**Influence of the reporter vector backbone on 2-deoxyglucose dependent promoter activation**

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**INTRODUCTION**

Reporter vectors are very often used as tools to investigate gene expression and intracellular reactions related to transcriptional activity in cells. Typically, a reporter gene is controlled by the examined promoter and after transfection of cells, the amount of a reporter protein, its enzymatic activity, or the amount of mRNA of a reporter gene is assessed. The ideal reporter system is characterized by high sensitivity and reproducibility. Importantly, the reporter protein is not endogenously expressed in the recipient cells. This feature enables the use of the reporter protein for monitoring for transfection efficiency in response to inhibition of glycolysis.

**Key words:** reporter vector, gene expression, metalloproteinase-2, 2-deoxyglucose

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Abbreviations: 2-DG, 2-deoxyglucose; MMP, metalloproteinase; IER, immediate-early response protein

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Reporter vectors are also used in studies focusing on the influence of metabolism on regulation of gene expression. Metabolites, such as acetyl-CoA, α-ketoglutarate, β-hydroxybutyrate, butyrate, crotonyl-CoA, FAD, fumarate, 2-hydroxyglutarate, nicotinamide, nicotinamide mononucleotide, S-adenosyl-methionine, and succinate are involved in chromatin remodeling (van der Knaap & Verrijzer, 2016). One of the interesting issues concerning interaction between the pattern of gene expression and cell physiology, is the influence of changes in the glucose metabolism on activation/repression of promoters. Glucose is metabolized via glycolysis to pyruvate, which can be metabolized to CO₂ in the tricarboxylic acid (TCA) cycle to generate large amounts of ATP through the process of oxidative phosphorylation (OXPHOS). Pyruvate can be also reduced to organic acids (e.g., lactate) in a process called fermentation (Lunt & Vander Heiden, 2011). Fast-growing cells primarily rely on glucose fermentation even under oxygen-rich conditions. This phenomenon was described almost 100 years ago by Otto Warburg and is called the "Warburg effect." Warburg observed that cancer cells rather utilize a high rate of glycolysis followed by lactic acid fermentation in the cytosol, than a low rate of glycolysis followed by OXPHOS in mitochondria, regardless of the oxygen availability. Aerobic glycolysis is not caused by a defect in mitochondrial respiration since respiration is not impaired in most cancer cells (Zu & Guppy, 2004; Fantin et al., 2006; Moreno-Sánchez...
et al., 2007). It is now well understood that aerobic glycolysis is an adaptation to facilitate uptake and incorporation of nutrients into the biomass needed by fast-growing or proliferating cells (Vander Heiden & DeBerardinis, 2017). Proliferating cells produce lactate to regenerate NAD⁺, however, lactate generation may have secondary benefits for tumor cells. The resulting acidification of the extracellular microenvironment promotes survival, growth and invasive behavior of cancer cells via suppression of anti-cancer T-cell immune response and upregulation of metalloproteinase activity (Fischer et al., 2007; Gilles & Gatenby, 2007; Gatenby & Gillies, 2008; López-Lázaro, 2008). Matrix metalloproteinases (MMPs) are enzymes capable of extracellular matrix degradation that are overexpressed during metastasis (Hanahan & Weinberg, 2011). Under physiological conditions, they play an important role in wound healing, embryonic growth and development, morphogenesis, regulation of the inflammatory response, angiogenesis, and apoptosis. Under pathological conditions, such as rheumatoid arthritis, cardiovascular diseases or tumors, MMPs are overexpressed and activated (Fanjul-Fernández et al., 2010; Kessenbrock, Wang, & Werb, 2015). Matrix metalloproteinase 2 (MMP-2) belongs to a group of gelatinases. It is constitutively expressed in different cell types and has a broad spectrum of substrates, including gelatin, collagen, elastin, pro-TNFα (tumor necrosis factor type alpha) and TGFβ (tumor growth factor type β) (Fanjul-Fernández et al., 2010; Kessenbrock et al., 2015). MMP-2 has been associated with progression of many cancers, including stomach, esophagus, breast, prostate, lungs, bladder, and ovaries cancer. It is particularly important for the invasiveness of gliomas (Ramachandran et al., 2017). Since inhibition of glycolysis is considered as one of the strategies for the treatment of gliomas (Wicks et al., 2015), we decided to study the influence of metabolic changes on expression of MMP-2 in a human glioblastoma cell line.

During our studies, we have discovered that the type of the chosen reporter vector greatly impacts the obtained results. The pattern of promoter activation regulation through cellular metabolism depends on the type of the reporter vector selected for the study.

**MATERIALS AND METHODS**

**Cell culture.** Human astrocytoma U-251 MG cell line was cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 g/L D-glucose (BioWest) supplemented with 10% (v/v) Fetal Bovine Serum (BioWest), at 37°C in a humidified atmosphere with 5% CO₂.

**RNA isolation and reverse transcription.** Total RNA was isolated from U-251 MG cells using the modified Chomczynski method (Chomczynski & Sacchi, 1987). RNA concentration was measured using the NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific). cDNA was obtained from 1 μg of RNA in a reverse transcription reaction using MML-V Reverse Transcriptase (Promega) and 500 ng of oligo(dT)₁₅ (Genomed) according to the manufacturer’s protocol.

**Real-Time PCR.** Real-time PCR was performed using SYBR A RT HS-PCR Mix (A&A Biotechnology) and primers specific to the analyzed transcripts (Genomed):

- **MMP-2-forward:** GTGACGGAAAGATGTTGCTG
- **MMP-2-reverse:** GGTGTAAGTTGTAATAGGTTGA
- **IER3-forward:** GCCGTCCGGTCTGAGATCTCTCA
- **IER3-reverse:** GAGCCCTTTTGGTGTTGGTTCGT
- **TBP-forward:** GCCAGCTTGAGGAGAGATGTGTTGA
- **TBP-reverse:** GCAGCTCAGAATGCAGATGGTCTTTA

mRNA level in each sample was analyzed in duplicate, and results were normalized to the reference gene (TBP). The relative level of transcripts was calculated using the ΔΔCₚ method (Pfaffl, 2001).

**Gelatin zymography.** U-251 MG cells were seeded in 24-well plates at a density of 5×10⁴ cells/well. The next day, the culture medium was exchanged to FBS-depleted medium and 2-deoxy-D-glucose (Cayman Chemical) was added (12.5 mM) for 24 hours. After that, media were collected, cellular debris was removed by centrifugation and samples for SDS-PAGE were prepared by addition of the loading buffer (5×: 312.5 mM Tris-HCl, 50% (v/v) glycerol, 10% (w/v) SDS, 0.05% (v/v) bromophenol blue, pH=6.8) to the prepared media. The electrophoresis was carried out in a Laemmli system (Laemmli, 1970) (5% stacking gel, 10% separation gel with 0.1% (w/v) gelatin). After electrophoresis, the gel was washed and incubated overnight in 2.5% (v/v) Triton X-100. The following day, the gel was washed and incubated for 48 hours at 37°C with incubation buffer (10 mM CaCl₂, 0.15 M NaCl, 50 mM TRIS, pH 7.5, 0.01% (v/v) NaN₃). Next, the gel was washed with distilled water and stained with a Coomassie Brilliant Blue solution (0.1% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid). The staining solution was removed and the gel was left in the destaining solution (40% (v/v) methanol, 10% (v/v) acetic acid) until discolored bands were observed. The image was recorded using the Fusion FX5 system (Vilber Lourmat).

**Cell migration assay, time-lapse video microscopy.** U-251 MG cells were seeded in 24-well plates at the density of 1.5×10⁴ cells/well. The following day, the cells were treated with 2-deoxyglucose (12.5 mM) for 24 hours. After that, the cell movement was recorded with a Leica DMi6000B time-lapse system equipped with interference modulation contrast optics, a cooled, digital DFC360FX CCD camera and a chamber maintaining temperature and the CO₂ level. Cell trajectories were constructed from the sequence of cell centroid positions recorded at 5-minute intervals for 8 hours. The distance from the starting point directly to the cell’s final position (i.e. displacement) were quantified with the Hiro program (Baran et al., 2009).

**Cell transfection.** U-251 MG cells were seeded in 24-well plates at a density of 5 x 10⁴ cells/well. The next day, the cells were transfected with reporter vectors using PEI MAX 40000 (Polysciences) reagent at a reagent to DNA weight ratio of 3:1. A total amount of 500 ng of the luciferase encoding plasmid and 5 ng of pEF/myc-His/LacZ (control) vector. The medium was exchanged 4 hours post-transfection. The efficiency of transfection was around 60–70% in all experiments.

**Construct generation.** The pGL3-MMP-2 construct was prepared by cloning the MMP-2 (NG_0089891) promoter region from position –3000 to +110. PCR product was prepared from genomic DNA (gDNA) using Hot Start Q5 polymerase (NEB). The following primers were used:

- **forward:** TCGACCTCAGTTTCCATGTGTCACACCCACACCAGC
- **reverse:** ATAAAAGCTTGTTCCAGTAACCTTGTG

PCR products were separated by gel electrophoresis, excised, purified (Gel/PCR ME Mini Kit, Syngen), cleaved with Nhel and HindIII (NEB) restriction enzymes and purified after enzymatic reaction completion. Thus obtained PCR product was inserted into pG3L-Basic plasmid linearized with Nhel and HindIII restriction enzymes and ligated using T4 DNA ligase (ThermoFisher). DELI
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MMP-2 promoter variants were prepared as described above with primers listed in Table 1. DEL1 was prepared from gDNA, DEL3 to DEL6 from pGL3-MMP-2-DEL1. pGL3-MMP-2-DEL2 was prepared by cleaving pGL3-MMP-2-DEL1 with EcoRI and SacI (NEB) restriction enzymes. Cleavage products were separated by gel electrophoresis, excised and purified, blunted with T4 polymerase (NEB) and ligated with T4 DNA ligase. pGL3-MMP-2-DEL7 was generated using Q5 Site-Directed Mutagenesis Kit (NEB) according to the manufacturer’s protocol, pGL3-MMP-2 was used as a template and specific primers were as follows: forward: CCCCCGTGTTTCCGCTGCAT, reverse: ACCCCACTTGCCTCTCTC.

Table 1. Primer pairs used to obtain deletion variants of the MMP-2 promoter.

| Promoter variant | Primers |
|------------------|---------|
| DEL1             | Forward: ATAGCTAGCAAACTGACTCTGGAAAGTCAGAGCAC, Reverse: ATAAAGCTTCAGGTCCTGGCAATCCCTTTG |
| DEL3             | Forward: ATAGCTAGCGAAGCAACCTGGGACCTTCC, Reverse: ATAAAGCTTCAGGTCCTGGCAATCCCTTTG |
| DEL4             | Forward: ATAGCTAGCGACGAGGTCGTGCACTGAG, Reverse: ATAAAGCTTCAGGTCCTGGCAATCCCTTTG |
| DEL5             | Forward: ATAGCTAGCGACGAGGTCGTGCACTGAG, Reverse: ATAAAGCTTAGGGCAGCCGCCAGACTTAG |
| DEL6             | Forward: ATAGCTAGCGACGAGGTCGTGCACTGAG, Reverse: ATAAAGCTTAGGGCAGCCGCCAGACTTAG |

and DEL3 to DEL6 MMP-2 promoter variants were prepared as described above with primers listed in Table 1. DEL1 was prepared from gDNA, DEL3 to DEL6 from pGL3-MMP-2-DEL1.

pGL3-MMP-2-DEL2 was prepared by cleaving pGL3-MMP-2-DEL1 with EcoRI and SacI (NEB) restriction enzymes. Cleavage products were separated by gel electrophoresis, excised and purified, blunted with T4 polymerase (NEB) and ligated with T4 DNA ligase. pGL3-MMP-2-DEL7 was generated using Q5 Site-Directed Mutagenesis Kit (NEB) according to the manufacturer’s protocol, pGL3-MMP-2 was used as a template and specific primers were as follows: forward: CCCCCGTGTTTCCGCTGCAT, reverse: ACCCCACTTGCCTCTCTC.

pGL2-MMP-2 and pGL3-IER3 were obtained by cleavage of pGL3-MMP-2 and pGL2-IER3 (described previously, Kochan et al., 2014) plasmids with KpnI and HindIII (NEB) restriction enzymes. After gel electrophoresis, the products were excised and purified. Then, plasmid backbones were dephosphorylated using rSAP (NEB) according to the manufacturer’s protocol. MMP-2 promoter and IER3 promoter were ligated with linearized and dephosphorylated pGL2 and pGL3 plasmids, respectively.

Obtained constructs were verified by restriction analysis and Sanger sequencing (Genomed).

**Analysis of luciferase activity.** After transfection and 2-DG treatment, the cells were lysed with Passive Lysis Buffer (Promega). The activity of Firefly luciferase was assessed using the Luciferase Assay System (Promega). The activity of β-galactosidase expressed from the pEF/myc-His/LacZ vector was measured using the Beta-Glo Assay System (Promega) and served as control of transfection efficiency.

**Sequence analysis.** Prediction of transcription factor binding sites was done using the AliBaba2.1 software available online [http://gene-regulation.com/pub/programs/alibaba2/] and LASAGNA-Search 2.0 (Lee & Huang, 2013) [https://biogrid-lasagna.engr.uconn.edu/].

**Statistics and graphs.** Statistical analysis and graphs were done using Graph Pad Prism (v. 5.0, GraphPad Software Inc.). Statistical analysis was performed using the following tests: One-way ANOVA with Tukey’s multiple comparison (Fig. 1A) and paired t-test (other figures), *p<0.05, **p<0.01, ***p<0.001. Schemes of MMP-2

**Figure 1.** Influence of glycolysis inhibition on expression of MMP-2 (A, B) and cell motility (C). U-251 MG cells were treated with 2-DG (12.5 mM) for 24 h or 48 h. A. RNA was isolated from cell lysates, then the mRNA level was assessed using RT-PCR; One-way ANOVA with Tukey’s multiple comparison test was used. The plot shows mean results from three independent experiments (n=3) ± S.D., **p<0.01, ***p<0.001. B. Culture medium from control and 2-DG treated cells was collected and put into analysis using gelatin zymography. White bands indicate the proteolytic activity of MMPs. C. Cell motility was visualized by time-lapse microscopy and analyzed with the Hiro program (written by W. Czapla). The plot shows mean results from three independent experiments (n=3) ± S.D., *p<0.05. The paired t-test was used. In each experiment, trajectories of 50 cells were analyzed.
RESULTS

Inhibition of glycolysis affects the MMP-2 mRNA level and activity, as well as cell motility

2-deoxy-D-glucose (2-DG) is a glucose analog that acts as an inhibitor of glucose metabolism. It enters cells via GLUT1 transporters and is phosphorylated by hexokinase to 2-DG-P. 2-DG-P cannot be further metabolized and blocks glycolysis (Pelicano et al., 2006). Using 2-DG we examined the influence of the inhibition of glycolysis on the MMP-2 mRNA level in the U251-MG astrocytoma cell line. The results indicate that 2-DG treatment leads to a decrease in the level of MMP-2 transcript (Fig. 1A). To verify the obtained results we examined the activity of the matrix metalloproteinases by zymography (Fig. 1B). This method allows for assessing the proteolytic activity of MMPs in a polyacrylamide gel containing gelatin which is a substrate for MMP-2 and MMP-9. Distinction between these two proteins is possible due to differences in their molecular weights. Changes in metabolism induced by the 2-DG treatment resulted in the decreased activity of both detected MMPs. It is well documented that inhibition of MMP-2 or MMP-9 leads to a decrease in cell migration (Webb et al., 2017). To confirm that the observed changes in the activity and mRNA level of MMP-2 have a physiological effect, we applied a time-lapse video microscopy. The obtained results indicate that cells pretreated with 2-DG show lower ability to displace the 2-DG treatment in pGL3-based vector series. A. Scheme of the pGL3-based reporter vector constructs containing the firefly luciferase gene under the control of various fragments of MMP-2. B. Response of the constructs shown in (A) to 2-DG. U251-MG cells were transfected with plasmids containing the reporter gene under MMP-2 promoter control. 24 h after transfection, the cells were treated with 2-DG (12.5 mM) or left untreated for 24 h. The following day, the cells were lysed and luciferase and β-galactosidase activity were measured. The plot shows mean results from three independent experiments (n=3) ± S.D., *p<0.05, **p<0.01, ***p<0.001. The paired t-test was used.

Analysis of MMP-2 promoter activity after 2-DG treatment

Changes observed at the mRNA level may result from alterations in the promoter activity or mRNA half-life. We decided to start with the examination of MMP-2 promoter activity following 2-DG treatment. For this purpose, we prepared a series of reporter vectors containing the Firefly luciferase gene under control of different deletion variants of the MMP-2 promoter (Fig. 2A). U251-MG cells were transiently transfected with the plasmids generated, and treated with 2-DG. Individual vectors (DEL1 – DEL6) that were used for transfection contain deletions of increasingly larger fragments of the promoter sequence, however, treatment of the cells with 2-DG led to a similar, significant decrease in luciferase activity for each of the used constructs. Based on the obtained results, we selected a potential regulatory region of the MMP-2 promoter (~192 to +18 bp) responsible for the regulation in response to inhibition of glycolysis. Importantly, this region contains a consensus binding site for the SP1 transcription factor, which is necessary for constitutive expression of MMP-2 in human glioblastoma cells (Qin, Sun, & Benveniste, 1999). To verify the importance of this region in 2-DG-dependent regulation of MMP-2 expression, we removed it from the wild type promoter, thus creating the DEL7 construct. However, MMP-2 promoter without the investigated fragment (~192 to +18 bp) still responded to 2-DG in a similar way as the wild-type promoter (Fig. 2B). Since further shortening of the MMP-2 promoter could lead to the removal of the minimal promoter region, we decided to discontinue this approach.

Analysis of MMP-2 promoter activity in response to glycolysis inhibition using different types of reporter vectors

Due to the failure to identify regulatory elements in the MMP-2 promoter responsible for the observed 2-DG-dependent inhibition, we decided to examine the response to 2-DG treatment of the vector itself. To do this, we re-cloned the wild type MMP-2 promoter into another reporter vector – pGL2-Basic, thus obtaining pGL2-MMP-2 vector. In contrast to the results obtained for the pGL3-MMP-2 vector, there were no significant differences in luciferase activity measured in the lysates from cells transfected with the pGL2-MMP-2 vector after 2-DG treatment, in comparison to the control (untreated) cells (Fig. 3A). To further investigate the influence of the vector itself on the 2-DG-dependent promoter activity, we employed two constructs containing the immediate early response 3 (IER3) promoter, namely pGL2-IER3 and pGL3-IER3. Since IER3 is not down-regulated by 2-DG (Fig. 3B), both vectors should not respond to 2-DG treatment, similarly to the pGL2-MMP2 and pGL3-MMP2 vectors. However, the luciferase activity in the cells transfected with the pGL3-IER3 construct was significantly decreased after 2-DG treatment, mimicking the pattern of regulation observed for the MMP-2 promoter (pGL3-MMP2 construct). Simultaneously, we didn’t observe such a decrease in luciferase ac-
tivity in lysates from cells transfected with pGL2–IER3 (Fig. 3A). The obtained results clearly indicate that the originally observed down-regulation of the MMP-2 promoter activity in response to inhibition of glycolysis by 2-DG was an effect of the response of the regulatory elements present in the originally used pGL3–Basic vector backbone rather than the elements present in the MMP-2 promoter itself.

DISCUSSION

Our study reveals the importance of the type of reporter vector used in research focused on investigation of the effect of metabolic changes on promoter activation. We have analyzed the outcome of the use of the pGL3 and pGL2 vectors for such studies. The pGL3 vector is one of Promega’s next generation expression vectors. In comparison to pGL2, its backbone has been altered to increase the luciferase expression (Promega Corporation, 2008a, 2008b). Four major modifications of the vector backbone were introduced: 1) the early poly(A) SV40 signal was replaced with a late signal to increase transcription termination efficiency and polyadenylation of luciferase transcripts (Carswell & Alwine, 1989); 2) synthetic poly(A) and a transcription pause site were placed above MCS to limit non-specific transcription that can be initiated within the vector backbone (Enriquez-Harris et al., 1991; Levitt et al., 1989); 3) the small T antigen intron has been removed to prevent a decreased expression of the reporter gene due to cryptic RNA splicing (Evans & Scarpulla, 1989; Huang & Gorman, 1990); 4) optimal Kozak consensus sequence was inserted to increase the efficiency of luciferase gene translation initiation (Kozak, 1989). In addition, the pGL3 vector carries a modified gene encoding Firefly luciferase (luc +). The main modifications that distinguish the luc + gene from the native luciferase gene include: 1) removal of the C-terminal tripeptide to eliminate peroxisome targeting; 2) improving the use of codons for expression in plant and animal cells; 3) removal of two potential N-glycosylation sites; 4) DNA sequence changes to remove internal restriction sites and eliminate consensus sequences recognized by regulatory proteins (Promega Corporation, 2008b; Sherf & Wood, 1994). Bioinformatics prediction of transcription binding sites using AliBaba2.1 software showed that the pGL3–Basic plasmid contains 370 recognition sites for transcription factors, whereas there are 446 such sites in the pGL2–Basic sequence.

Our work is part of a series of studies on the effect of metabolism on gene expression regulation. In 2013, Chang et al. showed that aerobic glycolysis is engaged in posttranscriptional regulation of specific cellular functions. T cells fully supported by oxidative phosphorylation have a severe defect in IFN-γ (interferon γ) production. This effect is due to a block in translation (Chang et al., 2013). Independently, in the same year, Tannahill et al. described the influence of glycolysis on expression of interleukin 1β (IL-1β) in mouse macrophages. Inhibition of glycolysis results in suppression of LPS (lipopolysaccharide) induced transcription of the IL-1β gene. This effect is caused by changes in the stability of the transcription factor HIF-1α (hypoxia-inducible factor 1α) which participates in stimulation of IL-1β expression. Expression of TNF in investigated cells was not regulated by glycolysis (Tannahill et al., 2013).

In this study, we made an attempt to identify the mechanisms underlying downregulation of MMP-2 expression after glycolysis inhibition. Originally, our results suggested that the observed regulation of MMP-2 by 2-DG occurred at the promoter level. However, despite preparation of a series of promoter deletion variants, we were not able to identify a sequence that could play a significant role in response to the 2-deoxyglucose treatment. All constructs, containing wild type and deletion variants of the MMP-2 promoter, and generated using the pGL3 vector as a backbone were downregulated in response to the 2-DG treatment. To verify whether the pGL3–based system is a proper experimental setup, we prepared a construct containing the wild-type MMP-2 promoter using a different reporter vector backbone, the pGL2–Basic vector. Surprisingly, the previously observed 2-DG induced down-regulation was no longer visible in cells transfected with the pGL2–based vector. Moreover, we detected a similar pattern of vector–backbone dependent regulation by 2-DG for the IER3 promoter, while IER3 expression is not down-regulated by this compound. Thus, the originally observed 2-DG-dependent regulation of MMP-2 expression at the promoter level didn’t rely on the sequences present in the investigated promoter but was due to the presence of regulatory elements in the pGL3 plasmid that was used.

Considering the reduction in recognition sites for transcription factors in pGL3–Basic backbone in comparison

![Figure 3A. Influence of the reporter vector backbone on the response of the construct to 2-DG.](image)

![Figure 3B. IER3 mRNA level after inhibition of glycolysis.](image)
to the pGL2-Basic backbone (370 versus 446, respectively), one would expect a lower influence of the plasmid sequence on the investigated promoter activation. However, our data suggest that the use of the pGL3 vector leads to a false decrease in activation of the investigated promoters in response to 2-DG. This study raises the problem of choosing the right model for experiments based on reporter vectors.

The authors declare no conflict of interest.

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