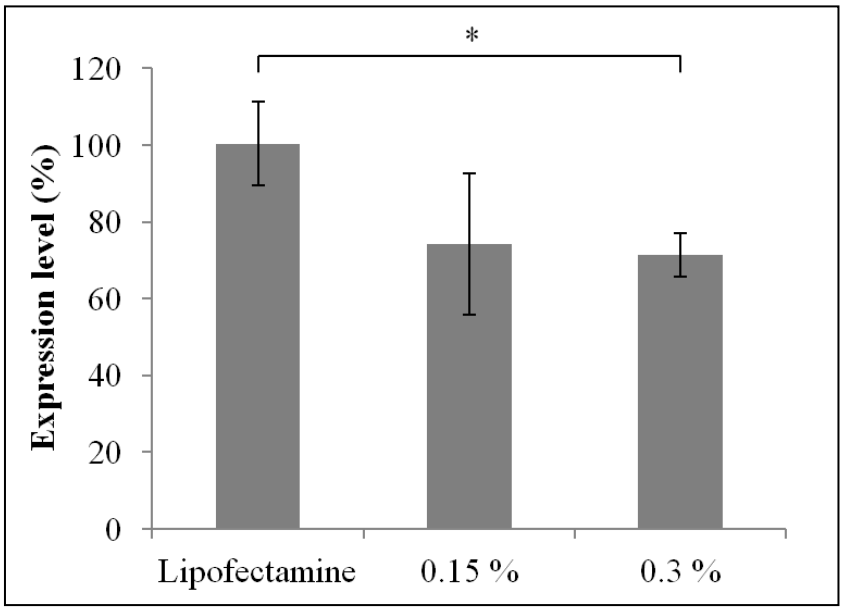

Figure 4. Relative expression levels of *NeuN* normalized against *ActB* in siRNA-treated NSC-34 seeded at 20 000 cells/mL, compared to a Lipofectamine® reagent -treated siRNA-free control, in two groups treated using different concentrations of Lipofectamine® reagent. n = 3. "*" indicates $p < 0.05$, one-tailed Kruskal-Wallis test with Bonferroni correction.

Differentiation

Using a decreased FBS concentration seemed to increase neurite length and number. FBS concentrations of < 1 % did not decrease proliferation enough, even when seeded at a low concentration of cells, to reliably verify differentiation in those media. FBS concentrations of > 1 % resulted in unstable growth and high levels of observed cell death or cell detachment. An FBS concentration of 1 % sufficiently slowed proliferation when seeded with 7 000 cells/mL (see figure 5) while seeding with 10 000 cells/mL or more resulted in too high levels of confluence to be able to examine neurites after 4-5 days. Using 1% FBS in DMEM resulted

in an extensive loss of cells after 1-3 days, while no increased loss of cells was noted with 1 % serum in α-MEM. The addition of RA did not noticeably increase cell loss. Seeding immediately in differentiation medium yielded less loss of cells due to detachment compared to seeding in growth medium and switching medium after 6 h, and a decreased rate of proliferation compared to switching after 24 h.

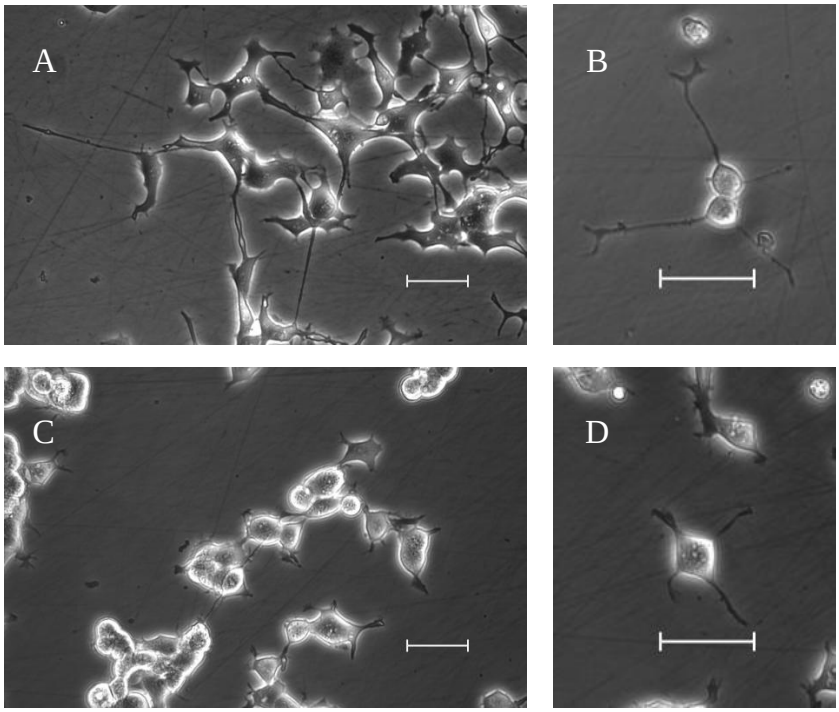

Figure 5. Cells seeded at 7 000 cells/mL and imaged at 7 days post seeding. Cells incubated in differentiation medium containing 1 % FBS and 1 µM RA in α-MEM (A and B) displayed longer neurites and a lower proliferation rate compared to cells incubated in growth medium (C and D). Scale bar: 50 µm.

Expression levels of *NeuN* differed neither between differentiated and undifferentiated cells for passages 5 and 13 (see figure 6) nor between the two differentiated groups on a statistically significant level. The mean ΔCt value in P5D was 15.00 ± 0.14 and in P13D it was 15.25 ± 0.20.

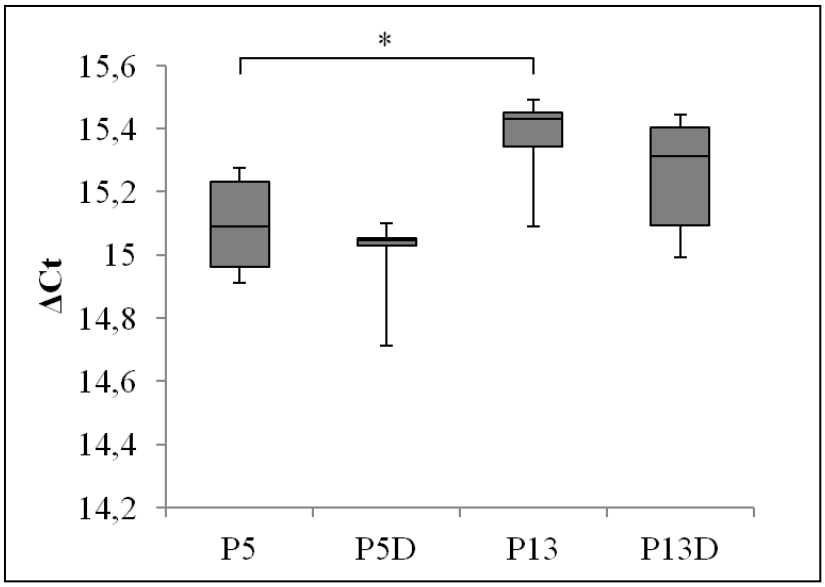

Figure 6. Expression levels of *NeuN* in undifferentiated and differentiated cells from passages 5 and 13 (P5 and P13, and P5D and P13D respectively) normalized against the expression level of *ActB*. For P5, P5D and P13D: n=6. For P13: n=5. ”*” indicates $p < 0.05$, two-tailed Mann-Whitney U test.

DISCUSSION

The aim of this study was to characterize expression levels of *NeuN* in the mouse neuronal NSC-34 cell line using RT-qPCR and immunocytochemistry. *NeuN* espression was evaluated in undifferentiated and differentiated cells and siRNA-treatment was used to alter gene expression. It was a proof-of-concept study and ultimately meant to establish the newly procured NSC-34 cell line in the Orthopaedics laboratory at Rudbecklaboratoriet in Uppsala.

In the initial investigation of *NeuN* expression during passages 5 to 15, mRNA is present in all passages in measurable amounts. The amount of input RNA was not kept equal between passages and the amount used for cDNA synthesis was often larger than recommended by the manufacturer of the kit. Comparing values from different passages should therefore be done with caution. The expression is put in relation to a housekeeping gene to alleviate some of the discrepancy, meaning that it should be possible to detect large differences. The results

therefore indicate that the expression level of *NeuN* does not vary greatly during passages 5 to 15.

In the follow-up RT-qPCR one early (P5) and one late (P13) passage were compared and the amount of input RNA was kept at 500 ng per cDNA reaction. The values are therefore more comparable, and the increased ΔCt translates to a decreased expression in P13 relative to P5 ($p < 0.05$). The purpose of this comparison was to investigate if future studies of *NeuN* in NSC-34 would benefit from using either early or late passages. The difference in expression was quite small, although since the baseline expression level of *NeuN* tended to be quite low (approximately 14 Ct above *ActB* in general) this suggests that early passages are appropriate to use. This has the added bonus of being practically and economically beneficial as well, with less time and materials required to grow the cells prior to the experiment.

That *NeuN* expression did not differ between undifferentiated and differentiated cells was not expected, since *NeuN* is sometimes used as a marker for matured differentiated neurons (Butts et al. 2017). However, it is used to differ between immature stem cells and mature neurons, meaning that undifferentiated cells seem to be equally mature in terms of *NeuN* expression as the differentiated cells.

The goal of the siRNA-treatments done during this study was to establish an appropriate concentration of Lipofectamine® reagent for use in treating NSC-34 cells, since higher concentrations of the reagent tend to give increased gene silencing. However, the reagent is supposedly toxic to the cells. Even though the reagent used in this study is less toxic than several other liposome-mediated transfection agents available (Wang et al. 2018), it is still toxic in high concentrations. However, different cell lines tolerate different concentrations and the procedure should be optimized for each new cell line used. No signs of toxicity were noted during this experiment, even with the highest concentration used, although knockdown

of *NeuN* did not increase much. Even though mRNA-levels are often used to measure the gene knockout effect of siRNA-treatments, the goal of the treatment is usually to inhibit the function that is provided by the protein encoded for by that gene. The level of mRNA knockdown needs to be high to reliably decrease the amount of the protein, although this depends on the target protein. Measuring the protein level could therefore give a more accurate indication of the success of the knockdown. With the limited knockdown seen in this study it is possible that the treatment is not potent enough to inhibit protein function. However, the aim in this study was to verify the method and not necessarily to inhibit NeuN function in the cells. Even though only the highest used concentrations resulted in statistically significant expression reduction ($p < 0.05$) in this study, the small differences in expression reduction between groups could indicate that other parameters is the cause for the low level of knockdown. With those parameters optimized it is possible that a concentration of 0.05% v/v Lipofectamine® reagent is sufficient for transfection of NSC-34 cells, although to sufficiently compare the smaller differences between the treatment groups the experiment could be redone with larger sample sizes. One such parameter could be the efficiency if the siRNA itself. To further optimise the siRNA treatment of the anti-*NeuN* siRNA on NSC-34 cells it is also necessary to optimise the siRNA concentration. The used concentration of 50 nM is already quite high, and the low knockdown effect could indicate that the affinity of this siRNA is not good enough and that a new siRNA should be designed for future studies. Another factor to consider is that the efficiency of siRNA-mediated gene knockdown seems to be affected by the expression level of the target gene (Hong et al. 2014). The low expression level of *NeuN* could therefore be a contributing factor to the low knockdown effect of the treatment. As indicated by the difference between using Lipofectamine® reagent compared to PBS as negative control on the relative knockdown of the 0.15 % treatment group, the reagent itself influences the expression of *ActB*. It would therefore be better to use Lipofectamine® as the

control in future studies, and it could be advantageous to use a different housekeeping gene that is not affected by the treatment.

The method used to illustrate relative expression was the comparative ΔΔCt method (Livak and Schmittgen 2001). A limitation of this method is that both primer pairs used, for the gene of interest and the housekeeping gene, need to have equal amplification efficiency in the qPCR so that an equal fold change in template for both genes results in an equal change in Ct value. When a validation of primer efficiency was performed during this study (data not shown), it was noted that the efficiency for the *NeuN* and *ActB* primers were not equal. It is possible that an optimisation of the qPCR protocol could correct the difference, if not it would be advisable to verify the findings in this study using a different method such as a standard curve.

The seeding concentration and medium composition deemed most successful for differentiation was 7 000 cells/mL in 1 % v/v FBS, 1 % v/v MEM-NEAA, 1% v/v P/S and 1 µM RA in α-MEM. The main difficulty with differentiation was the high proliferation rate of the cells. In several previous studies differentiation was initiated at a high level of confluence, commonly 80 % or above (Madji Hounoum et al. 2016; Matusica et al. 2008). When this was tried in this study, the cells reached > 95 % confluence in one or two days. A possible reason for this is that other studies report a large amount of cell death one to two days after the switch to differentiation medium, which was not seen in the present project. They also see a 60 % decrease in proliferation with the addition of RA (Madji Hounoum et al. 2016), which was not entirely reproduced in this study. Even though a decrease in proliferation was noted in this study as well it did not seem to be as large as 60 %. It is possible that the difference results from the use of a different medium base. A rather common medium base used for differentiation of NSC-34 is DMEM mixed 1:2 with Ham's F12 (Madji Hounoum et al. 2016; Maier et al. 2013). Using α-MEM as the base, as done in this study, has been reported to give

longer neurites than using DMEM/Ham's F12 (Madji Hounoum et al. 2016). However, since there is more documentation on DMEM/Ham's F12 in the literature it should be an option to consider in future studies. Evaluating the outcome of the differentiation trials was made more difficult by the lack of objective measurements of morphological differences. There are some studies that use the expression levels of certain mRNAs and proteins in combination with morphology to identify differentiated NSC-34 cells (Maier et al. 2013), which might be an option to consider for future experiments. If reliable markers can be found, it would make for a more objective classification of differentiation.

From the present study it can be concluded that *NeuN* is expressed in a stable, low level throughout passages 5 to 15 in the NSC-34 cell line with a possible slight decrease in expression levels in later passages. The expression does not change with differentiation. There is more to optimise regarding both siRNA-treatment and differentiation, although siRNA-treatment significantly decreased *NeuN* expression when using high concentration of reagent.

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