Correlations between pentose phosphate pathway and DNA replication in human fibroblasts

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DNA replication is one of the most important processes in all living organisms. In contrast to prokaryotic organisms, in Eukaryota, replication is carried out in the nucleus. In addition, the DNA synthesis proceeds much slower. However, it is compensated by the possibility of starting it in many places at the same time. The region where replication begins is called an origin of the replication. In most animals (except mammals), most of the origins of replication are active in the embryonic stage during DNA synthesis. This is to ensure very rapid duplication of the genetic material. On the other hand, very precise duplication of the genetic material is extremely important for the proper functioning of the cell. Replication is a multi-stage process, with many factors involved at all steps. Therefore, it is prone to introduction of different errors in the newly synthesized DNA. Disturbances in the correct course of this process may lead to the development of
a number of diseases, including cancer. Therefore, organisms developed many mechanisms that precisely control the replication and correct most of appearing errors.

DNA replication is an energy-dependent process, and a lot of available energy and pool of free nucleotides are needed to its proper functioning. Both of them might be provided by the central carbon metabolism (CCM). CCM is a set of enzymatic reactions providing energy (ATP, NADPH, FADH) and precursors of amino acids and nucleotides. The main source of the energy is glucose. The CCM consists of several pathways: glycolysis, the Krebs cycle, gluconeogenesis, and the pentose phosphate pathway. Abnormalities in the proceeding of any of the CCM pathways can cause problems in the functioning of the cell, including the DNA replication process.

Recent studies of the role of metabolic enzymes suggested that some of them may participate in the regulation of the replication process and affect the cell cycle in bacteria and yeasts.

The pentose-phosphate pathway (PPP) is an one of the metabolic pathways of the central carbon metabolism. This pathway includes a series of reactions leading to the oxidation of hexoses to pentoses. It consists of two phases: the oxidative phase - consisting of three irreversible reactions, and a non-oxidative phase - a series of reversible transformations catalyzed by allosteric enzymes. In the oxidative phase, glucose-6-phosphate dehydrogenase (G6PD) converts glucose-6-phosphate to NADPH and
6-phosphogluconolactone, which is then hydrolyzed by phosphogluconolactonase (PGLS) to 6-phosphogluconate (6PG). Two first steps can be also catalyzed by hexose-6-phosphate

dehydrogenase (H6PD). Next, 6PG is converted to NADPH and ribulose-5-phosphate (Ru5P) by 6-phosphogluconodehydrogenase (PGD), and then it is moved into the non-oxidative phase. Initially, ribulose-5-phosphate isomerase (RPI) and ribulose-5-phosphate epimerase (RPE) transform ribulose-5-phosphate to ribose-5-phosphate (R5P) and xylulose-5-phosphate (Xu5P), respectively. Then, depending on cell's needs, R5P can be transformed into nucleotide precursors. In this case, enzymes such as ribulose
5-phosphate pyrophosphokinase (PRPS), ribokinase (RBKS) and phosphoglucomutase (PGM) take action, which transform them to pyrophosphoryl pyrophosphate (PRPP),
D-ribose (D-Rib) and D-ribose1-phosphate (D-Rib-1P), respectively. In addition, R5P with Xu5P can be transformed by transketolase (TKT) to glyceraldehyde 3-phosphate (G3P) and sedoheptulose-7-phosphate (S7P). Then, transaldolase (*TALDO*) transforms S7P and G3P to fructose-6-phosphate (F6P) and erythrose-4-phosphate (E4P). G3P with acetaldehyde can be converted by deoxyribose-phosphate aldolase (DERA) to 2-deoxy-D-ribose-5-phosphate (2d-D-Rib-5P). Finally, RBKS and PGM transform 2d-D-Rib-5P into 2-deoxy-D-ribose (2d-D-Rib) and 2deoxy-D-ribose-1-phosphate (*2d-DRib-1P*).
If the cell is active and its demand for NAPDH increases, an elevated concentration of $NADP^+$ accelerates the rate of the cycle.

It has been observed that in prokaryotic cells, DNA replication depends on the availability of nutrients. Induced starvation inhibits the initiation of replication in *Escherichia coli* cells and elongation at specific sites in *Bacillus subtilis* chromosome. The rate of replication is influenced not only by the lack of nucleotides and energy. As it was shown in studies on the bacterial model, the efficiency of the replication process varies depending on the presence or absence of specific metabolic enzymes. Negative effects of the mutations in replication genes can be suppressed by introducing additional deletions in specific genes encoding metabolic enzymes. Similar relationships were also observed in other organisms, including yeasts.

The first aim of my work was the bioinformatic analysis of the possibility of linking the pentose phosphate pathway with the regulation of DNA replication (Konieczna et al. 2015). My next task was to check whether the silencing of the expression of individual metabolic genes of the pentose phosphate pathway would significantly affect the DNA replication process in eukaryotic cells. I performed all the planned experiments (being a part of my doctoral dissertation) employing human fibroblasts from a healthy adult person (HDFa). This is important because most of previous studies have been carried out on bacterial or cancer cells. In the latter case, as we know, the processes of metabolism and replication are often severely disturbed. In the next step, I examined the effect of double silencing of gene expression (both metabolic and replication genes) on the

efficiency of DNA replication and cell division.

I began all planned experiments by silencing the expression of previously selected genes in the examined cells. I did this with commercially available siRNAs. These are double-stranded RNA fragments that, when introduced into a eukaryotic cell, due to the presence of a suitable RNase in the cytoplasm, are cleaved into short single-stranded RNA segments, i.e. real siRNAs. In this form, they are transported to the nucleus, where their binding to homologous regions in the newly formed mRNA leads to its degradation. For the analysis of the cell cycle, it was necessary to synchronize the cells. Because the synchronization by chemical reagent often causes additional effects, e.g. influences processes like DNA replication or metabolic pathways, I needed to use techniques without using any of them. Finally, I used decreasing concentrations of bovine serum in the medium. As a result, the cells were arrested in the G0/G1 phase of the cell cycle. Then,

I determined the level of mRNA in the examined cells. I performed RNA isolation and reverse transcription reaction using commercially available kits. I checked the level of gene expression silencing using real-time PCR. To estimate the expression level of silenced genes, I compared their activities to the reference genes and in relation to non-silenced genes. The expression was silenced by about 70-85%, with the exception of the *G6PD* and *RBKS* genes (about 45-55%) (Fornalewicz et al., 2017). Then,

I performed a cell cycle analysis using the Muse Cell Analyzer. I determined the impact of the applied methods on the conditions of the cells by examining their number and viability with the Muse Count and Viability Assay Kit. Additionally, I determined the rate of cell proliferation by using the BrdU test, and Cell Proliferation ELISA which allows quantification of the level of DNA synthesis. Finally, I checked the level of PPP enzymes after silencing by Western blotting analysis (Fornalewicz et al., 2017). In the light of obtained results, I proved that the presence of the siRNAs and transfection reagent did not negatively affect the number of cells and their condition.

Under conditions of significant reduction of the expression of *H6PD, PRPS1* and *RPE* genes, I observed a decrease in the level of DNA synthesis and the number of cells entering the S phase of the cell cycle. In contrast, silencing of the expression of *G6PD, RBKS* and *TALDO* genes increased the number of cells entering the S phase of the cell cycle and significantly increased the level of DNA synthesis, with the exception of *RBKS* (no effect). Decreasing the expression of the *DERA* gene caused a delay in the entry of cells into the S phase of the cell cycle. The silencing of *PGLS, RPIA* and *TKT* gene expression did not cause any changes in the cell cycle progression (Fornalewicz et al., 2017).

In the next stage of my research, I examined the effects of silencing of DNA replication/repair genes coding for primase 2 (the *PRIM2* gene), DNA polymerase iota (the *POLI*

gene), ligase 4 (the *LIG4* gene), and topoisomerase IIIβ (the *TOP3B* gene). Silencing of the expression of the replication/repair genes caused a delay of the entry of cells into the S phase and significantly reduced the number of cells entering this phase (results obtained together with Aneta Wieczorek).

In the light of the results of my studies, as well as previously published data suggesting the existence of direct links between metabolic enzymes and DNA replication, I decided to check whether decreased expression of specific genes encoding PPP pathway enzymes could suppress the negative effects of silencing of the expression of the DNA replication/repair genes. For this purpose, I carried out analogous tests as described above. In the case of double silencing of the expression of metabolic genes (PPP pathway) and replication/repair genes: *POLI, PRIM2, TOP3B, LIG4*, I received the following results: the negative effects of the decreased *TOP3B* expression is abolished by the silencing of the expression of single metabolic genes, such as *RBKS, TKT* or *TALDO*. Interestingly, the silencing of the *RBKS* gene expression was the only one that suppresses the negative effects associated with the silencing of the *PRIM* gene. Moreover, the negative effects of silencing of the expression of *POLI* and *LIG* genes have been partly eliminated by additional reduction in the level of expression of *H6PD, RPIA, RBKS* and *TALDO* genes. In addition, the effects of impairing the expression of the *POLI* gene were partially compensated by reducing the expression level of *G6PD, PGLS, RPE* and *TKT* (Wieczorek et al., 2018).

In summary, in the light of the results of my studies and recently published data, it can be concluded that specific metabolic enzymes are involved in the regulation of the cell cycle and the DNA replication process. Such conclusion is supported by the fact that decreased expression of specific metabolic genes suppressed the negative effects of reduction of expression of individual genes coding for DNA replication/repair proteins, and also significantly affected the process of DNA synthesis in these cells. However, based on the data presented above, it is impossible to determine the exact mechanisms of the observed phenomenon. One can assume that due to the presence of certain enzymes in the cell nucleus, they may be directly involved in the regulation of the replication process through protein-protein interactions. It is also likely that they affect the rate of cell division by disturbing the proportion of available nucleotides (some of the enzymes participate in the synthesis of their precursors) or the energy balance in the cell. Therefore, in order to be able to answer these questions unambiguously, further analysis will be necessary.