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Enhanced transgene expression from chromatinized plasmid DNA in mouse liver

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Abstract

Plasmid DNA was chromatinized with core histones (H2A, H2B, H3, and H4) *in vitro* and was delivered into mouse liver by hydrodynamics-based administration. Transgene expression from the chromatinized plasmid DNA was more efficient than that from plasmid DNA delivered in the naked form. The use of acetylation-enriched histones isolated from cells treated with a histone deacetylase inhibitor (trichostatin A) seemed to be more effective. These results indicated that chromatinized plasmid DNA is useful for efficient transgene expression *in vivo*.

Keywords: exogenous DNA; histone; nucleosome; intranuclear disposition; expression efficiency

Abbreviations: TSA, trichostatin A; Q-PCR, quantitative PCR; RLU, relative light units; NLS, nuclear localizing signal.

1. Introduction

Plasmid DNA delivered with nonviral vectors binds to histones, which are the principal protein components of chromatin, to form nucleosomes (Hebbar and Archer, 2008; Reeves et al., 1985). The nucleosome core particle consists of ~147 bp of DNA wrapped around a histone octamer, containing two molecules each of H2A, H2B, H3, and H4 (Luger et al., 1997). The binding of histones generally limits the access of transcription factors to their recognition sites. Thus, the regulation of histone binding to the exogenous DNA would be an important factor for transgene expression, and hence for the intranuclear disposition of the plasmid DNA (Kamiya et al., 2003). Indeed, transgene expression was influenced by the introduction of DNA sequences that modulate histone dynamics into plasmid DNAs (Fukunaga et al., 2012; Kamiya et al., 2007, 2009; Nishikawa et al., 2003; Sumida et al., 2006).

Recently, histones have been used as vehicles for plasmid DNAs (Kaouass et al., 2006 and references therein). Moreover, Jans and co-workers reported transfection with reconstituted chromatin, obtained by mixing a histone octamer and plasmid DNA (Wagstaff et al., 2008). These reports suggested that the complexes of histone(s) and plasmid DNA are useful delivery tools. However, we previously showed that simply mixing plasmid DNA with a histone (H3) suppresses transgene expression after nuclear entry (Kamiya et al., 2010). Thus, the report by Jans and co-workers raised the question of whether chromatin-mediated gene delivery is advantageous, from the viewpoint of transgene expression efficiency in the nucleus.

In addition to binding to mammalian chromosomal DNA, various histone modifications, such as acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation, are believed to play a pivotal role in the modulation of chromatin structure and function (“histone code” hypothesis) (Strahl and Allis, 2000). The

acetylation of core histones is a well-characterized modification. Highly acetylated histones are regarded as a hallmark of nucleosomes at active genes, while those at repressed genes are hypoacetylated (Turner, 2000). The acetylation is considered to influence gene expression by modifying the chromatin conformation and/or the recruitment of regulatory factors.

It would be quite interesting to study transgene expression efficiency from plasmid DNA chromatinized *in vitro*, since nucleosome positioning is involved in gene transcription from genomic DNA (Bai and Morozov, 2010) and chromatinized plasmid DNA has been used as a delivery tool (Wagstaff et al., 2008). To examine the efficiency of transgene expression from the plasmid DNA, we delivered plasmid DNA chromatinized with histones into mouse liver. Moreover, plasmid DNA chromatinized with acetylation-enriched histones was also delivered. The results obtained in this study indicated that chromatinized plasmid DNA is useful for efficient transgene expression *in vivo*.

2. Materials and methods

2.1. Materials

Oligodeoxyribonucleotides were purchased from Life Technologies Japan (Tokyo, Japan) in purified forms. The pYK-CMV-luc plasmid DNA (Ochiai et al., 2006), containing the luciferase gene, was purified with a Qiagen (Hilden, Germany) EndoFree Plasmid Mega kit.

2.2. Animals

All mice were maintained in an environment with a controlled temperature and photoperiod (23°C, 12-h light and 12-h dark), with food and water provided *ad libitum*. All experimental procedures followed the Guideline for Animals Experimentation prepared by the Animal Care and Use Committee of Matsuyama University.

2.3. *Extraction and purification of core histones*

Core histones (H2A, H2B, H3, and H4) were extracted from mouse Hepa 1-6 cells and then purified with an Active Motif Histone Purification Mini Kit (Carlsbad, CA). Acetylated histones were obtained from Hepa 1-6 cells treated with trichostatin A (TSA, 200 ng/mL) for 18 h.

The acetylation of histone H3 was confirmed by dot blotting, using an anti-acetyl histone H3 antibody (catalog number 39139; Active Motif) and an anti-histone H3 antibody (catalog number 07-690; Upstate Biotechnology, Lake Placid, NY, USA), and an ECL Western Blotting Starter Kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

The pYK-CMV-luc plasmid DNA (2.5 µg) was chromatinized with the extracted core histones (2.7 µg), using recombinant nucleosome assembly protein-1 (NAP-1) and the ATP-utilizing chromatin assembly and remodeling factor (ACF) complex (Chromatin Assembly Kit, Active Motif), according to the supplier's recommendations.

2.4. *Hydrodynamics-based injection of chromatinized DNA*

The chromatinized plasmid DNA (1.25 µg as DNA) in 2 mL of saline was injected into the tail vein of six week-old female Balb/c mice within 5 s, by

hydrodynamics-based administration (Liu et al., 1999; Zhang et al., 1999). The livers were harvested from the injected mice at 24 h after injection, and the luciferase activity and the amount of the exogenous DNA were measured, as described below.

2.5. *Luciferase activity*

Livers were minced with scissors and homogenized completely in lysis buffer (100 mM Tris-HCl, 2 mM EDTA, 0.1% Triton X-100, pH 7.8). After centrifugation at 16,400 g for 10 min at 4°C, the supernatant was examined for luciferase activity, using a Luciferase Assay System kit (Promega, Madison, WI, USA).

2.6. *Isolation of nuclear DNA and quantitative PCR*

Livers were homogenized in extraction buffer (3 mM Tris-HCl, 0.1 mM EDTA, 250 mM sucrose, pH 7.4). After centrifugation at 800 g for 5 min at 4°C, the pellet was resuspended in DNA lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂, 0.5% (w/v) IGEPAL-CA630, pH 7.4) (Tachibana et al., 2002). After centrifugation at 1,400 g for 5 min at 4°C, the pellet was washed twice with DNA lysis buffer. The intranuclear DNA was extracted with the SepaGene reagent (EIDIA, Tokyo, Japan).

Quantitative PCR (Q-PCR) was performed using a Bio-Rad CFX96 Real Time PCR System (Hercules, CA, USA), and EvaGreen chemistry (Bio-Rad). A portion of the recovered DNA was analyzed by Q-PCR. The *luciferase* gene in the mouse liver was detected using the following primers: Luciferase upper, 5'-dGGTCCTATGATTATGTCCGGTTATG-3'; Luciferase lower, 5'-dATGTAGCCATCCATCCTTGTC AAT-3'. Data were expressed as the ratio to the *Gapdh* gene, which was determined using the following primers: Gapdh upper,

5'-dTGTGATGGGTGTGAACCAC-3'; Gapdh lower,
5'-dGTTGTCATGGATGACCTTGG-3'.

2.7. Statistical analysis

Statistical significance was examined by the Student's *t*-test. Levels of $P < 0.05$ were considered to be significant.

3. Results

3.1. Purification of core histones and nucleosome formation

We first isolated the core histones (H2A, H2B, H3, and H4) that constitute the nucleosome core particle, together with 147-bp of DNA, from mouse Hepa 1-6 cells. Histone acetylation is regulated by two families of evolutionarily conserved enzymes, histone acetyltransferases and histone deacetylases (Khochbin et al., 2001; Yang and Seto, 2007). We isolated acetylated histones from the same cell line after an 18 h treatment with a histone deacetylase inhibitor, TSA, for the chromatinization reactions. These conditions were determined by the ratio of acetylated histone H3 to H3 (Fig. 1A) and the cell viability (data not shown). We confirmed that the core histones isolated from the TSA-treated cells contained acetylated histone H3 (Fig. 1B).

We chromatinized the luciferase-plasmid DNA (pYK-CMV-luc, 8454 bp) (Ochiai et al., 2006), using a commercially available kit. Nucleosome assembly protein-1 (NAP-1) (Nakagawa et al., 2001), a histone chaperone, was incubated with the isolated core histones. The plasmid DNA was chromatinized by mixing the histone-NAP-1 solution with ATP-utilizing chromatin assembly and remodeling factor

(ACF) (Nakagawa et al., 2001). Successful chromatinization was confirmed by partial nuclease digestion, followed by agarose gel electrophoresis. DNA fragment ladders indicate formation of nucleosomes (Fig. 1C). No difference was observed for plasmid DNAs chromatinized with control and acetylated core histones (lanes 2 and 5).

3.2. *Luciferase expression from chromatinized plasmid DNA*

We delivered the chromatinized plasmid DNA (1.25 μ g as DNA) to the livers of female six week-old Balb/c mice by hydrodynamics-based administration (Liu et al., 1999; Zhang et al., 1999). The livers were harvested at 24 h after injection, and the luciferase activities and the amounts of the exogenous DNAs were quantitated.

As shown in Fig. 2, the luciferase expression from the chromatinized plasmid DNA was higher than that from the plasmid DNA delivered in the naked form (3.3×10^7 relative light units (RLU)/mg protein versus 1.5×10^7 RLU/mg protein) with statistical significance ($P < 0.05$). Meanwhile, the injection of a plasmid DNA solution containing the core histones without the chromatinization reactions did not affect the expression (data not shown). Therefore, the *in vitro* chromatinization of the plasmid DNA increased the transgene expression in the mouse liver.

When the plasmid DNA was chromatinized with acetylated core histones and delivered, the luciferase expression was further increased (3.9×10^7 RLU/mg protein). This luciferase activity was significantly higher than that for the plasmid DNA delivered in the naked form ($P < 0.01$). However, the difference between the luciferase activities from plasmid DNAs chromatinized with histones isolated from TSA-treated and -untreated cells was statistically insignificant ($P=0.38$).

3.3. *Expression efficiency from chromatinized plasmid DNA*

We then measured the amounts of the exogenous luciferase DNA by Q-PCR (Fig. 3A). The amount of the DNA appeared to be slightly higher in the case of the chromatinized plasmid DNAs. We divided the luciferase activities by the amounts of the exogenous DNAs. The calculated values were considered to indicate the amounts of the luciferase protein expressed from one copy of the transgene (expression efficiencies). As shown in Fig. 3B, the expression efficiency seemed to be slightly higher in the case of the plasmid DNAs chromatinized with the acetylated core histones. These results suggested that the enhanced transgene expression is due to the effects of the chromatinization, the increased amount of plasmid DNA, and the enhanced transgene expression efficiency.

4. Discussion

In this study, we examined the effect of *in vitro* chromatinization of plasmid DNA on transgene expression. The chromatinized plasmid DNA exhibited higher transgene expression than the naked plasmid DNA in mouse liver (Fig. 2). The enhanced expression can be attributed to increases in the amount of plasmid DNA and the expression efficiency (Fig. 3). Similar results were obtained with the use of core histones isolated from HeLa cells, instead of those from Hepa 1-6 cells (Supplementary Fig. 1).

Many proteins with endo- and/or exonuclease activity exist in the nucleus, as well as the cytosol and serum. The formation of nucleosomes on plasmid DNA could protect it from nucleases in serum and hepatic cells, and this could be a reason for the increased amount of plasmid DNA. Alternatively, histone binding could enhance the nuclear entry of the DNA. Histones are imported into the nucleus by multiple pathways

(Mühlhäusser et al., 2001). Thus, the nuclear uptake of the chromatinized DNA might occur by one of these pathways. In line with this hypothesis, the nuclear localizing signal (NLS) sequence of histone H2A is crucial for the transfection efficiency of the DNA–H2A complex, and the additional attachment of the NLS peptide enhances both the nuclear entry and transgene expression, in the case of the DNA–H2B complex (Balicki et al., 2002; Wagstaff et al., 2007). As a consequence of the stabilization and/or the improved nuclear entry, the amount of exogenous DNA might increase (Fig. 3).

Histone binding limits the access of transcription factors to DNA, and thus, nucleosome formation on plasmid DNA could reduce transgene expression. Alternatively, the expression efficiencies from the “naked” and “chromatinized” plasmid DNAs could be similar when the plasmid DNA, delivered as naked DNA, is rapidly bound to histones in the nucleus (Hebbar and Archer, 2008; Ochiai et al., 2007; Reeves et al., 1985). As shown in Fig. 3B, the efficiency of transgene expression from the plasmid DNA chromatinized with core histones was similar to that from the plasmid DNA delivered as naked DNA, and it seemed to be higher in the case of the DNA chromatinized with acetylation-rich core histones. In contrast to our expectations, our results suggested increased expression efficiency from the plasmid DNA chromatinized *in vitro*, at least that with acetylation-rich core histones. One possible explanation is that the positioning/dynamics of the nucleosomes on the plasmid DNA delivered as the complex were different from those on the plasmid DNA delivered as naked DNA. The former might be preferred to the latter, for transgene expression. However, further studies are necessary to determine the reason(s) for the enhanced transgene expression observed in this study.

We expected that chromatinization using acetylated histones would further enhance transgene expression, since a relationship between acetylated histones and

active genes has been reported, and acetylation is considered to influence gene expression by modifying the chromatin conformation and/or the recruitment of regulatory factors (Turner, 2000). Indeed, the expression seemed to be enhanced when the acetylation-rich core histones were used (Fig. 2). However, there was no statistically significant difference between the acetylation-rich histone and control histone experimental groups, for luciferase expression and efficiency (Figs. 2 and 3B). One possible reason for this observation is that the acetylated histones delivered with the plasmid DNA may have been exchanged with cellular histones after nuclear entry, and this might have already occurred at the time of transcription. Although the dynamics of cellular histones have been examined by many research groups, their details are still unknown. The simple complexation of plasmid DNA with acetylated histones did not enhance transgene expression, as compared with the “control” histones, and thus novel strategies based on future findings could overcome the present limitations of the chromatinized plasmid DNA.

Johnson-Saliba et al. (2000) reported that the affinity of importins α/β and β , important players in nuclear entry of proteins, for histone H3/H4 was diminished when H3/H4 was acetylated. The acetylation could mask NLS of H3/H4 and suppress their nuclear entry. As described above, the chromatinized DNA might be imported into the nucleus by NLS-dependent pathways. Thus, use of the acetylation-rich histones could reduce delivery of the chromatinized plasmid DNA. This is in line with the results shown in Fig. 3A and another possible reason for the slight enhancement of luciferase expression compared to the control chromatinized DNA (Fig. 2).

In conclusion, transgene expression from the chromatinized plasmid DNA was more efficient than that from plasmid DNA delivered in the naked form. The results obtained in this study indicated that chromatinized plasmid DNA is useful for

transgene expression *in vivo*. We used the hydrodynamic administration method for the delivery of the chromatinized plasmid DNA. The complex could be delivered by liposome-based nonviral vectors, such as the multifunctional envelope-type nanodevice (MEND) (Kamiya et al., 2003; Nakamura et al., 2012).

Acknowledgments

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Zhang, G., Budker, V., Wolff, J.A., 1999. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum. Gene Ther.* 10, 1735-1737.

FIGURE LEGENDS

Figure 1. Acetylation of histone H3 and analysis of chromatinized plasmid DNA. (A) Western blot analysis of H3 in TSA-treated Hepa 1-6 cells. Anti-acetyl histone H3 and anti-histone H3 antibodies were used to detect H3 in cells treated with TSA (200 ng/mL). Time points after the TSA-treatment are shown. (B) Dot blot analysis of H3, purified from Hepa 1-6 cells treated with TSA (200 ng/mL for 18 h) and untreated cells. (C) Partial nuclease digestion of chromatinized plasmid DNA. The pYK-CMV-luc plasmid DNA was chromatinized with the core histones (2.7 μ g) and treated with nuclease according to the supplier's recommendations. The treated DNAs were analyzed by agarose gel electrophoresis. Lanes 1 and 4, 2 μ g of pYK-CMV-luc; lanes 2 and 5, 2.5 μ g of pYK-CMV-luc; lanes 3 and 6, 3 μ g of pYK-CMV-luc; lanes M₁, marker DNA (100-bp ladder); lane M₂, non-digested pYK-CMV-luc. Lanes 1-3, acetylated core histones; lanes 4-6, control core histones. The plasmid DNA chromatinized under the conditions corresponding to lanes 2 and 5 were used in the expression experiments.

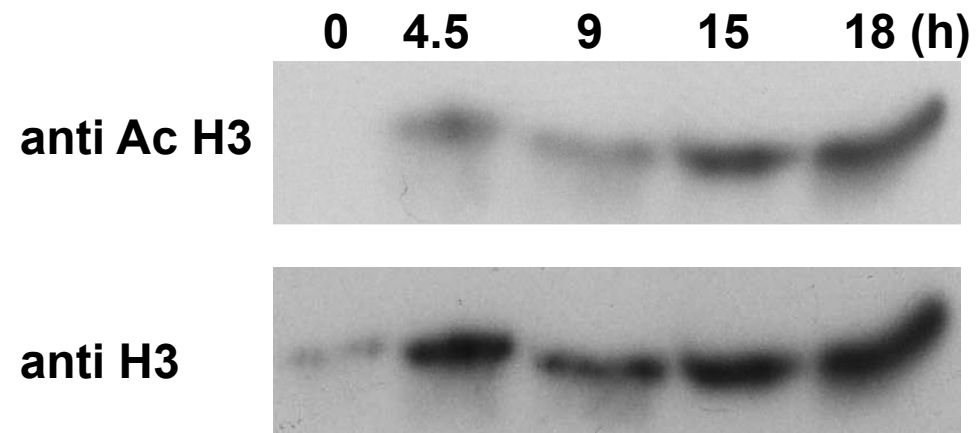
Figure 2. Expression of the luciferase gene from plasmid DNA chromatinized *in vitro*. Plasmid DNA (2.5 μ g) was chromatinized using recombinant NAP-1 and ACF, and one-half volume of the DNA solution (1.25 μ g as DNA) was mixed with saline and introduced into mouse liver. Naked plasmid DNA (1.25 μ g) was also injected. The luciferase activities were measured after 24 h. Six mice were analyzed per experimental group. Data are expressed as means + SE. White column, naked plasmid DNA; gray column, plasmid DNA chromatinized using histones isolated from TSA-untreated cells;

black column, plasmid DNA chromatinized using histones isolated from TSA-treated cells. $*P < 0.05$ and $**P < 0.01$ vs. naked DNA.

Figure 3. (A) Amounts of luciferase DNA in the nucleus and (B) expression efficiencies from plasmid DNA. Naked and chromatinized plasmid DNAs (1.25 μg as DNA) were introduced into mouse liver. (A) The amounts of the exogenous DNA were measured after 24 h by Q-PCR. (B) The luciferase activities, shown in Fig. 2, were divided by the amounts of the exogenous DNAs, shown in panel A. Six mice were analyzed per experimental group. Data are expressed as means + SE. White columns, naked plasmid DNA; gray columns, plasmid DNA chromatinized using histones isolated from TSA-untreated cells; black columns, plasmid DNA chromatinized using histones isolated from TSA-treated cells.

Figure S1. (A) Expression of the luciferase gene from plasmid DNA, (B) amounts of luciferase DNA in the nucleus, and (C) expression efficiencies from plasmid DNA. Plasmid DNA was chromatinized using HeLa core histones and the DNA solution (0.46 μg as DNA) was mixed with saline and introduced into mouse liver. Seven (naked plasmid DNA) and 8 (plasmid DNA chromatinized using histones isolated from HeLa cells) mice were analyzed. Data are expressed as means + SE. White column, naked plasmid DNA; gray column, plasmid DNA chromatinized using histones isolated from TSA-untreated HeLa cells. $*P < 0.05$ vs. naked DNA.

A)



B)

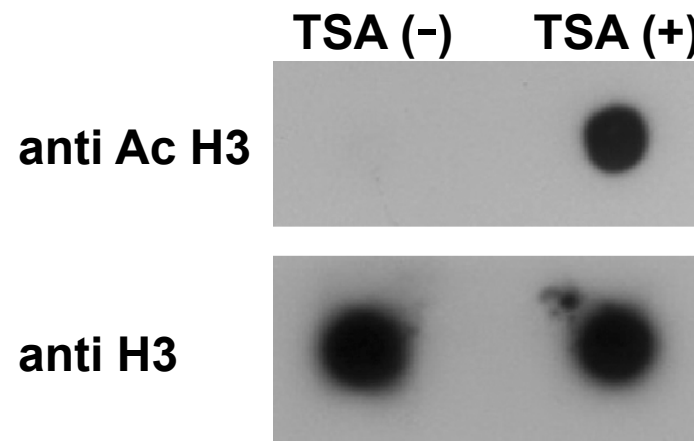


Fig. 1

C)

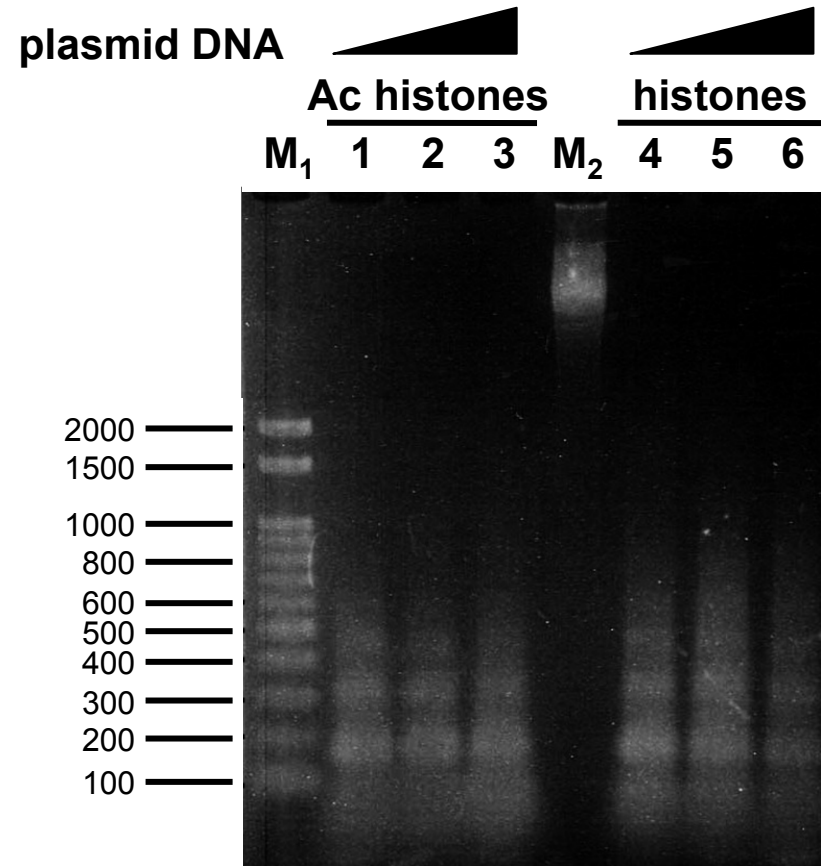


Fig. 1

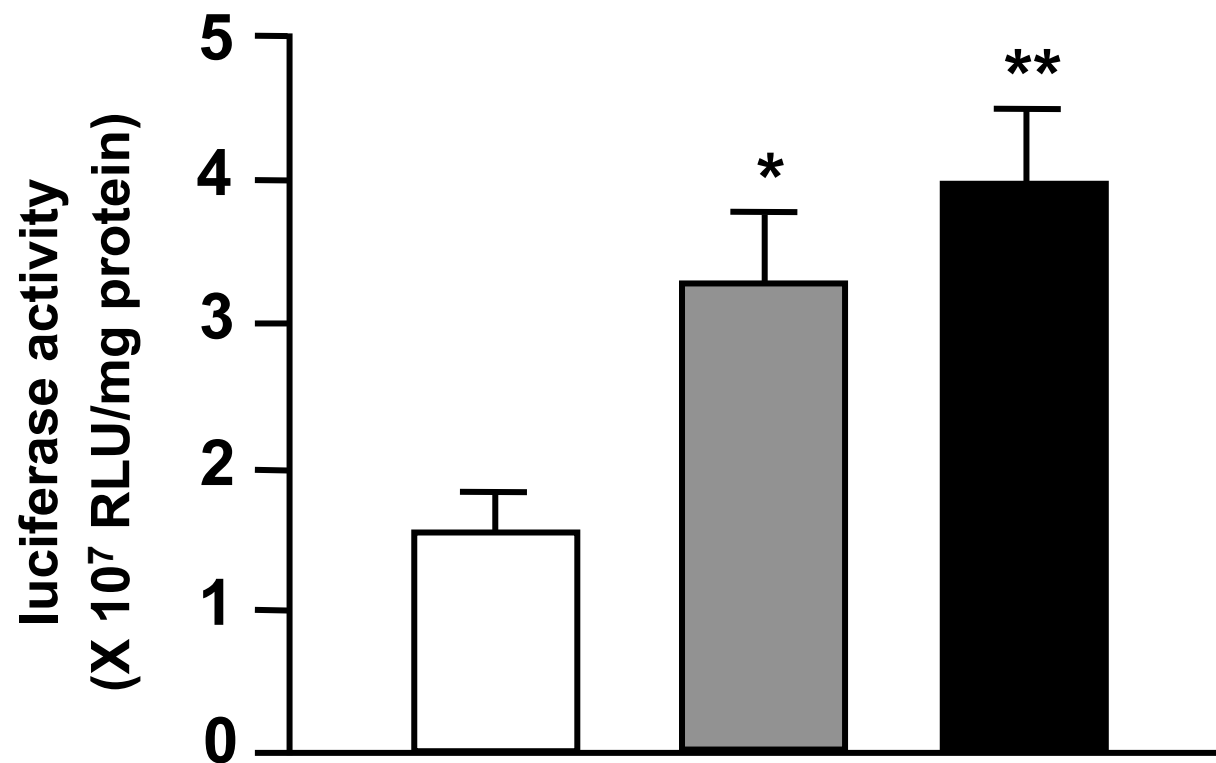


Fig. 2

A)

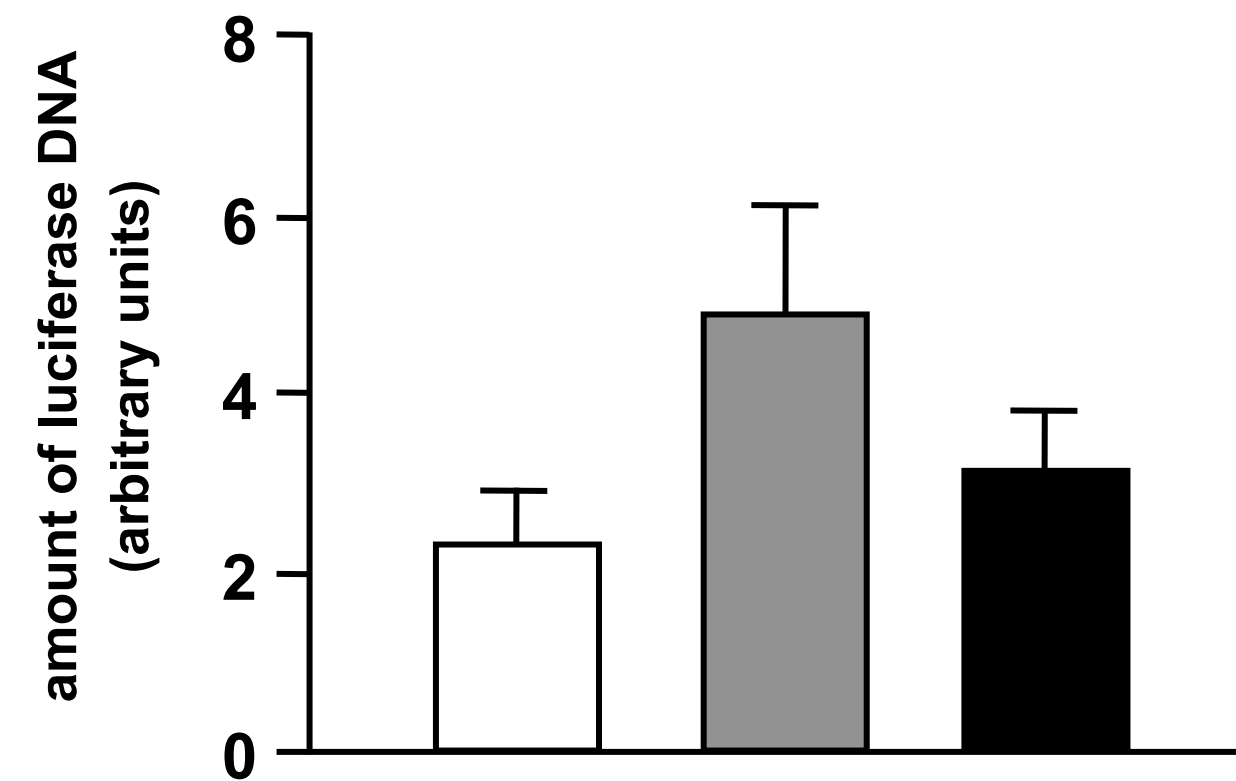


Fig. 3

B)

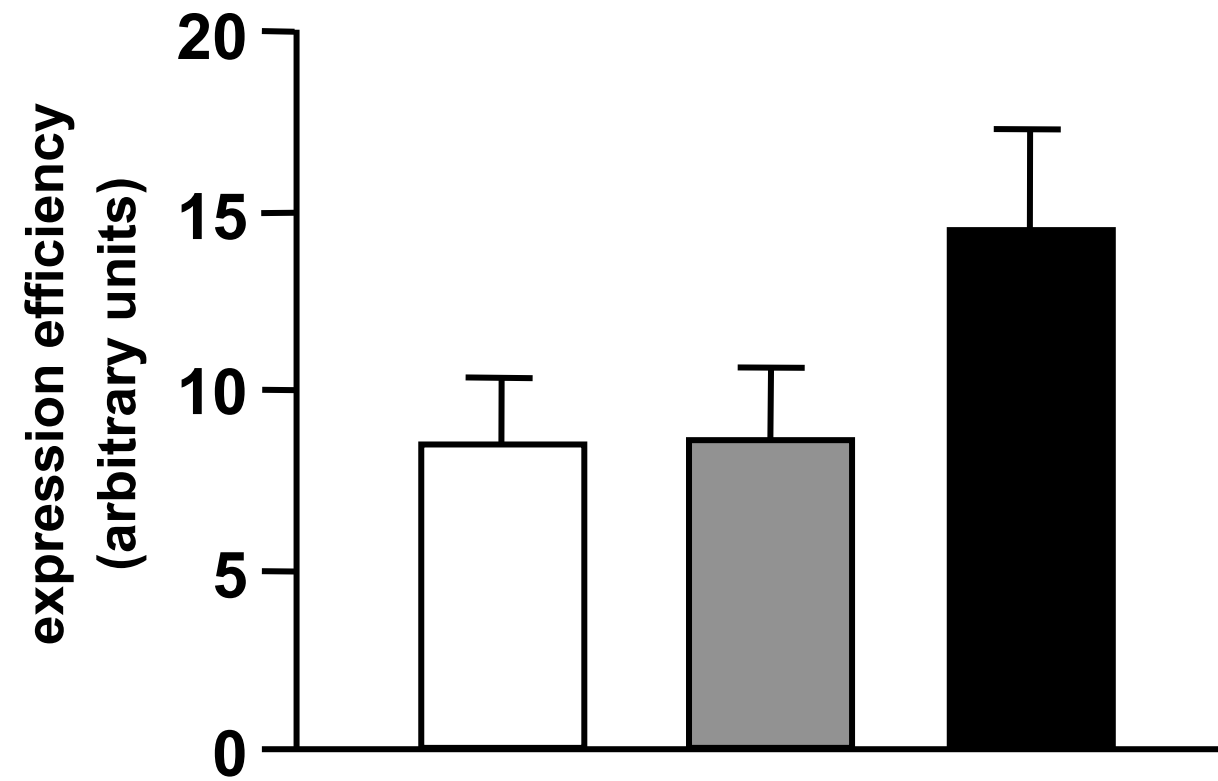
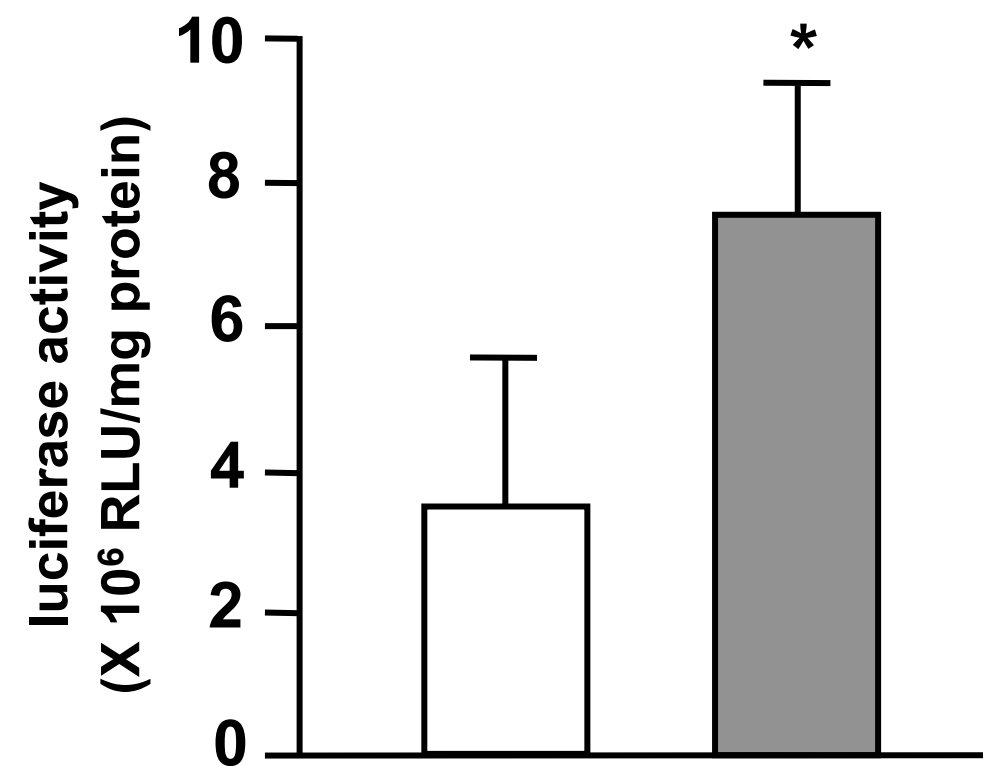


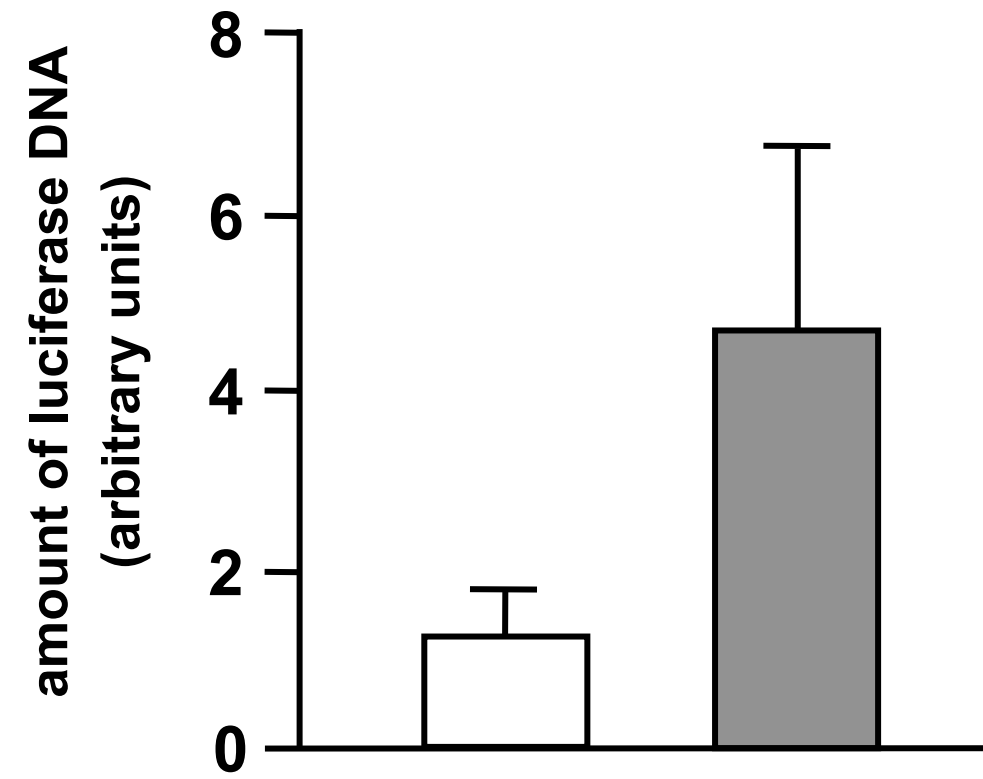
Fig. 3

A)



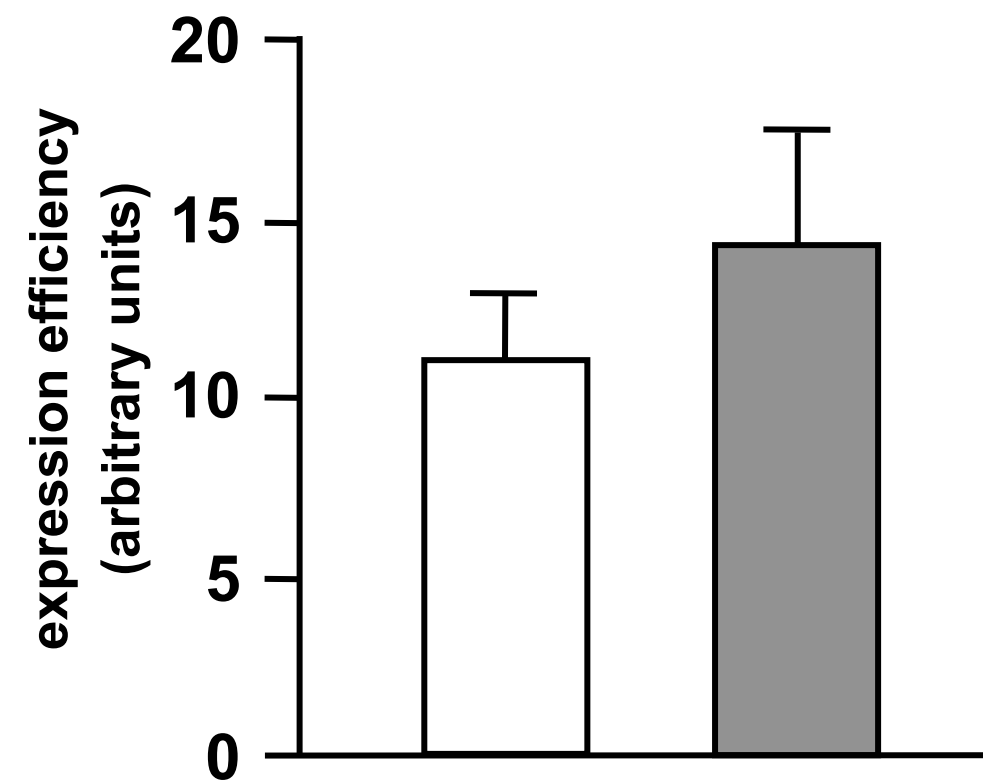
Supplementary Fig. 1

B)



Supplementary Fig. 1

C)



Supplementary Fig. 1