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Original Article

# Cadmium chloride down-regulates the expression of Rad51 in HC11 cells and reduces knock-in efficiency

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**ABSTRACT**

**Background:** Efficient gene editing technology is needed for successful knock-in. Homologous recombination (HR) is a major double-strand break repair pathway that can be utilized for accurately inserting foreign genes into the genome. HR occurs during the S/G2 phase, and the DNA mismatch repair (MMR) pathway is inextricably linked to HR to maintain HR fidelity. This study was conducted to investigate the effect of inhibiting MMR-related genes using $CdCl_2$, an MMR-related gene inhibitor, on HR efficiency in HC11 cells.

**Methods:** The mRNA and protein expression levels of MMR-related genes (*Msh2, Msh3, Msh6, Mlh1, Pms2*), the HR-related gene *Rad51*, and the NHEJ-related gene DNA *Ligase IV* were assessed in HC11 cells treated with 10 μM of $CdCl_2$ for 48 hours. In addition, HC11 cells were transfected with a CRISPR/sgRNA expression vector and a knock-in vector targeting Exon3 of the mouse-beta casein locus, and treated with 10 μM cadmium for 48 hours. The knock-in efficiency was monitored through PCR.

**Results:** The treatment of HC11 cells with a high-dose of $CdCl_2$ decreased the mRNA expression of the HR-related gene Rad51 in HC11 cells. In addition, the inhibition of MMR-related genes through $CdCl_2$ treatment did not lead to an increase in knock-in efficiency.

**Conclusions:** The inhibition of MMR-related gene expression through high-dose $CdCl_2$ treatment reduces the expression of the HR-related gene *Rad51*, which is active during recombination. Therefore, it was determined that $CdCl_2$ is an inappropriate compound for improving HR efficiency.

**Keywords:** cadmium chloride, CRISPR-Cas9 mediated knock-in efficiency, DNA mismatch repair, homologous recombination, non-homologous end joining

## INTRODUCTION

Gene targeting is a useful technique for inserting foreign genes into a specific locus in the genome. The major pathways of double-strand break (DSB) repair are homologous recombination (HR) and non-homologous end joining (NHEJ). The HR pathway is utilized to accurately insert foreign genes into the genome, but it occurs only during the S/G2 phase, and its efficiency for DSB repair is lower than that of NHEJ (Vasquez et al., 2001; Mao et al., 2008). Thus, studies have been performed to improve the efficiency of HR for accurate gene targeting. The HR-

related gene *Rad51* is involved in strand invasion and mediates homologous DNA pairing (Long et al., 2011). The NHEJ-related gene DNA *Ligase IV* is crucial for ligating DNA ends resulting from DSBs in the NHEJ pathway (Ochi et al., 2012). SCR7, an inhibitor of the NHEJ-related gene DNA *Ligase IV*, has been reported to increase HR efficiency in mammalian cells by up to 19-fold (Maruyama et al., 2015). RS-1 is a chemical compound that stimulates *Rad51* (Jayathilaka et al., 2008) and has been shown to increase knock-in efficiency in rabbit embryos by 2-5-fold (Song et al., 2016). However, the efficiency of gene targeting varies depending on the species or cell type, and further studies are needed for accurate gene targeting. Therefore, it is essential to examine DNA repair pathways closely related to the HR pathway.

The DNA mismatch repair (MMR) is known to be inextricably linked to HR (Spies and Fishel, 2015), and is reported as a post-replication repair pathway that occurs during replication or recombination (Li, 2008). The MMR-related MutS α (MSH2-MSH6) and MutS β (MSH2-MSH3) proteins bind to a mismatched DNA base pair, and MutL α (MLH1-PMS2) is an endonuclease that binds to MutS-heteroduplex complexes (Lahue et al., 1989; Habraken et al., 1996). The MMR maintains HR fidelity and prevents recombination between divergent sequences through heteroduplex rejection in the presence of extensive heteroduplexes during the recombination process (Goldfarb and Alani, 2005; Kunkel and Erie, 2015; Chakaraborty and Alani, 2016).

$CdCl_2$ treatment inhibits ATP hydrolysis of MutS α (MSH2-MSH6) in eukaryotic cells and specific binding to mismatched DNA (Clark and Kunkel, 2004). Meanwhile, it has been reported that knock-out of *Msh2* accelerates the DSB-promoted recombination by divergence substrates in gene targeting and intrachromosomal recombination (Elliott and Jasin, 2001). These reports suggest that the inhibition of MutS α and MutS β by $CdCl_2$ treatment may increase HR efficiency. However, it has been reported that low-dose $CdCl_2$ (1 μM) in Mac-T cells does not increase HR efficiency (Kim et al., 2022).

To address the conflicting reports, we aimed to determine the change in knock-in efficiency when MMR is suppressed through high-dose $CdCl_2$ treatment (10 μM), an MMR inhibitor, in HC11 cells. Furthermore, to assess the mechanism of HR inhibition by $CdCl_2$ treatment, we assessed the expression of MMR-related genes, the HR-related gene *Rad51*, and the NHEJ-related gene DNA *Ligase IV*.

## MATERIALS AND METHODS

### Construction of knock-in vector

The knock-in vector used in this study expresses hEPO protein using the GST fusion system at the mouse beta-casein Exon3 locus. The knock-in vector, which consists of a 5' homology arm (1.024 kb), GST-PreScission protease-hEPO, SV40polyA, CMV-EGFP, and a 3' homology arm (1.81 kb), was originally developed in our laboratory. The vector was modified in this study; CMV-EGFP, a positive selection marker, was replaced with PGK neo, a negative selection marker, and the 3' homology arm of 1.81 kb was modified to a 3' homology arm of 1.015 kb. As a result, the final knock-in vector was constructed as PBSK_1.024kb mBC5'arm_GST_hEPO_SV40polyA_PGK neo_1.015kb mBC3'arm.

### Cell culture and transfection

Mouse mammary epithelial cells (HC11) were cultured in the growth medium consisting of Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone), 1% penicillin/streptomycin (Hyclone), 0.1% gentamicin (Sigma, St. Louis, MO, USA), 0.1% EGF (Gibco, Thermo Fisher, USA), and 0.05% insulin (Sigma). HC11 cells were seeded at a density of $2.4 \times 10^5$ cells per well into 6-well plates (SPL, Gyeonggi-do, Korea) and incubated at 37℃ with 5% $CO_2$. The medium was replaced with 1 mL of fresh medium before transfection. The knock-in vector (3.75 μg) and pGuide it Zs Green1_sgRNA expression vector (1.875 μg) were transfected into HC11 cells using the Xfect transfection reagent (Takara, Tokyo, Japan). After 24 hours of transfection, the cells were treated with 10 μM $CdCl_2$ and incubated for 48 hours.

### Reverse transcription followed by quantitative polymerase chain reaction

Total RNA from HC11 cells was isolated using an RNeasy mini kit (QIAGEN, Hilden, Germany). A total of 3 μg of RNA was used as a template for cDNA synthesis, which was performed using random hexamers and M-MLV reverse transcriptase (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Quantita-

tive PCR was performed to measure the mRNA expression of DNA MMR-related genes (*Msh2, Msh3, Msh6, Mlh1, Pms2*), the HR-related gene *Rad51*, and the NHEJ-related gene DNA *Ligase IV* in a total volume of 20 μL, containing 20 ng of cDNA, 10 pmole of forward and reverse primer pairs (Table 1), and 10 μL of TOPreal™ qPCR 2X PreMIX (Enzynomics, Daejeon, Korea). All qPCR experiments were performed in triplicate using the Mx3000p instrument (Agilent Technologies, Santa Clara, CA, USA) through the following steps: denaturation at 95℃ for 10 minutes, followed by 40 cycles of amplification at 95℃ for 10 seconds, annealing at 60℃ for 15 seconds, and strand extension at 72℃ for 15 seconds. For comparative quantification, the mRNA expression levels of each gene were normalized to that of the mouse *Rplp0* transcript.

## Western blot analysis

Nuclear and cytoplasmic proteins from HC11 cells were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Total protein from HC11 cells was extracted using the PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. The nuclear and cytoplasmic protein extracts (15 μg) and total protein (30 μg) derived from the cells were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride membranes (Bio-Rad Co., Hercules, CA, USA). The membranes were then blocked for 2 hours at room temperature in 5% skim milk in TBST (Tris-buffered saline with 0.1% Tween 20), and blotted with a rabbit polyclonal anti-MSH2 antibody (ab-70270, Abcam, Cambridge, UK; dilution 1:2,000), mouse monoclonal anti-RAD51 (sc398587) and anti-DNA Ligase *IV* (sc271299, Santa Cruz Biotechnology, Dallas, TX, USA; dilution 1:1,000), or anti-β-actin (a5441, Sigma; dilution 1:10,000) overnight at 4℃. After overnight incubation, the membranes were washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (ab205718, Abcam; dilution 1:2,000), mouse immunoglobulin G (IgG) kappa binding protein (m-$IgG_k$ BP) conjugated to horseradish peroxidase (sc516102, Santa Cruz Biotechnology; dilution 1:1,000), or mouse IgG2a binding protein k (m-$IgG_{2a}$ BP)

Table 1. Primers used for GST_hEPO screening PCR analysis and quantitative PCR analysis of specific genes

| Gene | Primer name | Sequence (5' to 3') |
|---|---|---|
| Primers used for GST_hEPO screening PCR analysis | | |
| Mouse β-casein 5' arm | m C5 CV S5 β | ATAAGAACTGCAGTGGGATTCTTC |
| | m C3 GST AS9 β | AGCATTGAAATCTCTGCACGCTC |
| | m C5 CV S6 β | GTCTACCAATTCACTCTAGAAGTG |
| | m C3 GST AS7 β | ACGTATGATGGCCATAGACTGTG |
| Primers used for quantitative real-time PCR analysis of MMR-related gene, *Rad51*, and DNA *Ligase IV* gene | | |
| *Rplp0* | m*Rplp0* F | CGACCTGGAAGTCCAACTA |
| | m*Rplp0* R | ATCTGCTGCATCTGCTTG |
| MMR-related gene (*Msh2, Msh3, Msh6, Mlh1, Pms2*) | m*Msh2* CDS S | TGAAGTTGGACATGGCAGCA |
| | m*Msh2* CDS AS | TAATGCGGCCAGAGACTGAG |
| | m*Msh3* CDS S | CCATCGCCTATGCAACTCTA |
| | m*Msh3* CDS AS | TCACAGACTGGTGGATAGTG |
| | m*Msh6* CDS S | ATACTCAGGCATGCAACAGC |
| | m*Msh6* CDS AS | TCAAAAGTTGCGGTGCCTCT |
| | m*Mlh1* CDS S | AGATTAGTGAGCGGTGCCAT |
| | m*Mlh1* CDS AS | ACTGAGGATTCACACAGCCC |
| | m*Pms2* CDS S | ACTTCCAGGACGCCACAAAA |
| | m*Pms2* CDS AS | AACTGCCTGTCTGTTGCACT |
| HR-related gene *Rad51* | *Rad51* CDS S | AATTCCGAACTGGGAAGACAC |
| | *Rad51* CDS AS | TCACCTCCACCACGGTCAAT |
| NHEJ-related gene DNA *Ligase IV* | qPCR *Ligase IV* (CDS) S | TCCACAGGAAGGCTCTCTCA |
| | qPCR *Ligase IV* (CDS) AS | TCTCACCGTCAAGGATGCAC |

conjugated to horseradish peroxidase (sc542731, Santa Cruz Biotechnology; dilution 1:1,000) at room temperature for 2 hours. The membranes were washed with TBST. Msh2, Rad51, DNA Ligase *IV*, HDAC2, and β-actin protein bands were detected using the EZ-Western Lumi Pico Kit and EZ-Western Lumi Femto Kit (Dogen, Seoul, Korea). For comparative quantification of protein levels, Rad51, Msh2, and DNA Ligase IV protein bands were normalized to β-actin.

### Analysis of knock-in efficiency by polymerase chain reaction

The knock-in efficiency of the hEPO knock-in vector in HC11 cells was confirmed through first and second PCR experiments. The genomic DNA isolated from cells in each group was used as a DNA template for PCR. The first PCR was performed using the mβC5 CV S5 primer for regions outside the 5' arm and the mβC3 GST9 (Table 1) for the GST region using the amplification reagent Solg™ 2X Multiplex (Solgent, Daejeon, South Korea). The PCR consisted 25 cycles of amplification at 95℃ for 20 seconds, annealing at 62℃ for 40 seconds, and strand extension at 72℃ for 2 minutes. Subsequently, a nested PCR was performed using the mβC5 CV primer and the mβC3 GST AS7 primer using the amplification regent KOD FX Neo (Toyobo, Osaka, Japan) through the following steps: 25 cycles of denaturation at 95℃ for 20 seconds, annealing at 64℃ for 30 seconds, and strand extension at 72℃ for 2 minutes; followed by a final extension at 72℃ for 5 min. The PCR fragments were confirmed through electrophoresis on a 0.8% agarose gel. For comparative quantification, each DNA band was normalized to the mβCE7 region.

### Statistical analysis

Densitometric quantification of DNA or protein bands was performed by analyzing the data using UN-SCAN-IT gel Analysis Software (Silk Scientific Inc., Orem, UT, USA). Subsequently, a statistical analysis of the mRNA levels and densitometric quantification of DNA and protein bands was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). The data were analyzed in two ways. First, the data were analyzed with one-way ANOVA, followed by the Dunnett test, to compare all columns with reference to the control column. Second, a *t*-test was performed to compare the differences between the two columns. The confidence interval was set at 95%.

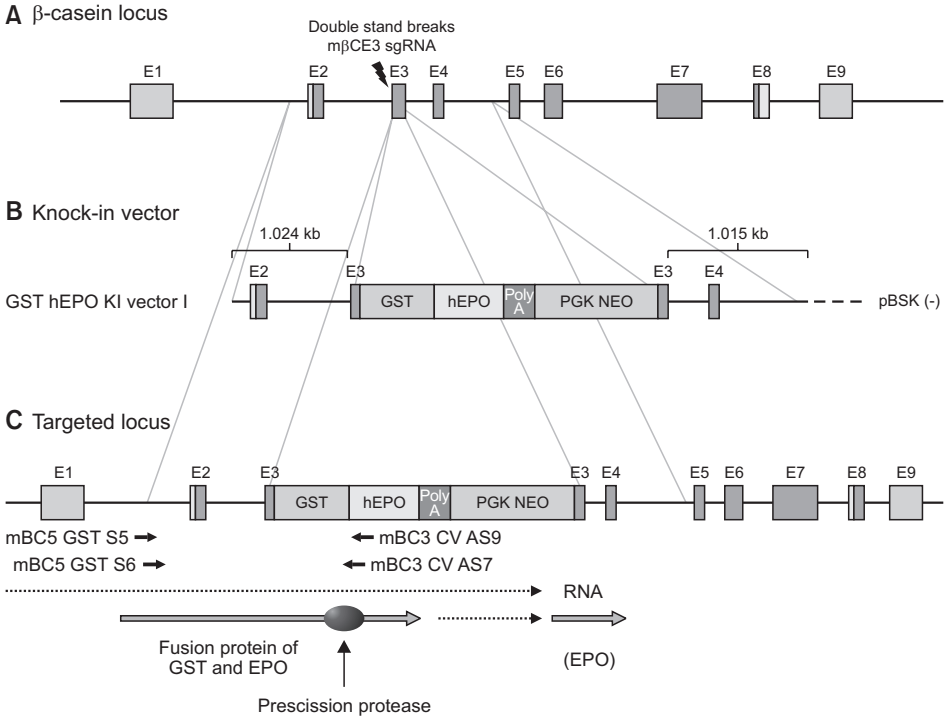


Fig. 1. The knock-in strategy at the mouse β-casein exon 3 locus for the expression of human erythropoietin. (A) Genome structure of the β-casein gene locus. (B) GST-hEPO knock-in vector I (Exon3). (C) Targeted locus of the knock-in vector by homologous recombination. The PCR primer pairs used for detecting HR events are indicated by arrows. PCR, polymerase chain reaction.

## RESULTS

### Construction of the knock-in vector for *hEPO* gene expression in the mouse beta-casein locus

The diagram illustrating the knock-in vector used for expressing the human erythropoietin (*hEPO*) gene in the mouse β-casein locus is shown in Fig. 1. The knock-in vector consisted of the 5'-homology arm, GST-hEPO fused gene, SV40 poly A signal, PGK-Neo, and the 3' homology arm. The *Neo* gene, lacking a polyA signal, was used as a positive selection marker. In this study, the knock-in vector functions as an *hEPO* expression vector that can be inserted into EXON 3 of the mouse beta-casein gene locus using the CRISPR/Cas9 system. If the knock-in vector is accurately introduced into the mouse beta-casein exon 3 locus through homologous recombination, the GST-*hEPO* gene can be expressed under the control of the gene regulatory sequence and promoter of the beta-casein, resulting in the production of the GST-hEPO protein. Additionally, because the GST-hEPO fusion protein contains a cleavage site for PreScission Protease, hEPO can be easily purified by removing the GST-tag.

### The mRNA and protein expression levels of DNA MMR-related genes, *Rad51*, and DNA *Ligase IV* in HC11 cells treated with $CdCl_2$

To determine the effect of high-dose $CdCl_2$ treatment (10 μM) on the mRNA expression of *Rad51*, DNA *Ligase IV*, and DNA MMR-related genes (*Msh2, Msh3, Msh6, Mlh1,* and *Pms2*), HC11 cells were treated with 10 μM $CdCl_2$ for 2 days. As shown in Fig. 2A, the mRNA expression levels of *Msh2, Msh6*, and *Pms2* decreased over time with cadmium treatment. Compared with the control group, the mRNA levels of *Msh2* and *Msh6* decreased by 84% and 74%, respectively, while *Pms2* mRNA level decreased by 31%. In contrast, the expression of *Msh3* mRNA rapidly increased on day 2 (5.4 fold; $p < 0.01$). However, *Mlh1* mRNA expression remained unchanged after the high-

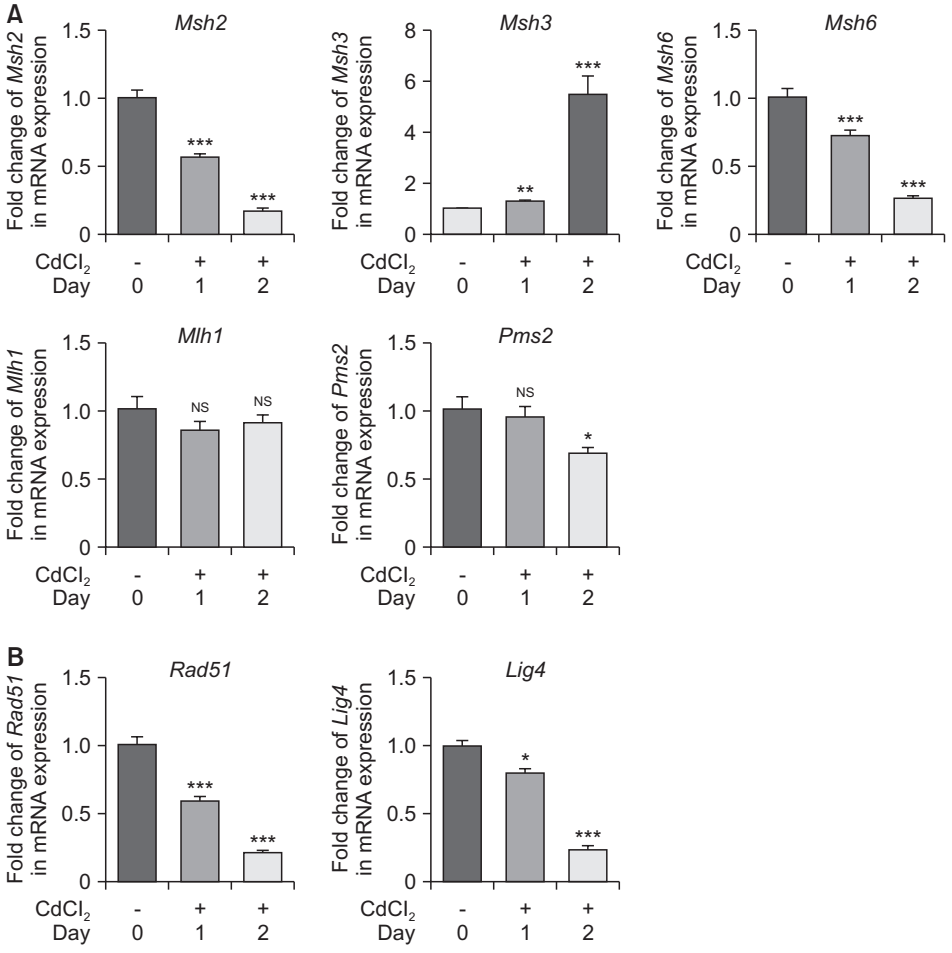


**Fig. 2.** The mRNA expression of MMR-related genes, the HR-related gene *Rad51*, and the NHEJ-related gene DNA *Ligase IV* in HC11 cells treated with $CdCl_2$. (A) The mRNA levels of MMR-related genes (*Msh2, Msh3, Msh6, Mlh1, Pms2*). (B) The mRNA levels of the HR-related gene *Rad51* and the NHEJ-related gene DNA *Ligase IV*. $CdCl_2$ treatment was performed at a final concentration of 10 μM. Day 0 indicates HC11 cells not treated with $CdCl_2$. $CdCl_2$, cadmium chloride; NS, not statistically significant. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$.

dose $CdCl_2$ treatment. As shown in Fig. 2B, the mRNA expression of the HR-related gene *Rad51* and the NHEJ-related gene DNA *Ligase IV* tended to decrease with increasing duration of cadmium treatment, and at 48 h, the mRNA expression of *Rad51* was about 80% ($p < 0.001$) compared to the cadmium-untreated control group, and DNA *Ligase IV* mRNA expression showed a significant decrease of about 76% ($p < 0.001$).

In order to determine the protein levels of Rad51, DNA Ligase *IV*, and MMR-related genes in HC11 cells treated with high-dose $CdCl_2$, a western blot analysis was carried out, and the protein levels were normalized to that of beta-actin. As shown in Fig. 3A and 3B, the nuclear levels of Msh2 decreased by 18% ($p < 0.01$) at 24 h after high-dose $CdCl_2$ treatment compared to the control group not exposed to cadmium. However, nuclear Msh2 levels did not decrease at 48 h. The NHEJ-related protein DNA Ligase IV (Lig4) did not exhibit any changes in the nucleus following high-dose $CdCl_2$ treatment. The expression of HR-related Rad51 showed a significant decrease of about 40% ($p < 0.05$) at 48 h after cadmium treatment compared to the control group. It is known that the expression of HDAC2, a histone protein, decreases with cadmium treatment. We confirmed that the HDAC2 protein expression decreased with increasing duration of cadmium treatment. In addition, the cytoplasmic protein levels of Msh2 and Lig4 remained unchanged after high-dose $CdCl_2$ treatment (Fig. 3A and 3C).

## The CRISPR/Cas9-mediated knock-in efficiency in HC11 cells treated with a high-dose of $CdCl_2$

To investigate the efficiency of CRISPR-Cas9-mediated knock-in, HC11 cells were exposed to high-dose $CdCl_2$ treatment and transfected with the GST-hEPO knock-in and the CRISPR-sgRNA expression vectors. The knock-in efficiency was analyzed after 48 h of $CdCl_2$ treatment (Fig. 4). The CRISPR-Cas9-mediated knock-in efficiency in the HC11 cells treated with a high-dose of $CdCl_2$ decreased by

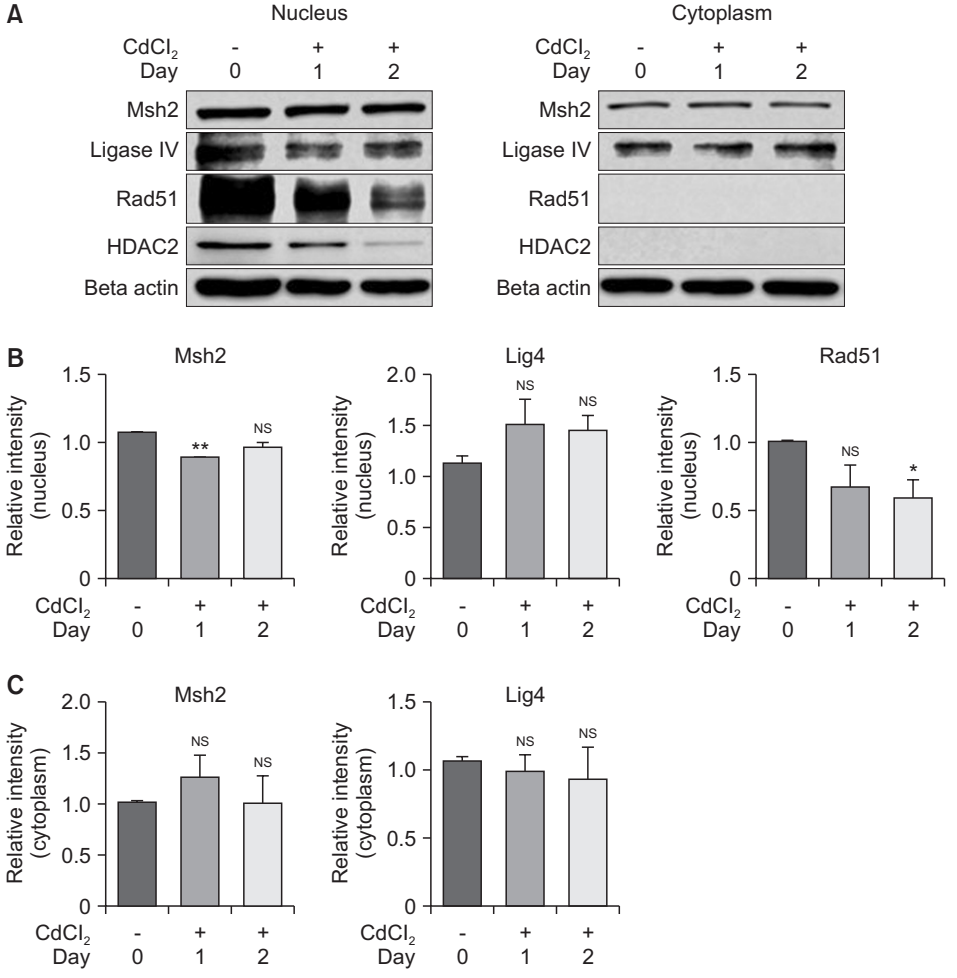


Fig. 3. Protein expression of the MMR-related protein Msh2, the HR-related protein Rad51, and the NHEJ-related protein DNA Ligase IV (Lig4) in HC11 cells treated with $CdCl_2$. (A) Nuclear and cytoplasmic protein levels of Msh2, Lig4, Rad51, and Hdac2 in HC11 cells treated with $CdCl_2$ at a final concentration of 10 μM. (B) The graph represents the densitometric quantification of the protein bands. (C) The graph represents the densitometric quantification of the cytoplasm protein bands. Protein expression levels were normalized by calculating the target gene/β-actin ratio. HDAC, histone deacetylase; NS, not statistically significant. *$p < 0.05$; **$p < 0.01$.

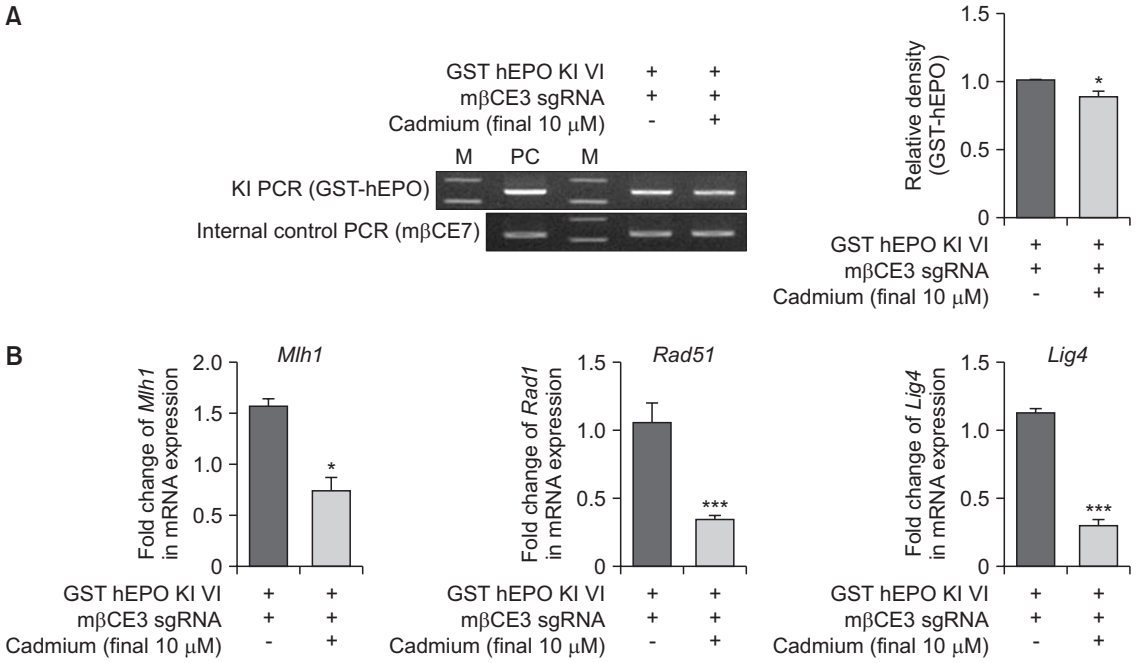


**Fig. 4.** The knock-in efficiency of gene targeting and mRNA expression of MMR-related, HR-related, and NHEJ-related genes in HC11 cells treated with $CdCl_2$. (A) Agarose gel electrophoresis of knock-in DNA band and densitometric quantification of PCR results. $CdCl_2$ treatment was performed at a final concentration of 10 μM. (B) The mRNA levels of MMR-related (*Mlh1*), HR-related (*Rad51*), and NHEJ-related (DNA *Ligase IV*) genes. $CdCl_2$ treatment was performed at a final concentration of 10 μM. PCR, polymerase chain reaction; M, DNA size marker (1-kb ladder); PC, positive control. *$p < 0.05$; ***$p < 0.0001$.

about 13% ($p < 0.05$) compared to the control group not treated with cadmium. Additionally, the mRNA expression levels of MMR-related genes (*Msh2, Msh3, Msh6, Mlh1, Pms2*), the HR-related gene *Rad51*, and the NHEJ-related gene DNA *Ligase IV* were examined using RT-qPCR. The results indicated that the mRNA expression levels of the MMR-related genes *Msh2, Msh6, Pms2*, and *Msh3*, as well as those of the HR-related gene *Rad51* and the NHEJ-related gene DNA *Ligase IV*, were comparable to their levels in the HC11 cells that did not undergo CRISPR-Cas9-mediated knock-in. However, the mRNA expression of *Mlh1* decreased by 52% ($p < 0.05$) compared to the control group not treated with $CdCl_2$ (Fig. 4).

## DISCUSSION

This study investigated whether the HR efficiency at the mouse beta-casein locus using CRISPR/Cas9 system was associated with the regulation of DNA mismatch repair (MMR) through high-dose $CdCl_2$ treatment in HC11 cells.

It has been reported that the efficiency of DSB-promoted recombination for divergence substrates is higher in *Msh2*-deficient cells compared to *Msh2*-proficient cells. In the same study, when intrachromosomal recombination is induced, MMR deficiency has been found to overcome the difficulty of divergence sequence recombination in DSB-promoted and spontaneous recombination (Elliot and Jasin, 2001). Yang et al. (2004) also reported an increase in RAD51 foci in human MSH2/MSH6-deficient cells. Based on these findings, we hypothesized that MMR deficiency could increase the efficiency of DSB-promoted HR recombination.

Therefore, to evaluate the expression of DNA MMR-related genes, the HR-related gene *Rad51*, and the NHEJ-related gene DNA *Ligase IV* after treatment with $CdCl_2$, an MMR inhibitor (Jin et al., 2003; Clark and Kunkel, 2004; Lützen et al., 2004; Banerjee and Flores-Rozas, 2005; Sherrer et al., 2018), HC11 cells were treated with 10 μM $CdCl_2$ for 48 hours. As a result, the mRNA expression of *Msh2, Msh6*, and *Pms2* decreased compared to the $CdCl_2$-untreated group, but the mRNA expression of *Mlh1* did not change. The result was similar to those reported by Clark and Kunkel in 2004, where $CdCl_2$ has been found to inhibit the Msh2-Msh6 heterodimer in yeast without effecting the Mlh1-Pms1 heterodimer. These results were also similar to the findings of Sherrer et al. (2018) report-

ing that $CdCl_2$ inhibits MutL alpha (Mlh1-Pms2 heterodimer), which acts as an endonuclease in the early stage of DNA MMR in eukaryotic cells.

Interestingly, the mRNA expression of the *Msh3* gene in $CdCl_2$-treated HC11 cells increased rapidly compared to the $CdCl_2$-untreated group. Since Msh2 and Msh3 are known to act as heterodimers (Fishel and Wilson, 1997), we expected that the mRNA expression of *Msh3* would also decrease. These results can be supported by the report that *Msh3* and *Msh2* are independently regulated according to the cellular response to DNA MMR (Selva et al., 1997), suggesting that the expression trends of *Msh3* and *Msh2* may differ. In addition, Marra et al. (1998) reported that overexpression of MSH3 causes an imbalance in the relative amounts of MSH3 and MSH6, reducing the expression of human MSH2/MSH6. It seems likely that the increased expression of *Msh3* in this study is the result of an imbalance between DNA MMR-related genes. However, the rapid increase in *Msh3* expression with $CdCl_2$ treatment can be interpreted in several ways; therefore, additional studies are needed.

In this study, we analyzed the effects of high-dose $CdCl_2$ treatment on the expression of the HR-related gene *Rad51* and the NHEJ-related gene DNA *Ligase IV* in HC11 cells. Specifically, we expected that the expression of *Rad51* would increase based on a previous research report by Yang et al. (2004) showing that Rad51 foci are increased in MSH2/MSH6 deficient cells. However, contrary to our expectations, *Rad51* expression was decreased in HC11 cells, despite the decreases in *Msh2* and *Msh6* levels observed in this study. These results are consistent with findings from studies on zebrafish liver cells (Chen et al., 2014) and Japanese medaka (Barjhoux et al., 2016), which also showed that $CdCl_2$ treatment reduces *Rad51* expression. Another study by Lützen et al. (2004) reported that $CdCl_2$ inhibits RPA, which has a multifunctional binding site to interact with Rad51. Additionally, mutation of the Rad51N binding site in RPA70A reduces the ability of Rad51 to replace RPA in ssDNA (Stauffer and Chazin, 2004). These findings suggest that $CdCl_2$ treatment can reduce the expression of Rad51 by inhibiting RPA. The high-dose $CdCl_2$ treatment in HC11 cells also reduced the mRNA expression of the NHEJ-related gene DNA *Ligase IV* compared to the non-$CdCl_2$-treated control. This result is consistent with a report by Li et al. (2015), which showed that $CdCl_2$ treatment reduces the expression of DNA Ligase IV in human cells.

In protein expression analysis, the nuclear levels of the Msh2 protein significantly decreased in HC11 cells treated with high-dose $CdCl_2$ at 24 h; Msh2 levels slightly decreased at 48 h, but there was no significant difference. These results indicate that treatment with 10 μM $CdCl_2$ does not completely inhibit the nuclear expression of Msh2. In addition, the nuclear levels of Rad51 significantly decreased at 48 h compared to non-$CdCl_2$-treated group. Chidambaram et al. (2017) reported that $CdCl_2$ treatment reduces histone deacetylase 2 (Hdac2) expression, and, in this study, the protein expression of Hdac2 decreased with increasing duration of $CdCl_2$ treatment. There was no significant difference in the cytoplasmic protein levels of Msh2 and DNA Ligase *IV* (Lig4) compared to the $CdCl_2$-untreated control group. The differences in the levels of Msh2, Rad51, and Lig4 in nuclear and cytoplasmic fractions are thought to be due to the fact that these proteins act mainly in the nucleus and are regulated by DNA repair pathways.

In this study, we performed CRISPR-Cas9-mediated gene targeting at the mouse beta-casein locus to determine the effect of high-dose $CdCl_2$ treatment on HR efficiency in HC11 cells. The results showed that high-dose $CdCl_2$ treatment slightly decreased the knock-in efficiency in HC11 cells compared to the $CdCl_2$-untreated group. Additionally, we examined the mRNA expression of MMR-related genes, the HR-related gene *Rad51*, and the NHEJ-related gene DNA *Ligase IV* during homologous recombination through high-dose $CdCl_2$ treatment and CRISPR-Cas9-mediated gene targeting in HC11 cells. $CdCl_2$ treatment led to a decrease in the mRNA expression of DNA MMR-related genes (*Msh2, Msh6, Mlh1, Pms2*) and an increase in *Msh3* mRNA expression. Furthermore, the mRNA levels of the HR-related gene *Rad51* and the NHEJ-related gene DNA *Ligase IV* were reduced compared to the $CdCl_2$-untreated group. These results were consistent with the MMR-related gene expression in HC11 cells treated only with high-dose $CdCl_2$ without CRISPR-Cas9-mediated gene targeting (Fig. 2). Interestingly, in HC11 cells, high-dose $CdCl_2$ treatment and CRISPR-Cas9 mediated gene targeting significantly reduced *Mlh1* mRNA expression. This suggests that these results may be attributed to the suppression of the MMR pathway initiator gene *Msh2*. A study by Kim et al. (2022) reported that low-dose $CdCl_2$ treatment does not inhibit MMR-related genes when

CRISPR/Cas9-mediated gene targeting is performed at the bovine beta-casein locus. Moreover, failure to suppress the expression of MMR-related genes has been reported as a potential cause of reduced knock-in efficiency.

In this study, high concentrations of cadmium were used to compensate for the problem of MMR-related gene inhibition. However, despite the suppression of MMR-related genes, the knock-in efficiency did not improve. Although it was believed that the inhibition of MMR-related gene expression through high-dose $CdCl_2$ would improve the HR mechanism, this was not the case.

Therefore, the high-dose $CdCl_2$ treatment can be used as a DNA MMR inhibitor that can suppress DNA MMR genes. However, it was deemed inappropriate as a method for improving the efficiency of HR due to the decrease in expression of the HR-related gene *Rad51*. In addition, further research is needed to determine whether the inhibition of MMR-related genes caused a decrease in *Rad51* expression, or whether the high-dose $CdCl_2$ treatment itself led to a decrease in *Rad51*.

## CONCLUSION

This study aimed to determine the potential changes in the efficiency of CRISPR-Cas9-mediated knock-in induced by treatment with high-dose $CdCl_2$, an MMR inhibitor. During homologous recombination, the expression of MMR-related genes was suppressed in HC11 cells treated with cadmium, and the mRNA and protein expression of the HR-related gene *Rad51* was decreased. Consequently, the efficiency of HR did not increase in cadmium-treated DNA MMR-deficient cells. Therefore, it appears that cadmium treatment, which induces DNA MMR deficiency and reduces the expression of the HR-related gene *Rad51*, is not an appropriate compound for improving HR efficiency.

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