Gene therapy for ovarian cancer using carbonyl reductase 1 DNA with a polyamidoamine dendrimer in mouse models

（カルボニル還元酵素 1 DNA とポリアミドアミンデンドリマー複合体を用いた卵巣癌マウスモデルの遺伝子治療）

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INTRODUCTION

Epithelial ovarian cancer is the seventh leading cause of cancer mortality among women around the world.[1] The stage of ovarian cancer is crucial since stage III or IV ovarian cancer has a poor prognosis.[2] Ovarian cancer does not present with symptoms in the early stages because the ovaries are located within the pelvis, so approximately 40-50% of cases of ovarian cancer are found in an advanced stage.[2] Cytoreductive surgery followed by adjuvant chemotherapy is commonly recommended as the primary treatment for advanced epithelial ovarian cancer. Postoperatively, the combination of paclitaxel with a platinum analogue is used as first-line chemotherapy.[3] Epithelial ovarian cancer is highly responsive to initial anticancer treatment, but approximately half of the cases of advanced cancer recur within two years and result in poor prognosis due to a decreased response to chemotherapy.[4] This hampers the prevention of peritonitis carcinomatosa, leading to a lower quality of life. Anticancer agents alone are not sufficient for advanced ovarian cancer, so a new treatment strategy is required.

We have found in previous studies that clofibric acid (CA), a peroxisome proliferator-activated receptor α ligand, inhibits growth of ovarian cancer.[5] Treatment with CA increases the expression of carbonyl reductase 1 (CBR1), reducing microvessel density and inducing apoptosis, thus inhibiting tumor growth.[5] In an experiment involving mice with a subcutaneous tumor, the size of the tumor increased over time in the control group while it

decreased in mice in which CBR1 expression had been induced.[6] However, injecting CBR1 siRNA into a tumor decreases expression of CBR1 and promotes ovarian cancer growth and metastasis.[7] Studies by the current authors suggest that CBR1 might be a new candidate for molecularly targeted therapy.

Over the last few years, the field of gene therapy has garnered attention, and an increasing number of studies have examined vectors for gene delivery. Numerous studies have reported that dendrimers are an effective vector for DNA.[8-10] Research is being conducted to use dendrimers to deliver DNA to cells without damaging or deactivating it. DNA is complexed with a dendrimer and the resulting complexes are then encapsulated in a water-soluble polymer. The encapsulated complexes are then deposited on or sandwiched in functional polymer films, facilitating transfection with no loss in DNA activity even when the DNA is dehydrated. Research based on this concept is being conducted to use complexes of a polyamidoamine (PAMAM) dendrimer and DNA as a functional biodegradable polymer to transport genes bound to a biochemical substrate.[11] Results have suggested that fast-degrading functional polymers can facilitate localized transfection.[11]

The current study was aimed to determine the optimal conditions for delivering CBR1 DNA to ovarian cancer cells via a PAMAM dendrimer and examine the therapeutic effectiveness of using a CBR1/PAMAM dendrimer against peritoneal carcinomatosis of ovarian cancer.

MATERIALS AND METHODS

Cell line and cell culture

HRA cells[12] and DISS cells,[5] derived from human epithelial ovarian adenocarcinoma, were generously provided by Dr. Y. Kikuchi (National Defense Medical College, Japan) and Dr. Saga (Jichi Medical School, Tochigi, Japan), respectively, and were grown in RPMI-1640 Medium (Sigma-Aldrich, St Lois, MO), supplemented with 10% fetal bovine serum (FBS), at 37°C in a water-saturated atmosphere with 5% CO_2/95% air. These 2 cell lines were verified in writing as being ovarian in origin and no mycoplasma contamination.[5, 12]

Plasmid DNA preparation

A pCMV6-AC-GFP vector (OriGene Technologies, Inc., Rockville, MD) that encodes the human CBR 1, GFP, and ampicillin resistance genes was used to optimize and obtain highly efficient transfection. For amplification, pCMV6-AC-GFP was transformed into E. coli-DH5α competent cells by heat shock transformation according to standard laboratory protocols. The transformed bacteria were amplified in LB-ampicillin medium. The plasmids were purified from cultured transformed bacteria using a PureLink HiPure Plasmid Filter Maxiprep Kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Plasmid DNA was diluted in TE buffer at a concentration of 1μg/μl.

Dendrimer/DNA complex formation

Polyamidoamine (PAMAM) dendrimer (generation 6 with 256 surface amino groups) was purchased from Sigma-Aldrich. Dendrimer/DNA complexes were formed by adding the dendrimer to phosphate-buffered saline (PBS) to reach an equal volume of plasmid DNA in PBS at an N/P ratio of 5:1, 10:1, 20:1, 25:1 or 30:1, followed by incubation for 30 min at room temperature. Here, N/P (the nitrogen content to the phosphorus content in the molecule) is the ratio of the total number of dendrimer terminal amine groups and the total number of DNA phosphate groups. PAMAM dendrimer concentration used in this study was maximally 0.158μM. When PAMAM dendrimer concentration is below 1.0μM, no cytotoxicity is found.[13]

In vitro transfection

One day before transfection, 0.5×10^7 plated HRA and DISS cells were cultured in 15 ml of RPMI-1640 Medium, supplemented with 10% FBS without antibiotics per 10-cm culture vessel, so that they would reach 80% confluence at transfection. Afterwards, a solution of dendrimer/DNA complexes containing 24 μg of human CBR1 DNA was added to the cells. Transfected cells were incubated at 37°C in a CO_2 incubator for 48 hrs and harvested. The control group was only given the dendrimer. Transfected cells were evaluated for green fluorescent protein (GFP) expression using a laser scanning confocal microscope (BZ-X700;

Keyence, Osaka, Japan) 24 hrs and 48 hrs after transfection.

Western blot analysis

Cell lysates were prepared from HRA and DISS cells cultured with a solution of dendrimer/DNA complexes for 24 and 48 hrs, electrophoresed on a 12.5% sodium dodecyl sulfate polyacrylamide gel, and blotted as described previously.[5] The protein concentration was determined using Bradford's method. The blots were probed with the following diluted antibodies for 2 hrs: CBR1 (Catalog number sc-390554, Santa Cruz Biotechnology Santa Cruz, CA) at 1:200 and β-actin (Catalog number A5441, Sigma-Aldrich) at 1:2000. The membranes were then incubated with anti-mouse or rabbit IgG HRP-linked Antibody (Cell Signaling Technology, Beverly, MA). Protein bands on the same membranes were visualized using enhanced chemiluminescence (ChemiDoc XRS, Bio-Rad, Hercules, CA) according to the manufacturer's protocol.

Cell proliferation assay

Cell proliferation was assayed using a Cell Counting Kit-8 (CCK-8; Dojin Laboratories, Kumamoto, Japan). HRA and DISS cells were cultured overnight in 96-well microplates at $1.0x10^4$ cells per well with 100 µl of medium. Afterwards, a solution of dendrimer/DNA complexes containing

0.1 μg of human CBR1 DNA was added to the cells. The control group was only given PBS. Transfected cells were incubated at 37℃ in a CO_2 incubator for 48 hrs. Cell proliferation was assessed 2 hrs after the addition of CCK-8 by measuring A_{450} with a Multiskan FC microplate reader (Thermo scientific, Yokohama, Japan). A preliminary study using this kit showed that absorbance was directly proportional to the number of cells. The experiment was conducted in triplicate.

Animal experiments

Animal experiments were approved by the Animal Research Committee of Hirosaki University and all animals were cared for and handled in accordance with the Rules for Animal Experimentation of Hirosaki University and animal practices as defined by national and local bodies governing animal welfare (Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health).

Model of peritoneal carcinomatosis

Eight-week-old female BALB/c nu/nu mice (CLEA Japan, Tokyo, Japan) were used in this study. All mice were group-housed in plastic cages with stainless steel grid tops in an air-conditioned room with a 12-h light/dark cycle in the Institute for Animal Experiments of Hirosaki University.

Mice were given water and food ad libitum. HRA cells (1.0×10^6 cells in 1000 µl of RPMI-1640 Medium) were injected into the abdominal cavity of mice with a 22-gauge needle. The mice were divided into 2 groups (n=5 in each group that could be statistically calculated[5]). All of the mice were numbered and housed separately. In the group receiving human CBR1 DNA, mice were administered a solution of dendrimer/DNA complexes in the abdominal cavity on alternate days. The solution consisted of a combination of diluted DNA (24µg of DNA in 1000µl PBS) and diluted dendrimer (appropriate amount of the dendrimer at an N/P ratio of 20:1 in 1000 µl of PBS). In the control group, mice were administered 2000 µl of PBS in the abdominal cavity on alternate days. Administration started 1 day after cells were injected. Mice were monitored for survival daily until reaching humane endpoints as defined by an inability to access food or water or increased effort due to worsening abdominal ascites. Health was observed daily and all efforts were made to minimize suffering.

Statistical analysis

Survival rates were calculated using the Kaplan–Meier method and the statistical significance of differences in the cumulative survival curves between the groups was evaluated using the logrank test. Other statistical analyses were performed using the Student's *t*-test. *P* values < 0.05 were considered statistically significant.

RESULTS

Comparison of levels of CBR1 expression

Levels of CBR1 expression were compared in HRA and DISS cells transfected with CBR1 DNA at various N/P ratios. The uptake of DNA increased over time for up to 48 hrs, and efficiency was highest at an N/P ratio of 20:1 (Figure 1). Western blot analysis showed that the level of CBR1 expression was higher in cells transfected with CBR1 DNA than in control cells. HRA and DISS cells transfected with CBR1 DNA at an N/P ratio of 20:1 for 48 hrs produced the highest level of CBR1 expression (Figure 2).

Antiproliferative action of CBR1 in vitro

Figure 3 shows changes in cell proliferation assessed by measuring A_{450} with a Multiskan FC microplate reader 2 hrs after the addition of CCK-8. HRA cell proliferation of transfection with CBR1 at N/P ratios of 5 to 25:1 was significantly reduced in comparison to the control cells (Figure 3A). HRA cell proliferation of transfection with CBR1 at N/P ratios of 20 or 25:1 was significantly decreased in comparison to N/P ratios of 5 or 10:1 (Figure 3A). There was no significant difference in HRA cell proliferation between transfection with CBR1 at N/P ratios of 20:1 and 25:1 (Figure 3A). On the other hand, DISS cell proliferation of transfection with CBR1 at N/P ratios of 20 or 25:1 was significantly decreased in comparison to the control cells and

N/P ratios of 5 or 10:1 (Figure 3B). There was no significant difference in DISS cell proliferation between the control cells and N/P ratios of 5 or 10:1, and between transfection with CBR1 at N/P ratios of 20:1 and 25:1 (Figure 3B).

Antitumor action of CBR1 in a mouse model of peritonitis carcinomatosa

A mouse model of peritonitis carcinomatosa was created to study the antitumor effect of CBR1 in vivo. The survival times of the control group (n=5) and the group receiving human CBR1 DNA (n=5) were compared. In the control group, all mice died by day 25, but all of the mice in the group receiving human CBR1 DNA survived at that point (Figure 4). Administration of a solution of dendrimer/DNA complexes to the group receiving human CBR1 DNA was discontinued after day 24 and survival was monitored thereafter. The first dead mouse in the group receiving human CBR1 DNA appeared on day 33; 8 days later of the last administration. The group receiving human CBR1 DNA had a significantly longer survival time compared to the control group ($P < 0.01$, Figure 4). The control group developed obvious malignant ascites and dissemination but the group receiving human CBR1 DNA did not show such symptoms or mild if present. The first dead mouse in the group receiving human CBR1 DNA had obvious malignant ascites and a mass where ovarian cancer cells had been injected, but other mice in the group had little ascites or dissemination in the abdominal cavity. There was no marked

weight loss as a result of receiving human CBR1 DNA. We tested the effects of PAMAM dendrimer alone or CBR1 DNA at an N/P ratio of 10:1 on a mouse model of peritonitis carcinomatosa, but the results were not significant (data not shown).

DISCUSSION

It is well established that CBR1 is involved in development of ovarian cancer.[6, 7, 14-17] In clinical studies, Umemoto et al. reported that decreased CBR1 expression in epithelial ovarian cancer was associated with retroperitoneal lymph node metastasis and poor survival.[16] Murakami et al. reported that decreased CBR1 expression in uterine cervical and endometrial cancer was associated with poor progression-free survival as well as overall survival.[14, 15] In animal studies, Ismail et al. reported that mouse cancer cells in which CBR1 was knocked down by transfection of an antisense CBR1 cDNA acquired a potent metastatic potential.[17] Osawa et al. reported that human ovarian cancer cells in which CBR1 was knocked down by transfection of small interfering RNA displayed significantly higher proliferative ability and invasive activity.[7] In addition, Wang et al. reported that murine ovarian cancer containing cells with increased CBR1 expression as a result of transfection of CBR1 cDNA undergo spontaneous regression.[6]

Because our previous study showed that oral administration of CA increased the expression of CBR1 in ovarian cancer and CBR1 suppressed tumor growth by inducing apoptosis and

reducing angiogenesis via decrease of prostaglandin E_2 level,[5] administration of CA was initially expected to be a useful candidate for the treatment of ovarian cancer. However, oral administration of high dose of CA causes gastrointestinal problems, weight loss, rhabdomyolysis and so on. Therefore, development of gene therapy using CBR1 DNA has been a matter of deep concern. This is the first report showing that gene therapy using a PAMAM dendrimer and transfection of CBR1 DNA could achieve a significantly longer survival in mice with peritonitis carcinomatosa. The transfection of CBR1 DNA also has the potential to reduce peritoneal dissemination of a tumor and production of malignant ascites. Although mice in the group receiving human CBR1 DNA had small peritoneal masses locally, those masses were absent in the abdomen or ascites was limited, suggesting that transfection of CBR1 DNA with a PAMAM dendrimer inhibited dissemination and proliferation of malignant cells in these animals. In the current study, there was no significant weight loss or adverse reactions to the administered solution.

As shown in Figure 1 and 2, DNA was successfully transfected into human epithelial ovarian cancer cells using a PAMAM dendrimer. The transfection efficiency changed with the N/P ratio, i.e. the ratio of the total number of dendrimer terminal amine groups and the total number of DNA phosphate groups. An N/P ratio of 20:1 was found to be the optimal ratio for the dendrimer to deliver DNA. Western blot analysis indicated that the level of CBR1 expression

was higher in cells transfected with CBR1 DNA than that in the control cells. HRA and DISS cells transfected with CBR1 DNA at an N/P ratio of 20:1 for 48 hrs produced the strongest expression of CBR1, supporting earlier study by Fu et al. who had reported that the efficiency of DNA transfection was better at an N/P ratio of 20:1 compared to ratios of 10:1 and 40:1.[11] Moreover, Zhou et al. reported that a ratio of 10:1 to 20:1 resulted in the most efficient dendrimer-mediated siRNA delivery.[18]

An experiment involving cell proliferation was conducted to assess whether the proliferation of ovarian cancer cells was controlled by transfection of CBR1 DNA with the dendrimer. As shown in Figure 3, cell proliferation was significantly reduced even at an N/P ratio of 5:1 to 10:1 in HRA cells and reached plateau at an N/P ratio of 20:1, indicating that an N/P ratio of 20:1 was the optimal condition for the dendrimer to deliver CBR1 DNA into the cells.

Over the past few years, various new cancer treatment strategies distinct from conventional chemotherapy have been developed. Bevacizumab, an anti-vascular endothelial growth factor (anti-VEGF), is a molecularly targeted drug for treatment of ovarian cancer. The GOG218 and ICON7 studies showed that progression-free survival (PFS) was significantly extended, but there were no significant differences in overall survival (OS).[19, 20] Other drugs that have been clinically applied are the multi-targeted tyrosine kinase inhibitor pazopanib,[21]

the mammalian target of rapamycin (mTOR) inhibitor temsirolimus,[22] and the angiopoietin inhibitor trebananib.[23] Although these molecularly targeted drugs are effective at extending PFS, no studies have indicated that they significantly extend OS.

An increasing number of studies are examining immunotherapies and the concept of boosting the immune system to target and destroy cancer cells. Typical forms of immunotherapy include lymphokine-activated killer cell immunotherapy and tumor-infiltrating lymphocyte therapy. Tsuda et al. reported that recurrent cervical cancer objectively regressed as a result of vaccination with peptides that had preexisting cytotoxic T lymphocyte precursors in the periphery (a fact that was verified prior to vaccination).[24] Clinical trials of advanced immunotherapies are being conducted to treat cancer of the prostate, pancreas, lymphoma, melanoma, and the kidneys, but a treatment has yet to be established.[25]

Gene therapy or introduction of a foreign gene into cancer cells is an attractive therapy. Viruses have been used as the vector for gene transfer in many clinical studies. Phase II trials of an adenoviral p53 gene therapy for treatment of squamous cell cancer of the head and neck[26] and phase I trials of p53 gene therapies for bladder cancer,[27] ovarian cancer,[28] and glioma[29] have been completed. A virus is a useful vector but it has several faults as well. Viruses carry a risk of infection, so greater care has to be taken with regard to safety. This means that extra facilities and measures are required, making treatment more complicated and expensive.

Therefore, the current study focused on dendrimers as a potential non-viral vector for gene transfer. In in vitro experiments, DNA was successfully transfected into human epithelial ovarian cancer cells using a dendrimer. Furthermore, use of a dendrimer to transfect CBR1 DNA in a mouse model of peritonitis carcinomatosa resulted in a significantly longer survival time in in vivo experiments. These findings suggest that dendrimers are a useful means of gene delivery, and that a gene transfer therapy with CBR1 DNA and a dendrimer may facilitate the treatment of advanced ovarian cancer with peritonitis carcinomatosa. The mechanism of delivery of dendrimer/DNA complexes into cells is via endocytosis.[10] PAMAM dendrimers are considered to be less toxic at the concentrations used for gene delivery.[30, 31] The toxicity of PAMAM dendrimers, in contrast to that of the large molecular weight polymers polyethylenimine (PEI) and poly-L-lysine (PLL), does not seem to stem from membrane damage assayed using LDH release or hemolysis.[32] The efficiency of nucleic acid transfection with a dendrimer and complexation depends on the dendrimer generation, the N/P ratio, and the concentration of nucleic acids.[18] In the current study, mice were intraperitoneally administered a solution of dendrimer/DNA complexes on alternate days. Although further investigation may be needed to improve the efficiency of dendrimer/DNA administration (e.g. via an intra-venous route), gene therapy using CBR1 DNA and a PAMAM dendrimer may be a promising strategy for treatment of advanced ovarian cancer.

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Legends for figures

Figure 1. Transfection of CBR1 DNA into HRA and DISS cells. Fluorescent photographs of HRA (**A**) and DISS (**B**) cells transfected with pCMV6-AC-GFP expressing CBR1 taken 24 and 48 hours after transfection. The efficiency of uptake was better at a N/P ratio of 20:1 than at other N/P ratios.

Figure 2. Western blot analysis for the CBR1 expression levels to confirm the optimal N/P ratio and incubation time. The level of CBR1 expression was the strongest in HRA and DISS cells transfected with CBR1 DNA at a N/P ratio of 20:1 for 48 hours.

Figure 3. Cell proliferation assay. **A.** HRA cells were transfected with CBR1 DNA at various N/P ratios. Cell proliferation of transfection with CBR1 at N/P ratios of 5:1 to 25:1 was significantly reduced in comparison to the control cells. Cell proliferation of transfection with CBR1 at a N/P ratio of 20:1 or 25:1 was significantly decreased in comparison to a N/P ratio of 5:1 or 10:1. There was no significant difference in cell proliferation between transfection with CBR1 at N/P ratios of 20:1 and 25:1. **B.** HRA cells were transfected with CBR1 DNA at various N/P ratios. DISS cell proliferation of transfection with CBR1 at N/P ratios of 20 or 25:1 was significantly decreased in comparison to the control cells and N/P ratios of 5 or 10:1. There was no significant difference in DISS cell proliferation between the control cells and N/P ratios of 5 or 10:1, and between transfection with CBR1 at N/P ratios of 20:1 and 25:1. * $P < 0.02$ versus control, † $P < 0.0001$ versus control, ‡ $P < 0.0001$ versus N/P ratio 5 or 10:1.

Figure 4. Antitumor effect of CBR1 in peritonitis carcinomatosa mouse model. In the control group, all mice died by day 25, whereas all mice receiving CBR1 DNA survived at the same

period. Dendrimer/DNA complex solution administration was discontinued after day 24. The first dead mouse in the group receiving human CBR1 DNA appeared on day 33; 8 days later of the last administration. The group receiving human CBR1 DNA had a significantly longer survival time compared to the control group ($P < 0.01$).

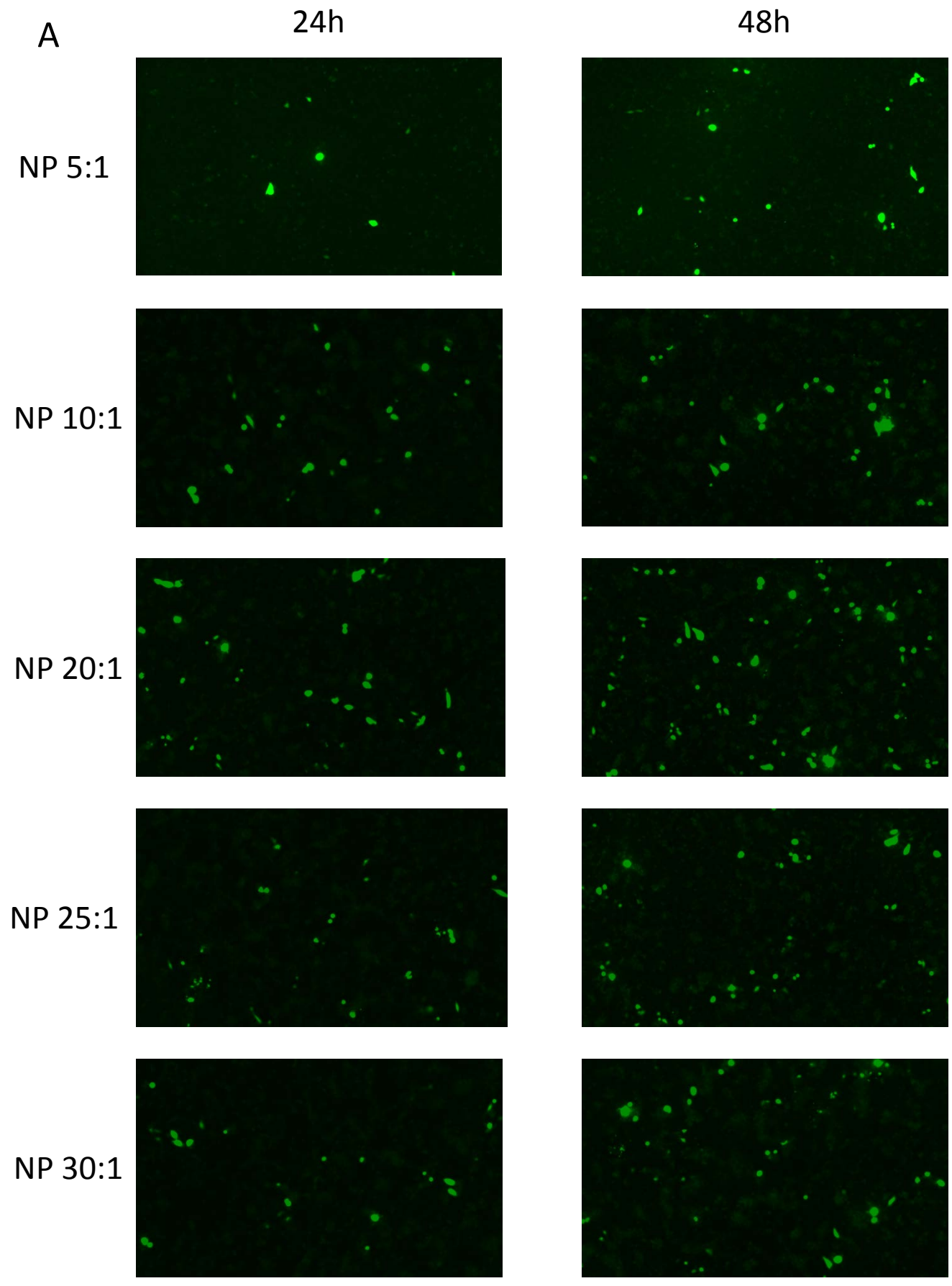

Figure 1

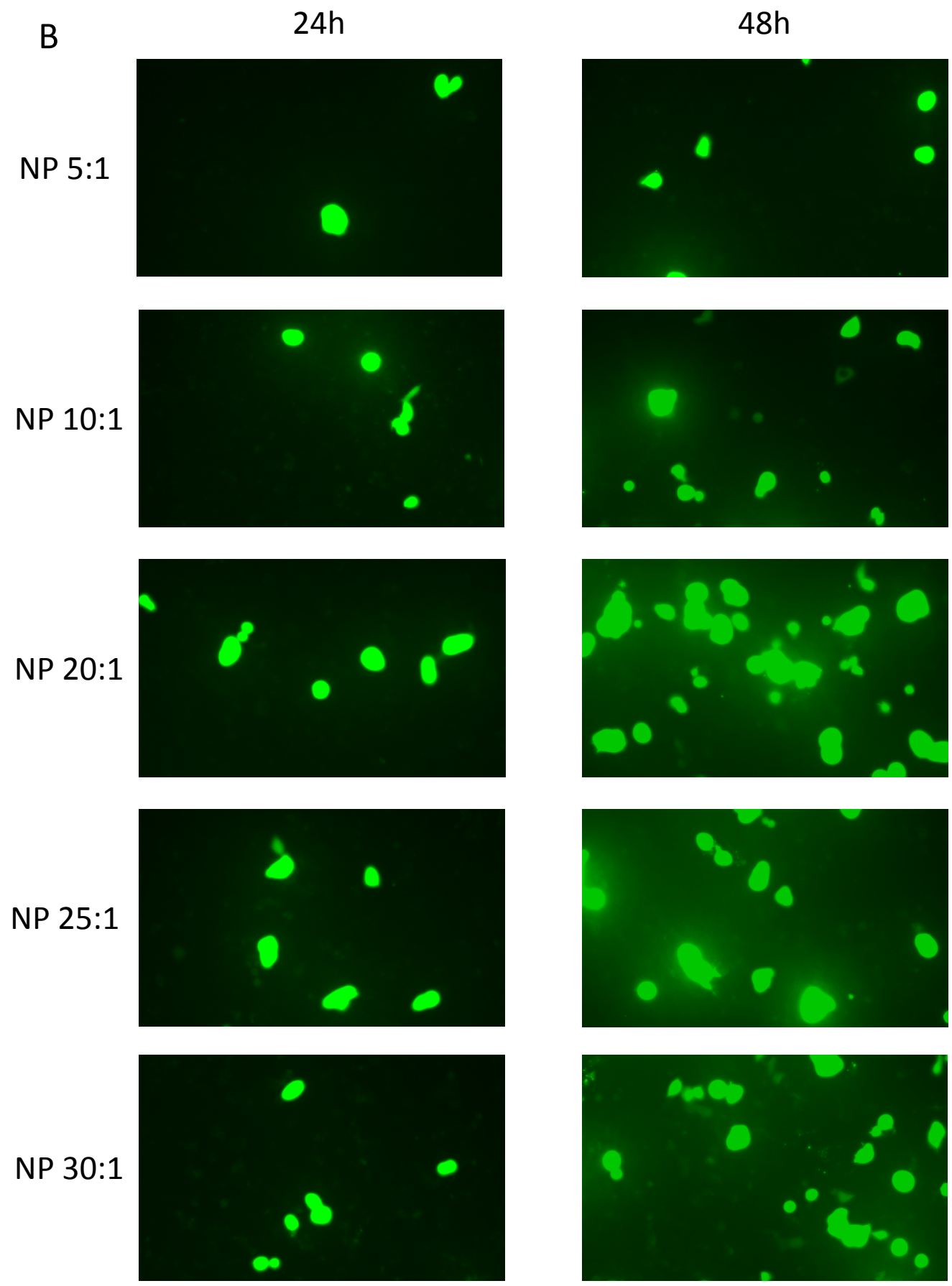

Figure 1

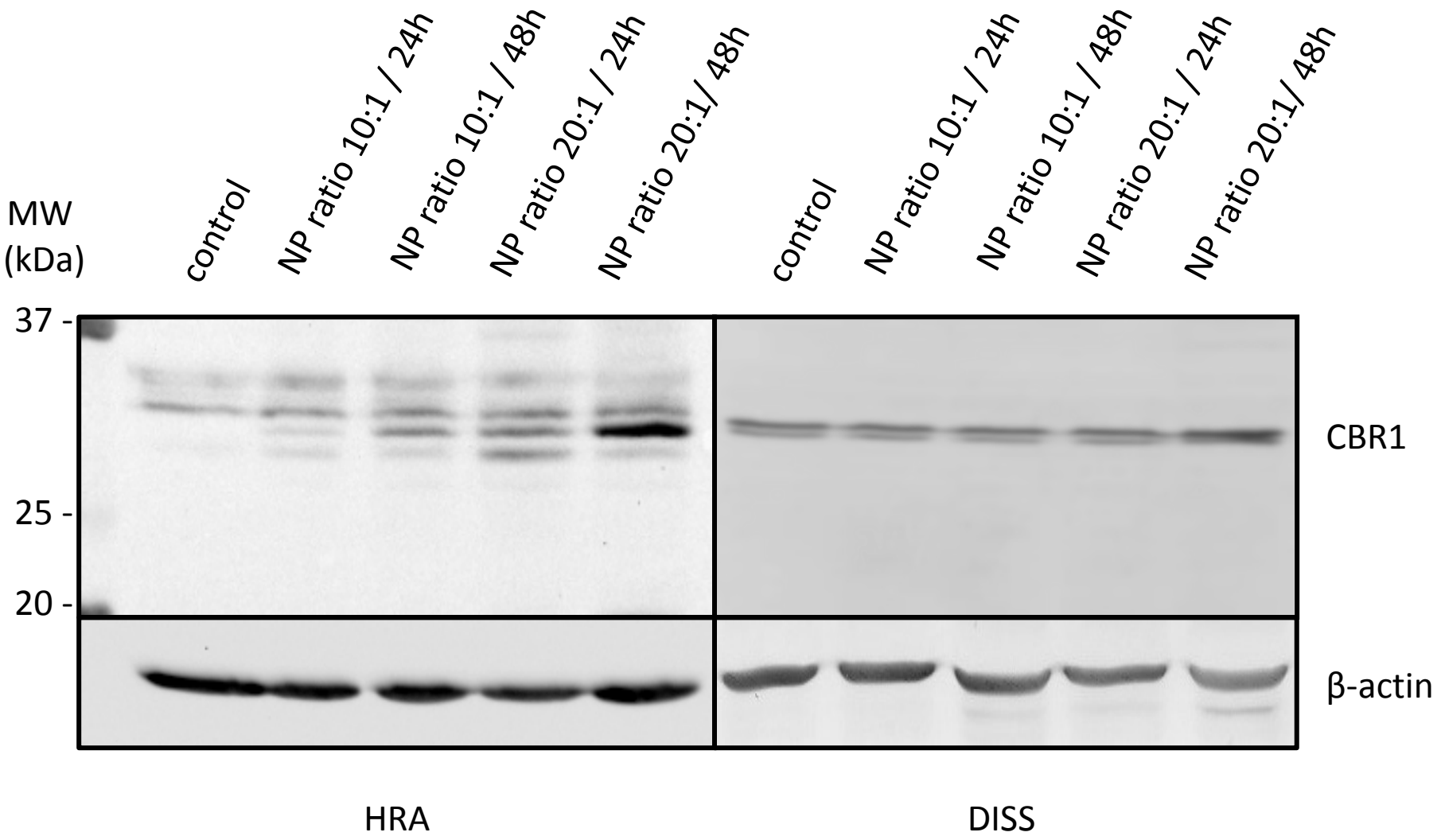
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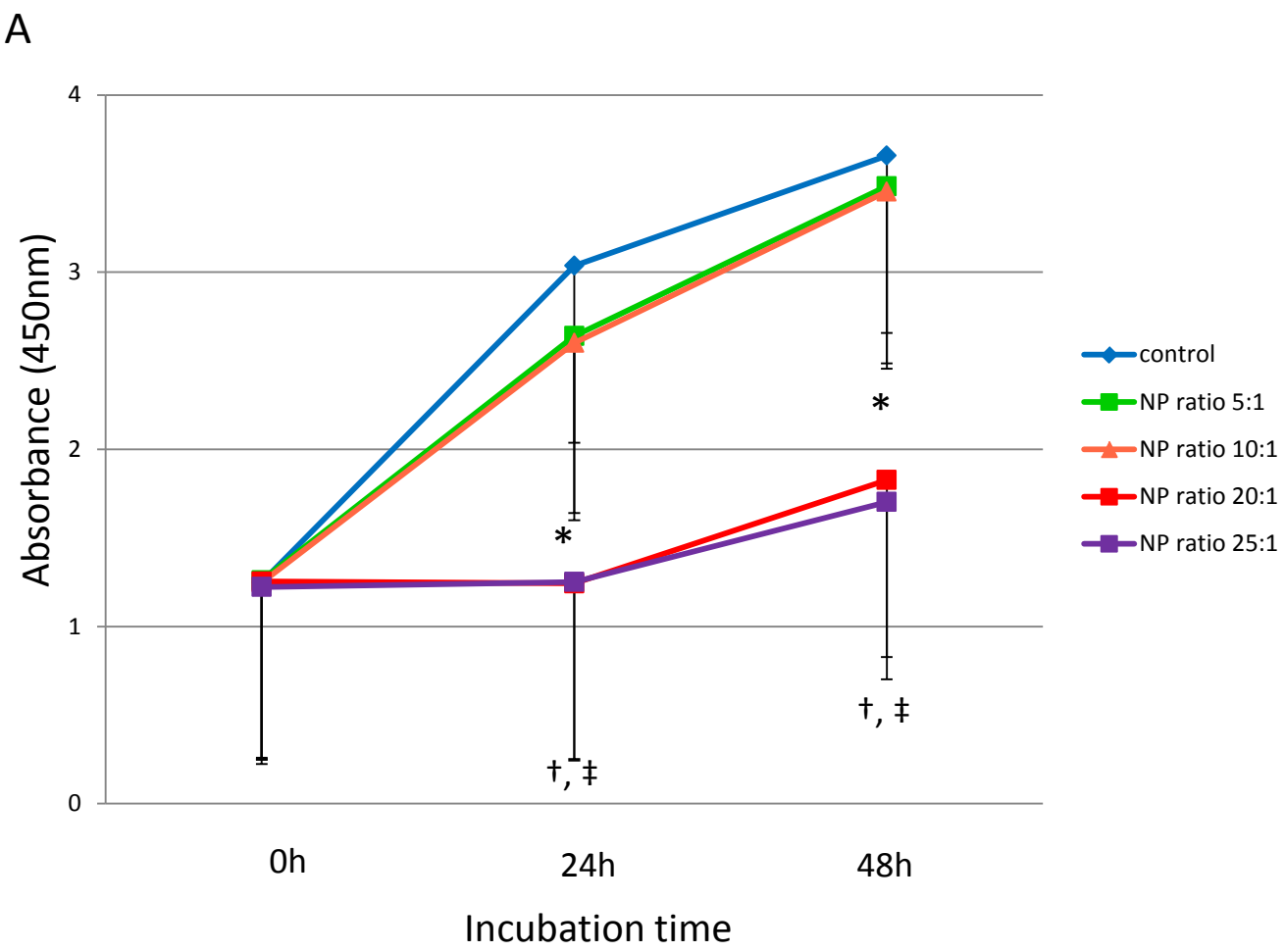
Figure 2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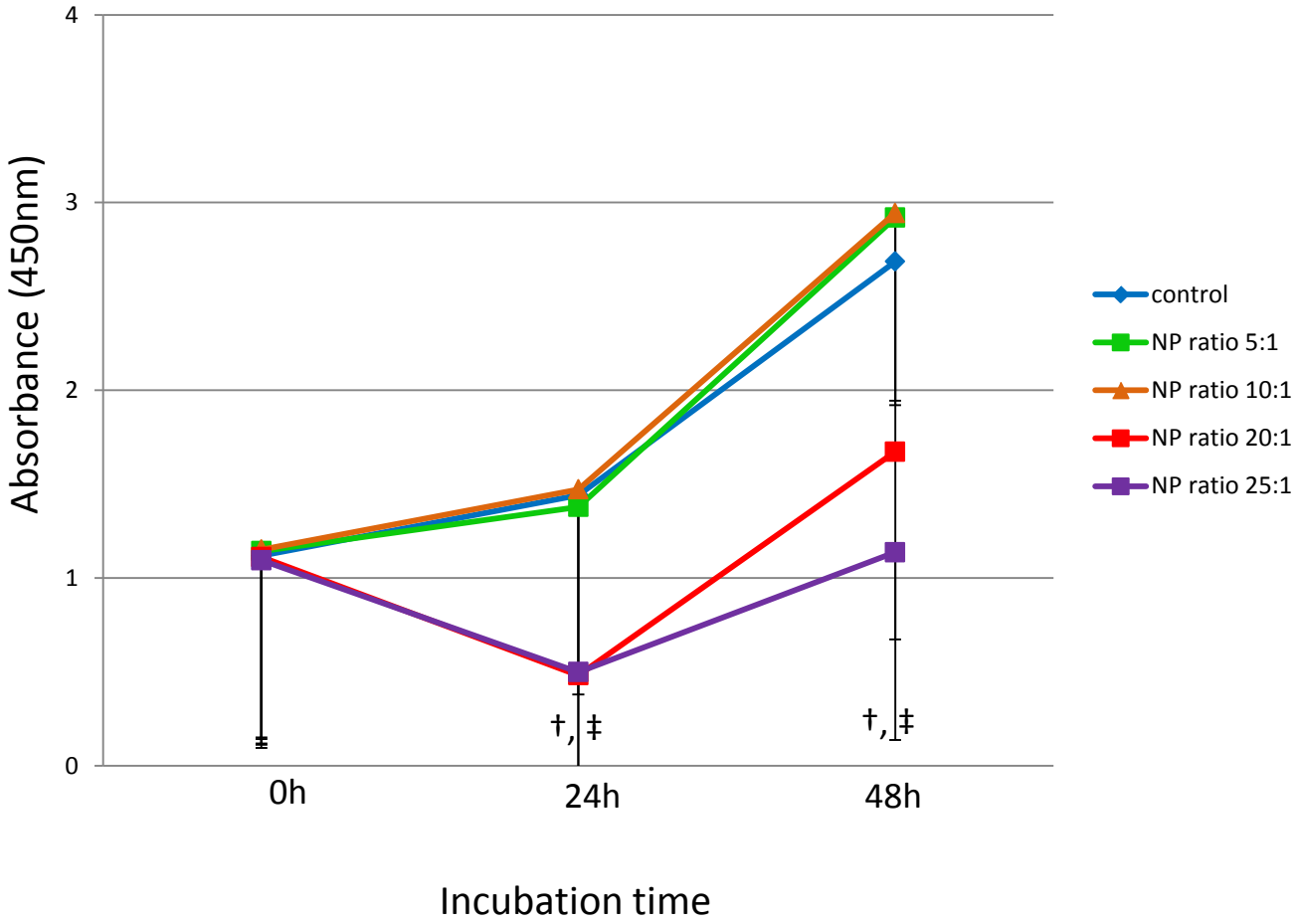
0h
24h
48h
Incubation time
*
*
†, ‡
†, ‡
control
NP ratio 5:1
NP ratio 10:1
NP ratio 20:1
NP ratio 25:1

Figure 3

B


4
3
2
1
0
Absorbance (450nm)
0h
24h
48h
†, ‡
†, ‡
Incubation time
control
NP ratio 5:1
NP ratio 10:1
NP ratio 20:1
NP ratio 25:1

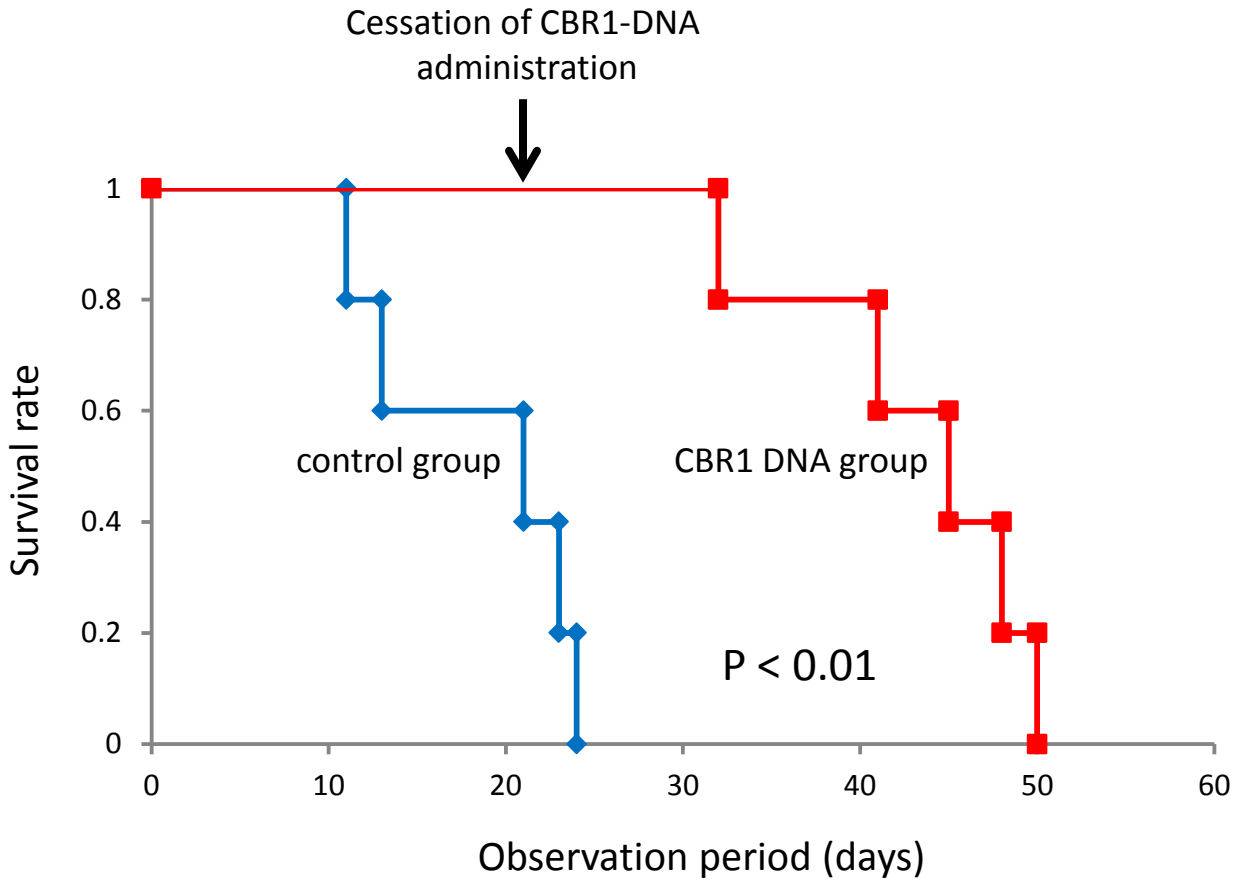

Cessation of CBR1-DNA administration
Survival rate
1
0.8
0.6
0.4
0.2
0
control group
CBR1 DNA group
P < 0.01
0
10
20
30
40
50
60
Observation period (days)

Figure 4