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Roles of neuronatin in hepatic glucose metabolism

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Molecular Biology and Biotechnology

Abstract

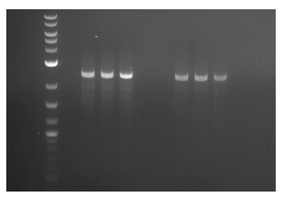
Neuronatin (NNAT) is a proteolipid in calcium signaling regulation, which is associated with several metabolism-related disease. To find out the NNAT regulation mechanism, this research focused on the modulation of glucose metabolism in hepatocytes by NNAT. By construction of NNAT and H6PD, determination of the interaction, and examination of NNAT ablation, a novel disease mechanism of the aberrant metabolic changes in diabetic liver may be identified.

Introduction

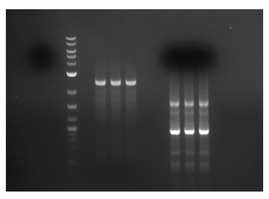
Genetic association studies found that abnormal NNAT expression is found in several diseases like diabetes, cancer, obesity, Lafora disease, and retinal degeneration. Nevertheless, its physiological roles still remain largely unclear. A previous study reported that NNAT interacts with hexose 6-phosphate dehydrogenase (H6PD) but the functional outcome of their interaction has not been explored. In this research, we aim to investigate if NNAT modulates glucose metabolism in hepatocytes. The cloning of NNAT and H6PD was fundamentally carried out for the construction of myc-tagged NNAT and mammalian GST-tagged H6PD. Hence, in further experiments, determination of NNAT and HGPD interaction in vitro and in vivo is possible by using GST pull-down assay and immunoprecipitation. Extracellular flux analysis would be applied as a terminal step for the examination of NNAT ablation on hepatic glycolysis.

Methods and Results

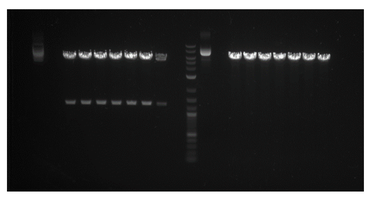
This research has successfully done the molecular cloning of myc-tagged NNAT and mammalian GST-tagged H6PD. 25 cycle PCR was applied for amplifying the target gene. Linearized vectors were prepared by restriction enzyme cut. To be specific, using SalI and NotI for the mGST vector, while EcoI1 and Not1 for the pCDNA3.1 vector. 1% and 0,8% agarose gel was set for running PCR product and RE cut product respectively.



mGST-tagged H6PD

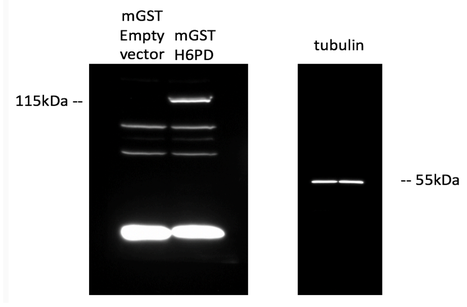


myc-tagged NNAT



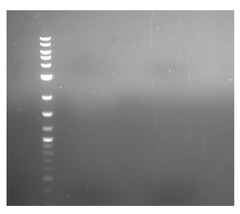
mGST and pCDNA vectors

After gel extraction, the PCR product mGST-tagged H6PD and myc-tagged NNAT were recombined with the RE cut product linearized vectors with complementary sticky ends. Transform the recombination product into the competent DH5α cells and incubate. By clone-checking PCR, appropriate clones with expected DNA length are further inoculated for miniprep and glycerol stock. Sequencing is used to check the target DNA consistency.
In the next step, target gene was transfected into the human embryonic kidney HEK293 cells. Western blot shows that the mGST-tagged H6PD overexpression system has successfully built up.

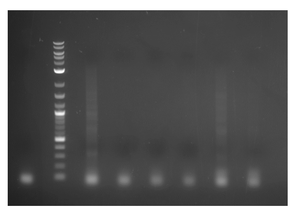


Discussion

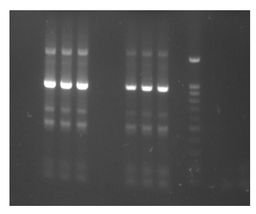
The PCR protocol for mGST-tagged H6PD is relatively matured. However, NNAT-related research is quite limited, which leading to the difficulty in finding optimal conditions for the amplification of NNAT. We tried to increase the template cDNA concentration from 7ng per 50µl reaction to 150ng per 50µl reaction. DMSO was also added into the master mix, but no expected band showing. Annealing temperature was also considered to be a possible influence factor. Control experiments shows that 48℃ and 53℃ give possibilities for successfully PCR while higher temperatures would fail. Then we assumed the low expression of NNAT in hepatocytes was the key element in solving the problem. Hence, to increase the amplification amount of NNAT, we did a second PCR by using 5 µl out of the total 50µl first round PCR product as DNA template. Together with the other adjusted elements, NNAT was finally successfully amplified.



Low DNA concentration



Higher concentration with 48/53/58℃

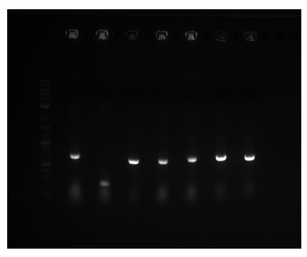


NNAT with 2nd PCR cycle

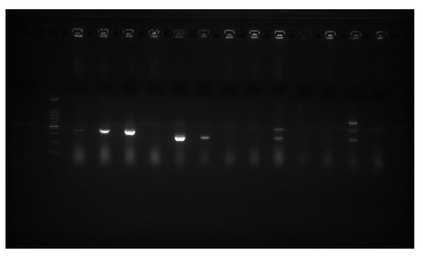
Simultaneously, the explored 50℃ annealing temperature and DMSO condition was also applied for H6PD to harvest a significant amplification product.

In the transformation step, no clones shown on the Ampicillin-containing plate for the first several trial. We first suspected the accuracy of PCR products, so another set of primer with longer complementary sequence was ordered. To find out whether it was the operation mistake or not, we tried a previous successful PCR product together with the target amplification product with identical set of vectors and competent cells. No clone shown in neither group. As a result, we were conscious of the efficiency of competent cell DH5α. After several times of re-preparation, we tripled the competency from 2×10^5 cfu/µg into 6×10^5 cfu/µg. Using the newly prepared competent cells, hundreds of clones was grown.

By the PCR checking, we chose appropriate clones with expected DNA length, and extracted the target fragment for further sequencing. The H6PD shown the same sequence as expected with two set of primer sequencing checking. However, NNAT clones gave rise to a set of totally different sequence. Since sequencing was time-requiring, we firstly used a pair of middle primers inside the NNAT to identify which of them was NNAT instead of gene from contaminated resistant cells. Then only a few clones with clear bands were sent to be sequenced.



NNAT check clone



Check clone with middle primer

However, the sequence we got was a mixture of NNAT isoform 1 and isoform 4, instead of a pure isoform. The reason may lie under the liver RNA extraction and cDNA reverse transcription, but further experiment is still needed.

Western bolt was applied to check the result of recombination gene transfection into HEK293 cells. As shown on the SDS-PAGE, the mGST-tagged H6PD overexpression system was successfully built up. After solving the isoform problem, the myc-tagged NNAT overexpression system will also be built up and checked. In further research, it is feasible to apply GST pull-down assay and immunoprecipitation in the determination of NNAT and HGPD interaction in vitro and in vivo. Eventually, NNAT ablation on hepatic glycolysis can be checked by extracellular flux analysis. Although the experiment is not fully completed yet, the overall result may give rise to identify a novel disease mechanism of the aberrant metabolic changes in diabetic liver.