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Expression studies of genes related to myelin in oligodendrocytes.

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Summary

A recent study concerning the genetic background underlying schizophrenia has shown chromosoame 6q25-6q27 to include a candidate gene, the *quaking homolog, KH domain RNA binding (mouse) (QKI*). Although the function of this gene has not been studied in humans, its homolog in mouse has been well studied. Mouse QKI regulates the alternative splicing of myelin genes such as those encoding myelin-associated glycoprotein (MAG), myelin proteolipid protein (PLP1) and myelin basic protein (MBP). The aim of the study was to determine how human QKI is involved in myelination and how it interacts with myelin-associated genes throughout the different stages during oligodendrocyte maturation. Even though it was not possible to differentiate the oligodendrocytes, QKI and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were silenced using Small interfering RNA (siRNA). The results show an interesting link between QKI and GAPDH gene. Despite the lack of differentiation they have shown MBP expression, leading an increase in MBP mRNA levels when QKI gene is silenced.

Introduction

Schizophrenia and the QKI gene

Schizophrenia is a psychiatric disorder characterized by delusions, hallucinations, reduced interest and drive, altered emotional reactivity, and disorganized behavior with a prevalence of 1% in the population. Heritability is the proportion of phenotypic variation in a certain population that can be attributed to genetic variation among individuals. In schizophrenia the heritability varies depending on the study, from 0.4 as high as 0.8 (McGrath et al. 2004).

During the last few years multiple studies support the idea that myelin abnormalities are involved in schizophrenia. Some of these findings include for instance alterations in white matter volume, mRNA expression studies and linkage studies that point out loci containing myelin-related genes (Aberg et al. 2006, Maier et al. 1996, Hakak et al. 2001, Batzokis 2002). Myelin is made of glial cell membrane that wraps around neurons. The myelin sheath is compact and isolates parts of the axon from the extracelullar medium. The space between two adjacent myelin sheaths is called the Node of Ranvier, which allows saltatory conduction among axons and therefore faster transmission of the electric impulse.

A recent linkage study in one of the world's largest pedigrees with individuals affected with schizophrenia has shown a schizophrenia-susceptibility locus at 6q25-6q27 (Aberg et al. 2006), which contains only one known gene described in the public database, the *quaking homolog, KH domain RNA binding (mouse)* (QKI) gene (http://genome.ucsc.edu/index.html, 2004 assembly) (Kent et al. 2002).

Mouse *quaking (qki)* is a classical example of recessive dysmyelination mutation (Sidman et al. 1964). This gene is known to give rise to six different transcripts: 5 kb-A, -B, 6 kb, 7 kb-A, -B and qki$^{\Delta KH}$ (Kondo et al. 1999) but recent data obtained from the public cDNA database suggest that there are four additional exons expressed in humans, exons 1b, 2b, 7d and 8b (figure 1).

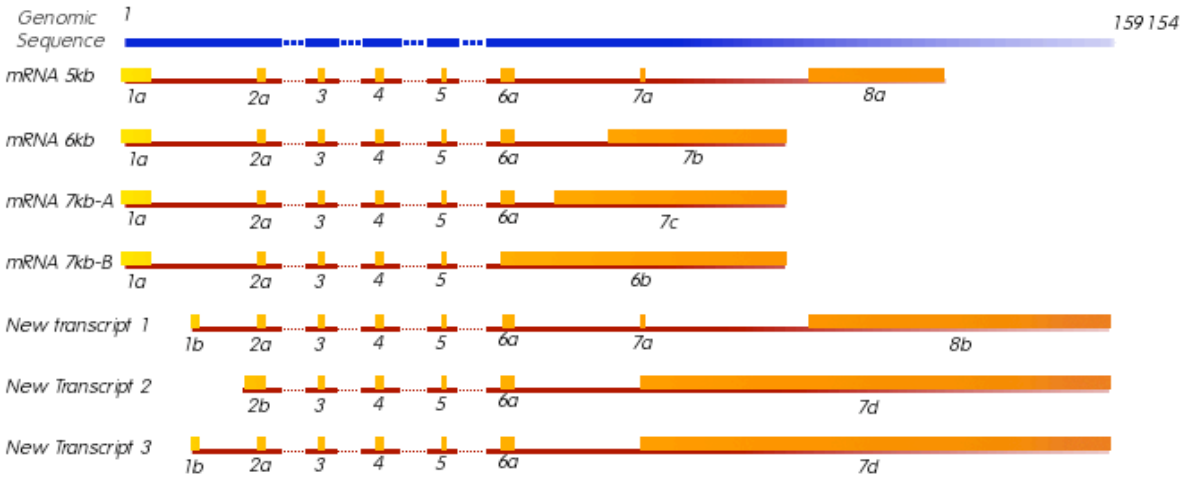

Figure 1. An overview of the genetic structure of the human QKI gene using recent data from the public database.

The amino acid sequences of all QKI isoforms share a KH domain, a protein motif which is common in a diverse family of proteins that binds to single-stranded RNA and therefore are thought to be involved in mRNA regulation (Lukong et al. 2003). The expression of QKI isoforms are differently regulated in distinct cell types and also follow a developmental pattern, for instance QKI-5 is known to be actively expressed during the embryogenesis and the neonatal stage and decreased thereafter (Hardy et al. 1996).

There are several targets for QKI gene product. The most studied target is the MAG transcript (Myelin-Associated Glycoprotein) The MAG protein is a myelin-specific transmembrane protein thought to be involved in the formation and maintenance of the myelin sheath (Schachner et al. 2000). QKI gene product have shown to interact with myelin basic protein (MBP) mRNA (Li et al. 2000). There are MBP alternatively spliced isoforms specifically expressed in myelin-producing cells, and the total amount of MBP mRNA is under a rigorous control during development (Campagnoni and Macklin, 1988), although how QKI regulates MBP mRNA homeostasis in response to developmental signals is a process that remains unknown. Other myelin-related genes such as cyclin-dependent kinase inhibitor 1 B (CDKN1B), sex determining region Y (SRY), myelin proteolipid protein (PLP1) and human tissue factor (TF) seem to have potential QKI binding motifs and may be regulated in a similar way (Aberg et al. 2006). Therefore QKI may play an important role in oligodendrocyte differentiation.

Silencing genes

One way to modify mRNA levels is to use the Small Interfering RNA (siRNA) silencing method. siRNA is a small oligonucleotide which has a complementary sequence to an mRNA target for a particular gene, therefore binds to the mRNA in the cell inhibiting its translation.

Gene silencing outcome can be measured by real-time polymerase chain reaction (real-time PCR). real time PCR is a strategy developed to measure mRNA levels. The most common real time PCR technique used nowadays is TaqMan™Probes originally developed by Applied Biosystems. In brief, the procedure consists of a fluorescently-labeled probe which binds to the target of interest. The fluorescent molecule is located at the 5' end of the probe and is cleaved from the probe by *Taq* polymerase as long as the target is being amplified from sequence-specific primers (Figure 2). The free 5' molecule emits a specific wavelength recorded by the optic thermocycler system. As the target is being amplified and recorded in each sample the software plot the results in a logarithmic scale. What the software measures in each sample is the cycle number at which the fluorescence level crosses above a certain threshold (to avoid background noise). This crossing point is called Ct value.

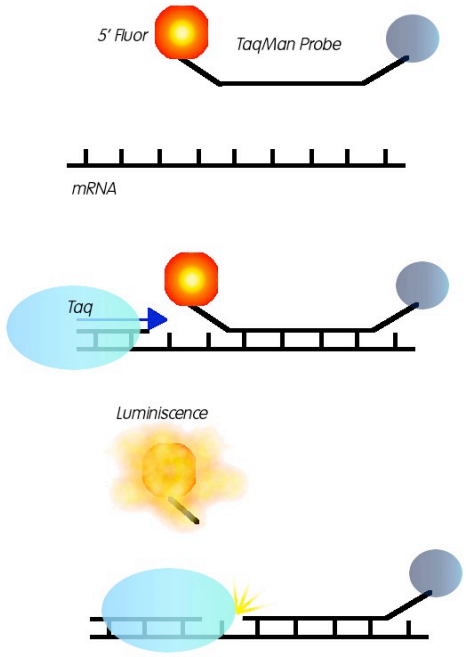

Figure 2. TaqMan™ real time PCR reaction scheme.

Since the total amount of mRNA for a certain gene can be different for different samples, due to the variation of the cell concentration, the relative amount of mRNA for a certain gene is used to compare the samples. To calculate the relative amount of mRNA for a certain gene the total amount of mRNA for that gene is divided by the total amount of a house keeping gene. House keeping genes are constitutive genes which products are needed for maintenance of the cell, and are commonly used as standards in quantitative PCR since it is assumed that their expression is not affected by experimental conditions.

Aims

The goal of this study is to silence QKI expression and that way determine QKI's effect of myelination-related genes in mature oligodendrocytes.

Results

Modification of QKI expression by gene silencing.

I have made all the experiments in human oligodendroglioma (HOG) cells due to their ability to grow indefinetely. siRNA was used to silence gene expression. After the exposure of the cells to siRNA (transfection) I measured the total amount of mRNA for the selected gene with real time PCR. I used β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as house keeping genes to calculate the ratio and determine the relative amount of mRNA for the gene of interest.

I performed different assays for QKI silencing. Considering the average value of the control cells as the maximum expression, gene silencing value was obtained using the average of the cells treated with siRNA to calculate which percentage of the maximum expression it represents. Say Ac = average of control cells and Asi= average of cells treated with siRNA, gene silencing is calculated using the formula GS = ((Asi*100)/Ac)-100.

In the first assay (figure 2) the ratio QKI/β-actin showed that the relative QKI expression was reduced 71% [((0.43*100)/1.48)-100]. Nevertheless I decided to repeat the experiment since there was a large variation between the samples. In the second assay I performed QKI silencing and GAPDH silencing (as positive control). mRNA levels of β-actin, GAPDH, QKI and MBP were measured. In the cells silenced with siRNA that targeted QKI, the relative mRNA levels were reduced 62% [((0.42*100)/1.11)-100], compared to the controls that were not silenced. GAPDH relative mRNA levels in cells silenced with GAPDH siRNA were reduced 41% [((0.45*100)/1.11)-100] (figure 3a). Despite the variation, the data indicated a clear decrease in QKI relative mRNA levels, both in the samples treated with QKI siRNA and in the samples treated with GAPDH siRNA. Figure 3b shows the ratio GAPDH/β-actin, indicating that GAPDH silencing succeeded to decrease the relative GAPDH mRNA levels 50% [((1.3*100)/2.6)-100]. In figure 3c the ratio QKI/GAPDH is represented. The ratio was reduced in the cells treated with QKI siRNA, but in the cells treated with GAPDH siRNA the average increased again, reaching values closer to those in the control samples. Data analyses of the real time PCR suggest that relative QKI expression can be reduced by 50-70%.

Myelin Basic Protein (MBP) mRNA levels was also measured. Analyses of the results suggest that there was an increase in MBP expression in cells treated with QKI siRNA and GAPDH siRNA (figure 3d).

I repeated the same experiment with GAPDH siRNA in order to further evaluate the possibility of QKI mRNA level depletion in cells exposed to GAPDH siRNA. In this experiment the ratio QKI/GAPDH showed the same tendency as before. That is, cells exposed to QKI siRNA the QKI/GAPDH ratio decreased 64% [((0.12*100)/0.33)-100] (QKI expression reduced but GAPDH expression maintained) and cells exposed to GAPDH siRNA showed a QKI/GAPDH ratio similar to the control cells (QKI expression and GAPDH expression are both reduced) (figure 4) Therefore this results show that QKI expression could be related to GAPDH expression since a decreased GAPDH expression lead to a reduced QKI mRNA levels.

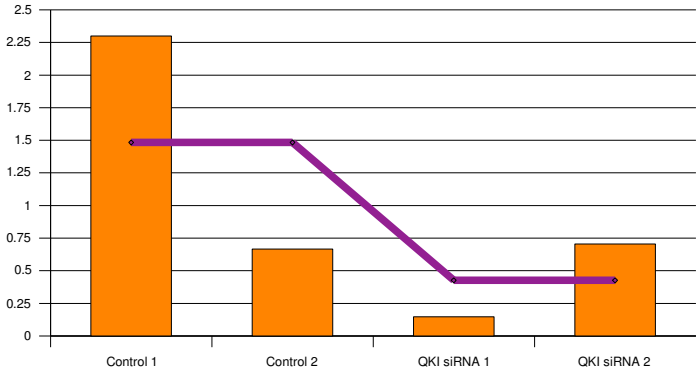

Figure 2. Result of the real time PCR showing the amount of QKI mRNA relative to β-actin in untreated cells (controls) and cells exposed to QKI siRNA (using 2 µl QKI siRNA per sample). The bars represent the relative amount of QKI RNA and lines represent the average.

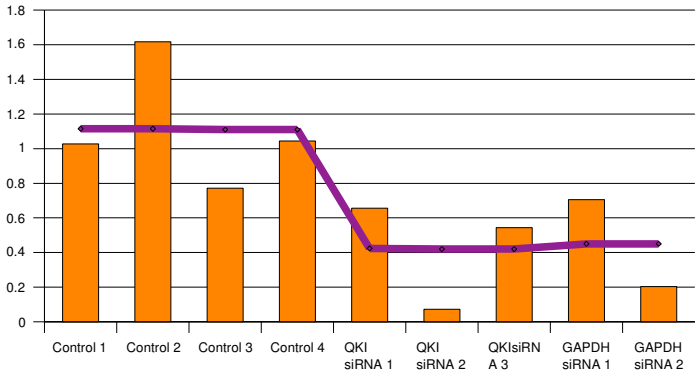

Figure 3a. Result of the real time PCR showing the amount of QKI mRNA relative to β-actin in untreated cells (controls) and cells exposed to QKI siRNA (using 8 µl QKI siRNA per sample) and GAPDH siRNA (using 2 µl QKI siRNA per sample). The bars represent the relative amount of QKI mRNA and lines represent the average.

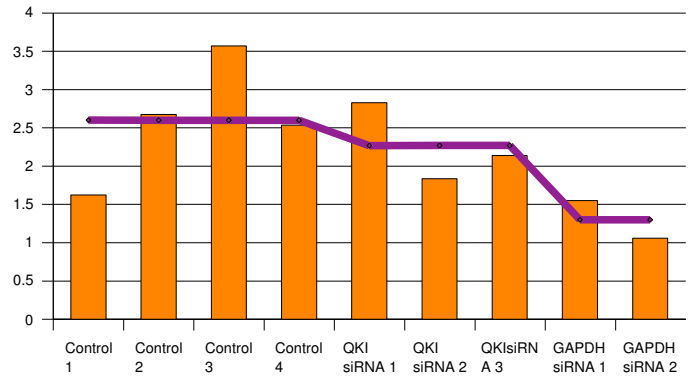

Figure 3b. Result of the real time PCR showing the amount of GAPDH mRNA relative to β-actin in untreated cells (controls) and cells exposed to QKI siRNA (using 8 µl QKI siRNA per sample) and GAPDH siRNA (using 2 µl QKI siRNA per sample). The bars represent the relative amount of GAPDH mRNA and lines represent the average.

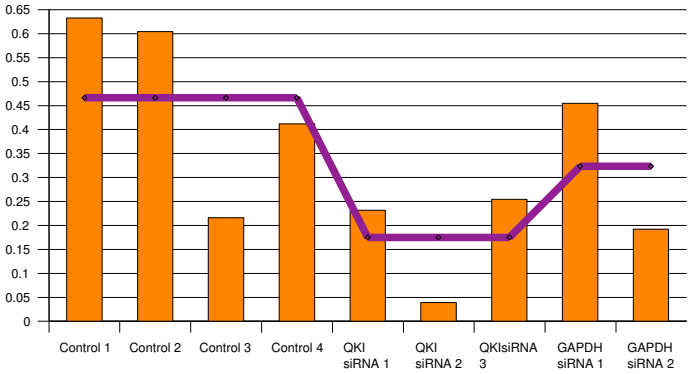

Figure 3c. Result of the real time PCR showing the amount of QKI mRNA relative to GAPDH in untreated cells (controls) and cells exposed to QKI siRNA (using 8 µl QKI siRNA per sample) and GAPDH siRNA (using 2 µl QKI siRNA per sample). The bars represent the relative amount of QKI mRNA and lines represent the average.

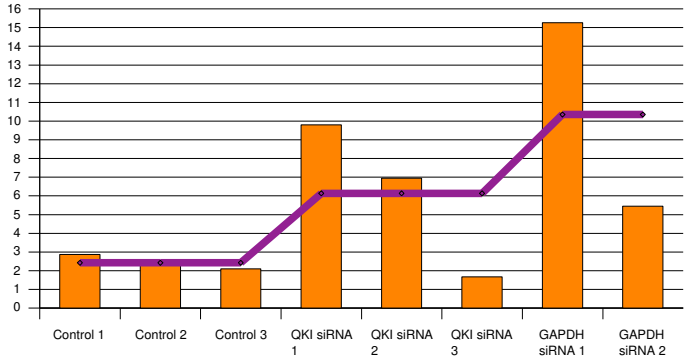

Figure 3d. Result of the real time PCR showing the amount of MBP mRNA relative to β-actin in untreated cells (controls) and cells exposed to QKI siRNA (using 8 µl QKI siRNA per sample) and GAPDH siRNA (using 2 µl QKI siRNA per sample). The bars represent the relative amount of MBP mRNA and lines represent the average.

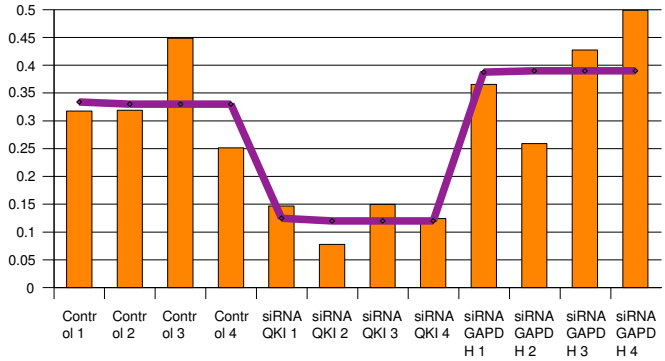

Figure 4. Result of the real time PCR showing the amount of QKI mRNA relative to GAPDH in untreated cells (controls) and cells exposed to QKI siRNA (using 8 µl QKI siRNA per sample) and GAPDH siRNA (using 2 µl QKI siRNA per sample). The bars represent the relative amount of QKI mRNA and lines represent the average.

Differentiation of oligodendrocytes.

There are two different aspects to cover in order to study the impact of QKI gene product has on other myelin-related genes. The first one is to succeed with the gene modification technique, which is described in the paragraph above, and the second one is to induce oligodendrocyte maturation and myelination.

In this study I have evaluated two different differentiation media, Mieke's differentiation medium (Buntinx et al. 2004), which has been tested before by Eva Lindholm in HOG cells during 7 days without success (data not published), and Bottenstein-deSato (Sato) medium kindly provided by Åsa Fex Svenningsen (Institutionen för neurovetenskap, Genetisk utvecklingsbiologi, Uppsala Universitet, Sweden). To detect whether the cells differentiated two different experiments were performed: immunohistochemistry and mRNA quantification.

Expression of RIP and NG2 in oligodendrocytes, an inmunohistochemical study. One way to detect whether the oligodendrocytes differentiated is to use fluorescently-labeled antibodies that target early and late markers for oligodendrocyte differentiation and observe the result in a fluorescent microscope. In this experiments the cells grow in Sato medium and Mieke's differentiation medium for a period of 7 days are fixed and then incubated with fluorescently-labeled antibodies that target the markers selected. There are several possible markers to use , all of them are proteins that are expressed only in the immature (early marker) or mature (late marker) state of the oligodendrocyte differentiation. I used RIP as a late marker, which is a protein expressed in the premyelinating oligodendrocyte, and NG2 as an early marker, which is an early myelination-related protein.

Pictures of the immunohistochemistry experiment reveal that there were mostly unspecific binding for both the early and the late marker since the fluorescence emitted is weak and diffuse (Figure 5a and 5b). This means that the cells had not differentiated enough to show the markers for oligodendrocyte maturation or maybe the immunohistochemistry failed although I followed a procedure that is known to work.

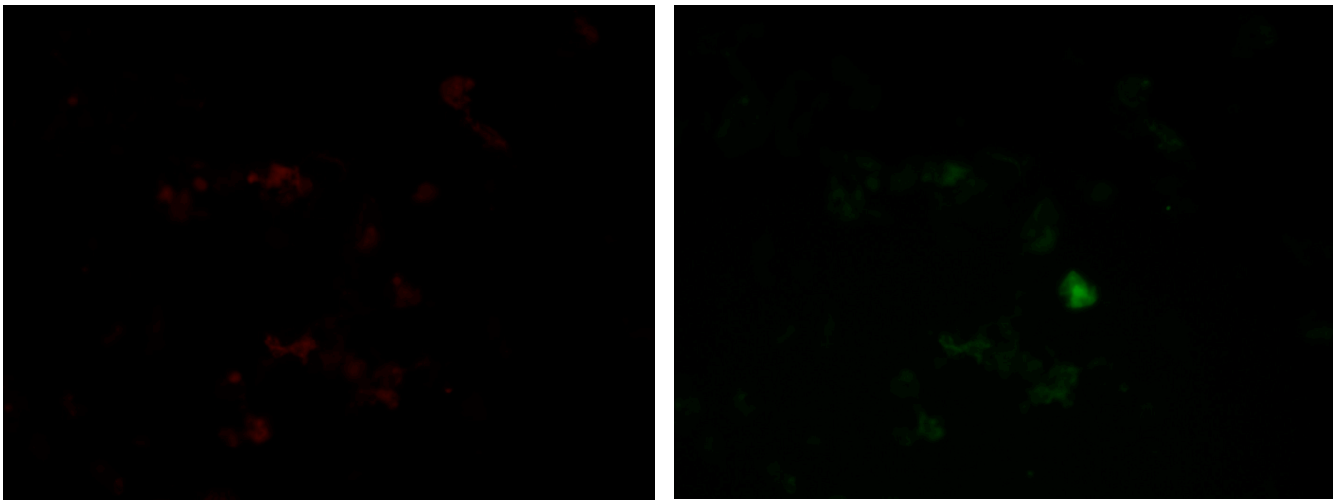

Figure 5a. Pictures of the inmunohistochemistry test showing the RIP marker (left) and the NG2 marker (right) in cells grow in Mieke's differentiation medium during a period of 7 days.

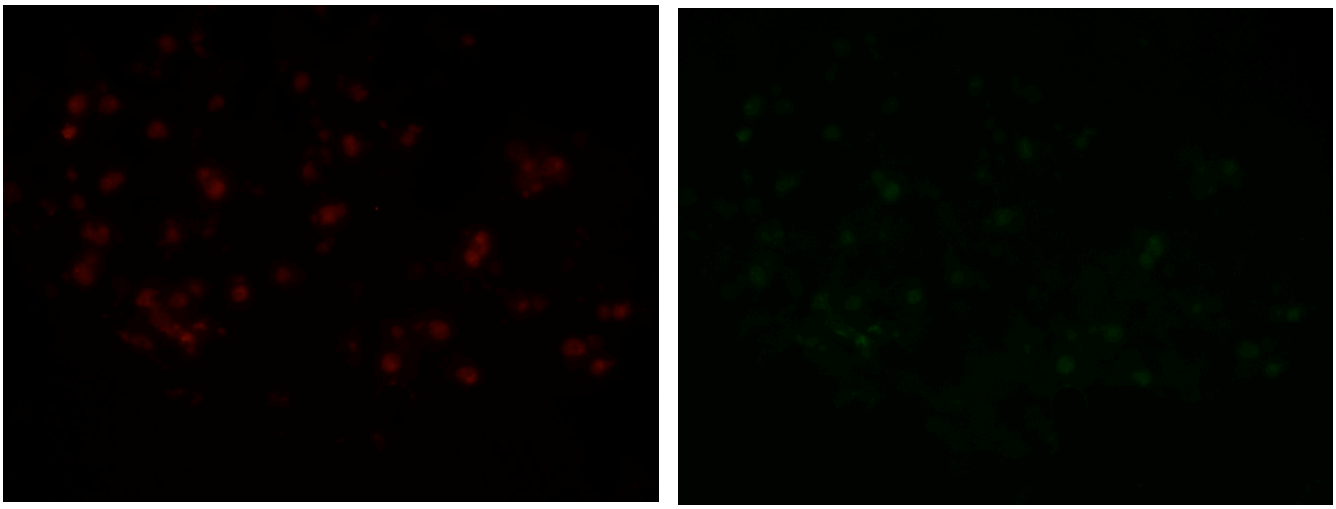

Figure 5b. Pictures of the inmunohistochemistry test showing the RIP marker (left) and the NG2 marker (right) in cells grow in Sato differentiation medium during a period of 7 days.

mRNA quantification. To rule out the possibility that the reason I did not see any expression of markers for oligodendrocyte differentiation was experimental failure, I measured mRNA levels of two gene markers for oligodendrocyte differentiation, MAGa and MAGb, in controls (cells in growth medium) and cells grown in Sato medium and Mieke's differentiation medium using real time PCR. I did not get any amplification of any of those genes, suggesting that the cells were still in an immature state. Nevertheless, it is important to note that the immature cells have the ability to express MBP, a gene that is expressed late during oligodendrocyte maturation (figure 3.d).

Discussion

The GAPDH-QKI link

In this study a possible link between QKI and GAPDH gene that has never been reported before is shown. This connection could be the result of a side effect due to GAPDH silencing. There are several reasons that could explain this link. Since the GAPDH enzyme catalyzes an important energy-yielding step in carbohydrate metabolism it is possible that QKI expression could be hindered due to the metabolic halt in the cell produced by the carbohydrate starvation. Another possibility is that GAPDH siRNA directly inhibits QKI mRNA translation by binding to it. Further analysis and more robust evidence are required in order to explain the results I have shown.

MBP expression increased in QKI-silenced cells

Surprisingly, immature oligodendrocyte cells showed MBP expression although MBP is a gene expressed only in mature oligodendrocytes. Since the cells I was working with have been cultured and passaged many times it is possible that the cells have changed.

This modification of the cell line sets a model for the study of MBP mRNA levels and the relationship with the QKI gene product. QKI protein has shown to stabilize MBP mRNA in myelin-producing cells during active myelinogenesis, allowing the rapid accumulation of MBP mRNA in the cell (Youyi et al. 2003). In this study I have shown that MBP mRNA expression is increased when QKI gene is silenced. This MBP mRNA increase has been observed before in schizophrenic patients, who have a decreased QKI mRNA levels in brain cells (Arberg et al. 2006). One possible explanation for this phenomenon is that the destabilization of MBP mRNA in the absence of QKI gene product lead to an increased production of MBP mRNA in compensation for the MBP mRNA degradation.

HOG differentiation, drawbacks and future perspectives

It is important to remember that cell lines have a tendency to de-differentiate if they are cultured and passaged many times. Since the HOG cell line is a cancer cell line, it is possible that the cells were de-differentiated from the beginning. The lack of success is most probably due the cell line itself rather than to an incorrect procedure since the same procedure has been tested before with positive results in HOG cell line (Buntinx et al. 2004).

The lack of differentiation prevented the progression of the study and therefore the goals that were established at the beginning could not be reached. The continuation of the present work should include a differentiation assay performed during a longer period of time (21-30 days) as described in another work by Gu et al. in mixed glial cultures. If this procedure could not reach the mature state then the best solution to succeed in oligodendrocyte differentiation is probably to change the cell line I was working with and use other fresh HOG cells from a different source, another cell line or primary cells from rat or mouse.

Materials and Methods

Oligodendroglial cell cultures

The primary oligodendrocyte HOG cell culture was provided by Anthony T. Campagnoni (University of California, Los Angeles). The cells were incubated at 37ºC and 5% CO_2 in 10 cm^2 cell-culture plates with 10 ml Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Sweden) supplemented with Earle's salts containing 5% fetal calf serum (FCS) (Invitrogen, Sweden) and 100 µg/ml penicillin and 100 µg/ml streptomycin (PEST) (Invitrogen, Sweden). The culture was split and the medium changed every 4 days.

The two differentiation media used were Sato medium, which contains 13.4 g/L DMEM-powder, 2 g/L NaHCO3, 10 mg/L TF, 10 mg/L insulin, 0.16 mg/L putrescine, 0.0062 mg/L progesterone, 0,34 mg/L T3, 0.035 mg/L sodium selenite, 0.403 mg/L L-thyrosine and 25 mg/L Gentamicin; and another differentiation medium originally developed by Buntinx et al. (13) (referred to as Mieke's differentiation medium) which is DMEM with tri-iodotironine (T3) 30 nM, transferrin 50 µl/ml, insulin 0.5 µl/ml, sodium selenite 30 nM and FCS 0.05%.

siRNA assay

QKI siRNA silencing was performed using DHARMACON QKI siGENOME Smart Pool and GAPDH siRNA silencing was peformed using Ambion Silencer™ GAPDH siRNA.

Transfection of cells. The protocol used was modified from Lina Emilsson.

Two plates of HOG cultures were used with about 70% confluence in each of the plates. The medium was discarded and 10 ml PEST-free medium was added to resuspend the cells.

For the preparation of transfection mixes in the first siRNA assay, I used 3 tubes: mix 1 with 4 µl siRNA + 496 µl Optimen (Invitrogen, Sweden); mix 2 with 500 µl Optimen and mix 3 with 40 µl Lipofectamine™ 2000 Reagent (Invitrogen, Sweden) + 960 µl Optimen. After 5 minutes incubation at room temperature I mixed 500 µl mix 1+ 500 µl mix 3 (siRNA-mix) and 500 µl mix 2 + 500 µl mix 3 (control-mix) and they were incubated for 20 minutes at room temperature.

From the 10 ml cell suspension I plated 1,5 ml in each of four 6 cm^2 culture dishes, and 500 µl siRNA-mix was added to two of them (siRNA samples) whereas 500 µl control-mix were added to the other two (control samples). The cells were incubated at 37ºC and 5% CO_2 overnight.

In the second siRNA assay I performed GAPDH silencing as a positive control for the transfection procedure since I know that it had worked before using the protocol I followed for siRNA transfection. The transfection mixes were prepared as follows: mix 1a with 16 µl QKI siRNA + 984 µl Optimen, mix 1b with 8 µl GAPDH siRNA + 992 µl Optimen, mix 2 with 1000 µl Optimen, mix 3 with 120 µl Lipofectamine™ 2000 Reagent + 2880 Optimen. After 5 minutes of incubation at room temperature I mixed 1000 µl of mix 1a + 1000 µl of mix 3 (QKI siRNA mix), 1000 µl of mix 1b + 1000 of mix 3 (GAPDH siRNA mix) and 1000 µl of mix 2 + 1000 µl of mix 3 (control mix)

The procedure followed was the same as described above, adding 500 µl of QKI siRNA-mix, GAPDH siRNA-mix and control-mix to each sample.

Another modification was introduced the third time I performed the siRNA assay. Since possible cell clumps and early cell attachment to the plate can make transfection inefficient, after the addition of the transfection mixes the cell cultures were shaken for 50 minutes at room temperature in a Tamro IKA® KS 130 basis shaker before the incubation.

24 hours after transfection the medium was discarded and 1 ml serum-free and PEST-free was added to each cell culture dish.

48 hours after transfection the cells were collected. The medium was discarded and 1 ml Phosphate-Buffered Saline (PBS) (Invitrogen, Sweden) was added to each plate in order to resuspend the cells. The cell suspension was transferred to an eppendorf tube and centrifuged at 1500 g, 5 min. The supernatant was removed and 500 µl Trizol Reagent (Invitrogen, Sweden) was added. The pellets were resuspended and stored at -70ºC waiting for mRNA extraction.

RNA extraction. 100 µl chloroform was added to the tubes containing Trizol Reagent with the cell suspension, and mixed gently for about 15 seconds. Then they were incubated 3 minutes at room temperature and centrifuged for 15 minutes, 12000 g.

After the centrifugation all subsequent steps were carried out on ice. The aqueous phase (which contains RNA) was transferred to a new tube and 250 µl isopropanol (at room temperature) was added to the cells. The samples were incubated for 10 minutes at room temperature and then centrifuged for 10 minutes at 12000 g. The supernatant was discarded and the pellets were washed with 200 µl 70% EtOH (ice cold). The samples were centrifuged one more time at 7500 g for 5 minutes. The EtOH was discarded and the samples were let dried for approximately 10 minutes. 10 µl RNase-free H_2O (Promega Corporation, Madison, USA) was added and incubated for about 10 minutes at 55ºC until dissolved.

Reverse transcriptase (RT) reaction. The RT reaction was prepared using 1 µl real time-Buffer (Applied Biosystems, New Jersey, USA) , 2.2 µl $MgCL_2$ (Applied biosystems, New Jersey, USA), 2 µl dNTP:s, (Applied Biosystems, New Jersey, USA) 0.5 µl Oligo dT (Invitrogen, Sweden), 0.2 µl RNase Inhibitor (Applied Biosystems, New Jersey, USA) and 0.5 µl MultiScribe® Reverse transcriptase (Applied Biosystems, New Jersey, USA) per sample. By adding 3.85 µl of the sample I had a total volume of 10 µl. The real time was performed on an MJ thermocycler with the following program: 25°C for 10 min, 48°C for 1 h, 95°C for 10 min and 12°C for ever.

Real time PCR. The reaction was performed using an ABI PRISM 7000 Sequence detection System (Applied Biosystems, Foster City, USA) The reaction mix was 9.2 µl H_2O, 9.8 µl Taqman® Universal PCR Master Mix (Applied Biosystems, New Jersey, USA), 0.66 µl forward primer, 0.66 µl reverse primer and 0.66 µl probe per sample. The primers had a concentration of 10 uM and the probe had a concentration of 5 uM. β-actin, GAPDH and QKI primers (reverse and forward) were obtained from CyberGene (Stockholm, Sweden) and their corresponding probes from Eurogentec (Belgium). The total volume of the reaction was 25 µl. Their sequence were as follows:

GAPD-Forward: 5'- gga agc tca ctg gca tgg c -3'
GAPD-Reverse: 5'- tag acg gca ggt cag gtc ca -3'
GAPD-Taqman probe: 5'- ccc cac tgc caa cgt gtc agt g -3'
β-actin-Forward: 5'-gag cta cga gct gcc tga cg -3'
β-actin-Reverse: 5'-gta gtt tcg tgg atg cca cag gac t -3'
β-actin-Taqman probe: 5'cat cac cat tgg caa tga gcg gtt cc -3'

MBP mRNA levels detection was performed using 7.75 µl H2O, 7.5 µl Taqman® Universal PCR Master Mix and 0.75 µl Assay on Demand MBP primer/probe (catalogue number: Hs00921943_m1. Applied Biosystems, New Jersey, USA) per sample, with a total volume of 20 µl per sample. The program used was as follows: 2 minutes at 50ºC, 10 minutes at 95ºC and 40 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. The data obtained was converted to threshold cycle values (Ct-values) by ABI Prism 7000 Sequence detector system software. The Ct-value of each sample was calculated using a standard curve made from reference samples of which the total amount of RNA was known.

Immunohistochemical study

Preparation of the cultures. For this experiment I used one plate with about 95% confluence. The cells were resuspended and the cell suspension was placed in a falcon tube to centrifuge it for 5 minutes at 1500 g. The supernatant was discarded and 6 ml PBS was added to the pellet to resuspend it. The cell suspension was split into 3 falcon tubes (1 tube for Sato medium, another for Mieke's differentiation medium and the third one for growth medium) 2 ml each. I made another centrifugation to spin down the cells. I added 500 µl of the appropriate medium to the falcon tubes and the cells were resuspended. Then 75 µl of each cell suspension was added to different wells in a Nunclon™ surface plate (NUNCTM, Nalge Nunc International, Roskilde, Denmark) in which glass slides were placed beforehand. The cell cultures were incubated 20 minutes to let the cells attach to the glass slides and then 100 µl of cell suspension was added to every well plus 325 µl of the appropriate medium.

The medium was changed every third day.

Fixation of the cultures. The cultures were fixed at day 0, day 2, day 4 and day 7. The medium was removed and 300 µl fix solution added to each well. The wells were incubated 20 minutes at room temperature and washed several times with PBS until the yellow colour of the fixation solution disappeared. The samples were stored covered in PBS for the immunohistochemistry test.

Inmunostaining procedure. Mikahel Corell prepared a mix containing 400 µl block solution (PBS with 0.25% Triton X-100 + 0.25% BSA) + 0.8 µl Monocolonal anti-RIP antibody (DSHB, Iowa University, US) + 0.8 µl Polyclonal anti-NG2 rabbit (MAB5320, Chemicon, Mediqip, Sweden). Drops of 45 µl were placed on a parafilm. The slides were placed with the side with the fixed cells against the drop and all the slides were incubated in a box at 4ºC overnight. The next day the glass slides were placed again in the Nunclon wells and washed with PBS 0.25 % Triron X-100 wash solution two times with 15 minutes waiting period between each. Then the second antibody was added: alexa 488 (mouse; 1:200; Molecular Probes) which is an anti-RIP antibody, diluted in blocking solution (green colour) and rhodamine red-X (RRX; rabbit, 1:400; Molecular Probes, Gibco-Invitrogen, Sweden) which is an anti-NG2 antibody (red colour). They were incubated 60 minutes at room temperature and washed again two times for 15 minutes. Finally, the samples were mounted and I took pictures.

Acknowledgments

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