Basic helix-loop-helix transcription factor DEC1 regulates the cisplatin-induced apoptotic pathway of human esophageal cancer cells

（DEC1 は食道癌細胞においてシスプラチン誘導アポトーシスを制御する）

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ABSTRACT

DEC1 [basic helix-loop-helix (BHLH)E40/Stra13/Sharp2) and DEC2 (BHLHE41/Sharp1) are BHLH transcription factors that are associated with the regulation of apoptosis, cell proliferation, and circadian rhythms, as well as malignancy in various cancers. However, the roles of DEC1 and DEC2 expression in esophageal cancer are poorly understood. In this study, we examined the roles of DEC1 and DEC2 in human esophageal cancer TE 5 and TE 10 cells that had been treated with *cis*-diamminedichloroplatinum (II) (cisplatin: CDDP). Expression of DEC1 and DEC2 was decreased with CDDP treatment in TE 5 cells; however, knockdown or overexpression of DEC1/DEC2 had little effects on CDDP-induced apoptosis in TE 5 cells. DEC1 expression was up-regulated in CDDP-treated TE 10 cells, whereas DEC2 expression was unchanged. DEC1 knockdown by siRNA in TE 10 decreased the amount of cleaved poly (ADP-ribose) polymerase (PARP) after treatment with CDDP, whereas DEC2 knockdown had no effects on the amount of cleaved PARP in both the presence and absence of CDDP. We also demonstrated that DEC1 overexpression promoted cleaved PARP expression, whereas DEC2 overexpression had no effects on the amount of cleaved PARP in TE 10 cells. These results suggested that DEC1 has pro-apoptotic effects on human esophageal cancer TE 10 cells of well-differentiated type.

INTORODUCTION

Esophageal cancer is the eighth most common cancer worldwide, and the sixth most common cause of death from cancer in 2012 (8). Squamous cell carcinoma is one of the most prevalent histological types of esophageal cancer. Traditionally, esophageal squamous cell carcinoma (ESCC) has been treated by multimodality therapies, including surgery, radiation, and chemotherapy. Recently, preoperative chemotherapy followed by radical esophagectomy has been the standard therapeutic approach to operable ESCC (1). CDDP (cisplatin) is a well-known anticancer agent clinically used for the treatment of various malignant tumors. CDDP regulates DNA repair, transcription inhibition, cell cycle arrest, and apoptosis induced by the expression of mitogen-activated protein kinases (MAPKs), p53, Bax, Bcl-2, Bcl-xL, Bim, caspases, and the cleavage of poly (ADP-ribose) polymerase (PARP) (4, 6, 12, 19, 20, 21, 26).

DEC1 [basic helix-loop-helix (BHLHE) 40/Stra13/Sharp2) and DEC2 (BHLHE41/Sharp1) are members of the BHLH superfamily of transcription factors that are related to the regulation of apoptosis, cell proliferation, circadian rhythms, and the response to hypoxia (11, 15, 23). DEC1 is overexpressed in the tumor tissues of breast, colon, and oral cancers (3, 5, 14). Previously, we investigated the role of DEC1 and DEC2 in CDDP-induced apoptosis in human oral squamous cell carcinoma CA9-22 and HSC-3 cells, and showed that DEC2 has anti-apoptotic effects on CDDP-induced apoptosis in HSC-3 cells involving Bim down-regulation (24). We also showed that

DEC1 and DEC2 have opposite properties in regulating apoptosis, *i.e.*, DEC2 has anti-apoptotic, whereas DEC1 has pro-apoptotic effects on human breast cancer MCF-7 cells (15, 23). However, the roles of DEC1 and DEC2 in apoptosis induced by antitumor drugs in ESCC are still unknown.

In the present study, we examined the effects of DEC1 and DEC2 on CDDP-induced apoptosis in two different types of human ESCC cells: TE 10 (well-differentiated ESCC) and TE 5 (poorly differentiated ESCC) cells. Our results suggest that DEC1 has pro-apoptotic effects on TE 10 cells.

MATERIALS AND METHODS

Cell culture and treatment

Human ESCC TE 10 and TE 5 cells were obtained from the Japanese Cell Resource Center for Biomedical Research. TE 10 and TE 5 represent well-differentiated and poorly differentiated human ESCC cells, respectively. Cells were cultured in RPMI-1640 medium (Gibco-BRL, Breda, The Netherlands) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO_2. In some experiments, cells were incubated with CDDP (Sigma-Aldrich Co., St. Louis, USA) at various concentrations for 24 h.

Short interference RNA (siRNA)

siRNA against DEC1 and DEC2 was synthesized by QIAGEN (Hilden, Germany). The sequences of DEC1, DEC2, and the negative control siRNA have been described previously (15). We used a different siRNA against DEC1 (DEC1 siRNA-2). The sense and antisense sequences of DEC1 siRNA-2 were as follows: 5′-GAAGCAUGUGAAAGCACUATT-3′ and 5′-UAGUGCUUUCACAUGCUUCAA-3′. For the siRNA transfection experiments, cells were seeded at a concentration of 5×10^4 cells per 35-mm well. After 24 h, siRNA were transfected into the cells using the Lipofectamine RNA iMAX reagent (Invitrogen, Carlsbad, CA, USA). After transfection, cells were incubated for 48 h and subjected to western blotting.

DEC1 and DEC2 overexpression

DEC1 and DEC2 overexpression was induced using the pcDNA vector as described previously (15). After transfection, cells were incubated for 24 h and subjected to western blotting.

Western blotting

Cells were lysed using M-PER lysis buffer (Thermo Scientific, Rockford, IL, USA), and their protein concentration (10 μg) determined using the bicinchoninic acid assay. The lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and the proteins transferred to

polyvinylidene fluoride membranes (Immobilion P, Millipore, Tokyo, Japan), which were incubated with antibodies. The ECL, ECL-plus, and ECL-advance Western Blotting Detection System (Amersham, Uppsala, Sweden) was used for detection.

Antibodies

The membranes for western blotting were incubated with antibodies specific to rabbit anti-human DEC1 (1:5,000; Novus Biologicals, Inc., Littleton, USA), rabbit anti-human DEC2 (1:5,000; H-72; Santa Cruz Biotechnology, Inc., Dallas, USA), rabbit anti-human PARP (1:1,000; Cell Signaling Technology, Inc., Danvers, USA), rabbit anti-human cleaved caspase-3 (1:5,000; Cell Signaling Technology, Inc.), rabbit anti-human cleaved caspase-8 (1:5,000; Cell Signaling Technology, Inc.), rabbit anti-human cleaved caspase-9 (1:10,000; Epitomics, Belmont, CA, USA), rabbit anti-human Bax (1:10,000; Santa Cruz Biotechnology), rabbit anti-human Bad (1:20,000; Cell Signaling Technology, Inc.), rabbit anti-human Bcl-2 (1:1,000; Cell Signaling Technology, Inc.), rabbit anti-human Bcl-xL (1:1,000; Cell Signaling Technology, Inc.), and rabbit anti-human actin (1:20,000; Sigma-Aldrich Co., St. Louis, USA), followed by horseradish peroxidase-conjugated secondary antibody (IBL, Fujioka, Gunma, Japan). Can Get Signal immunoreaction enhancer solution (Toyobo, Osaka, Japan) and Immunoshot immunoreaction enhancer solution (Cosmobio, Co, Ltd., Tokyo, Japan) were used to dilute the primary antibodies.

Cell viability assay

The cell viability assay was performed using the MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo phenyl)-2H-tetrazolium] assay as described previously (19).

Staining

TE 10 and TE 5 cells were seeded in a 4-chamber slide glass and incubated with or without CDDP (Sigma) for 24 h. Cell stain was carried out using the CnT-ST-100 stain kit (CellnTEC Advanced Cell Systems AG, Bern, Switzerland), in accordance with the manufacturer's instructions.

RESULTS

Effects of CDDP on DEC1/DEC2 expressions in TE 10 and TE 5 cells

We analyzed the effects of CDDP on DEC1/DEC2 expression in TE 10 and TE 5 cells. CDDP induced cell death in time- and dose-dependent manners in TE 10 and TE 5 cells using the MTS assay (Fig. 1A and 1B). The MTS assay showed that 50 μM and 100 μM of CDDP increased cell death to a greater extent in TE 10 cells than in TE 5 cells, suggesting that TE 5 cells are originally more CDDP-resistant than TE 10 cells. We also examined morphological changes using the CnT-ST-100 stain kit (Fig. 1C). CDDP treatment shrinked cell size and decreased number of colonies formed in both TE 10 and TE 5 cells. These changes were observed to a greater extent in TE 10 cells than in TE 5 cells. CDDP treatment for 24 h increased the amount of DEC1, cleaved PARP, cleaved caspase-3, cleaved caspase-8, Bax, and Bad, whereas it decreased Bcl-2 and Bcl-xL expression in TE 10 cells. The expression levels of DEC2 and cleaved caspase-9 were not affected by CDDP treatment in TE 10 cells (Fig. 2A). In TE 5 cells, cleaved PARP, cleaved caspase-3, cleaved caspase-8, and Bad expressions were increased, whereas DEC1, DEC2, and Bcl-xL expressions were decreased. Cleaved caspase-9, Bax, and Bcl-2 expressions were not affected by CDDP treatment in TE 5 cells (Fig. 2B).

Apoptotic effects in CDDP-treated TE 10 cells by DEC1 knockdown and overexpression

We examined whether knockdown or overexpression of DEC1/DEC2 affects apoptosis in TE 10 cells.

DEC1 knockdown in the presence of CDDP (50 μM) significantly decreased the amount of cleaved PARP, caspase-3, caspase-8, Bax, and Bad, whereas it increased Bcl-2 and Bcl-xL expressions (Fig. 3A). DEC1 overexpression in the presence of CDDP (50 μM) increased the amount of cleaved PARP, caspase-3, caspase-8, Bax, and Bad, whereas it decreased Bcl-2 and Bcl-xL expressions (Fig. 3B). Regardless of CDDP (50 μM) treatment, DEC1 knockdown or overexpression had no effects on the expressions of caspase-9 and DEC2. On the other hand, DEC2 knockdown or overexpression had little effects on the expressions of DEC1, cleaved PARP, caspase-3, and caspase-8 in TE 10 cells (Fig. 4A and 4B).

Apoptotic effects in TE 5 cells by DEC1/DEC2 knockdown and overexpression

We examined whether knockdown or overexpression of DEC1/DEC2 affects apoptosis in TE 5 cells. DEC1 and DEC2 knockdown or overexpression in the presence or absence of CDDP (50 μM) had no or little apoptotic effects on the expression of cleaved PARP, caspase-3, caspase-8, caspase-9, Bax, Bad, Bcl-2, and Bcl-xL in TE 5 cells. Any regulatory effects between DEC1 and DEC2 were not detected (Fig. 5A, 5B, 6A and 6B).

DISCUSSION

We previously examined the effects of DEC1 and DEC2 on CDDP-induced apoptosis in the human

oral squamous cancer cell lines HSC-3 and CA9-22. Our results suggested that DEC2 had anti-apoptotic effects and DEC1 had little effects on the CDDP-induced apoptosis in HSC-3 cells, whereas DEC1 and DEC2 had little effects on the CDDP-induced apoptosis by CDDP in CA9-22 cells (24). However, the role of DEC1 and DEC2 on apoptosis of esophageal cancer cell remains poorly understood. This is the first study focused on the functional analysis of DEC1 and DEC2 during CDDP-induced apoptosis in human esophageal cancer TE 10 and TE 5 cells.

DEC1 is expressed in various tumors and regulates responses to hypoxia, apoptosis, and the cell cycle (3, 5, 7, 14, 15). There are many environmental and endogenous factors affecting DEC1 expression, such as ionizing-radiation, hypoxia, hormones, growth factors, and cytokines (9, 10, 15, 17, 18, 22, 25). In the present study, we demonstrated that CDDP increased DEC1 expression and apoptosis-related proteins when it induced apoptosis in TE 10 cells, suggesting that CDDP treatment up-regulated the transcription of DEC1. CDDP, an antitumor drug, is thought to be one of the upstream factors regulating DEC1 expression in TE 10 cells, and DEC1 may regulate CDDP-induced apoptosis via sequential activation of caspase-8 and caspase-3 in TE 10 cells. We also demonstrated that DEC1 regulates Bcl-2, Bcl-xL, Bax, and Bad in TE 10 cells, indicating that DEC1 is related to CDDP-induced apoptotic cell death through mitochondrial pathways. However, our results showed that the expression of caspse-9, which was processed prior to mitochondrial cytochrome *c* release in response to TNF-receptor occupation in Bcl-xL-expressing cells (2, 9, 13, 16), was not significantly

changed. On the other hand, DEC1 had little effects on CDDP-induced apoptosis in TE 5 cells. MTS assay and apoptotic morphological changes showed that TE 5 cells were more resistant to CDDP, compared with TE 10 cells. Moreover, our previous reports have demonstrated that DEC1 has pro-apoptotic effect in MCF-7, a well-differentiated human breast adenocarcinoma cell line (23). Thus, we proposed that different effects on the DEC1-related apoptosis may depend on the differences in cellular differentiation (TE 10 cells and MCF-7 cells are well-differentiated carcinoma; and TE 5 cells are poorly differentiated type).

Our previous reports showed that DEC2 has anti-apoptotic effects in human oral cancer HSC-3 and human breast cancer MCF-7 (23, 24). Furthermore, DEC1 and DEC2 had opposite effects on apoptosis in MCF-7; *i.e.*, we speculated that a balance between DEC1 and DEC2 may contribute to the apoptotic pathways. However, DEC2 had little effects on the CDDP-induced apoptosis in both TE 5 and TE 10 cell lines, and any interaction between DEC1 and DEC2 mutually regulating each other's expression was not observed. These different effects of DEC2-related apoptosis and relationships between DEC1 and DEC2 may depend on the differences of cell lines and/or gene expression.

In conclusion, we have shown for the first time that the expression level of DEC1 is up-regulated by CDDP treatment in TE 10 cells. DEC1 may play an important role in the regulation of CDDP-induced apoptosis in human esophageal cancer TE 10 cells.

Acknowledgments

This study was supported by Grants-in-Aid for Science from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, a Grant for Hirosaki University Institutional Research, and the Fund for the Promotion of International Scientific Research.

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Figure legends

Figure 1. CDDP-induced time- and concentration-dependent cell death in TE 10 and TE 5 cells.

The cells were treated in Materials and Methods, and cell viability was measured using the MTS-assay (A: TE 10 cells, B: TE 5 cells). The values are shown as a percentage of each control. Each value represents the mean ± SE (bars) of three independent experiments (*P < 0.05, compared with the control). These cells were stained with the CnT-ST-100 stain kit, and observed in bright field (C).

Figure 2. Expression of DEC1/DEC2 and apoptosis-associated proteins by CDDP treatment.

TE 10 and TE 5 cells were treated with various concentrations of CDDP for 24 h. Cells were lysed, and the lysates were subjected to western blot analysis of DEC1, DEC2, cleaved PARP, cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, Bax, Bad, Bcl-2, Bcl-xL, and actin (A and B).

Figure 3. Expression of apoptosis-associated proteins in TE 10 cells by DEC1 knockdown or overexpression.

(A) TE 10 cells were transfected with control siRNA or siRNA against DEC1 and incubated for 48 h. Subsequently, cells were treated with or without CDDP (50 μM) and incubated for 24 h. (B) TE 10 cells were transfected with empty vector (pc DNA) or DEC1 expression plasmid (DEC1 pcDNA) and incubated for 24 h. Next, the cells were treated with CDDP (50 μM) and incubated

for 24 h, before being lysed.

Figure 4. Expression of apoptosis-associated proteins in TE 10 cells by DEC2 knockdown or overexpression.

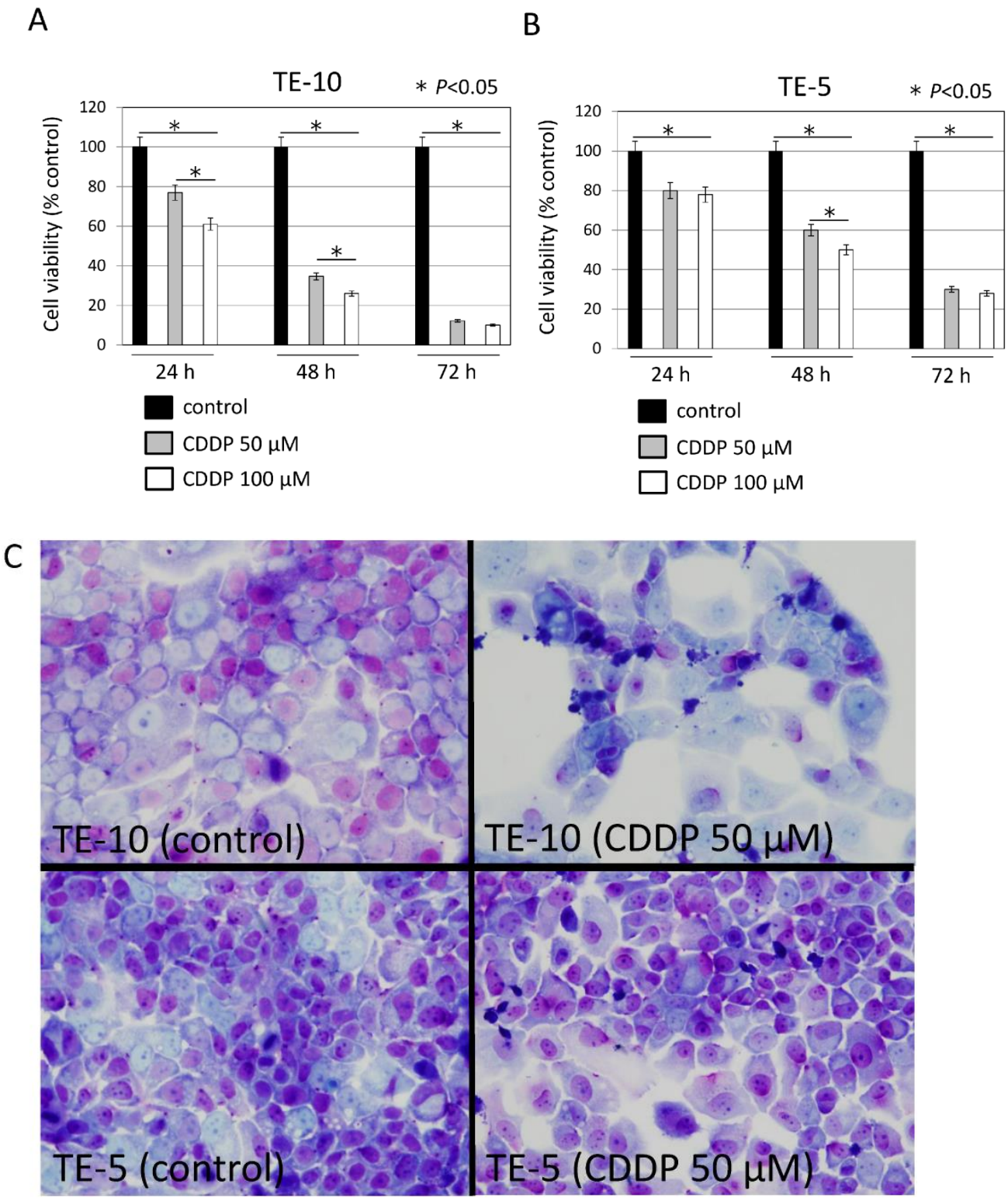
(A) TE 10 cells were transfected with control siRNA or siRNA against DEC2. (B) TE 10 cells were transfected with empty vector (pc DNA) or DEC2 expression plasmid (DEC2 pcDNA).

Figure 5. Expression of apoptosis-associated proteins in TE 5 cells by DEC1 knockdown or overexpression.

(A) TE 5 cells were transfected with control siRNA or siRNA against DEC1. (B) TE 5 cells were transfected with empty vector (pc DNA) or DEC1 expression plasmid (DEC1 pcDNA).

Figure 6. Expression of apoptosis-associated proteins in TE 5 cells by DEC2 knockdown or overexpression.

(A) TE 5 cells were transfected with control siRNA or siRNA against DEC2. (B) TE 5 cells were transfected with empty vector (pc DNA) or DEC2 expression plasmid (DEC2 pcDNA).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CDDP 100 μM
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* P<0.05
Cell viability (% control)
120
100
80
60
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24 h
48 h
72 h
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Figure 1

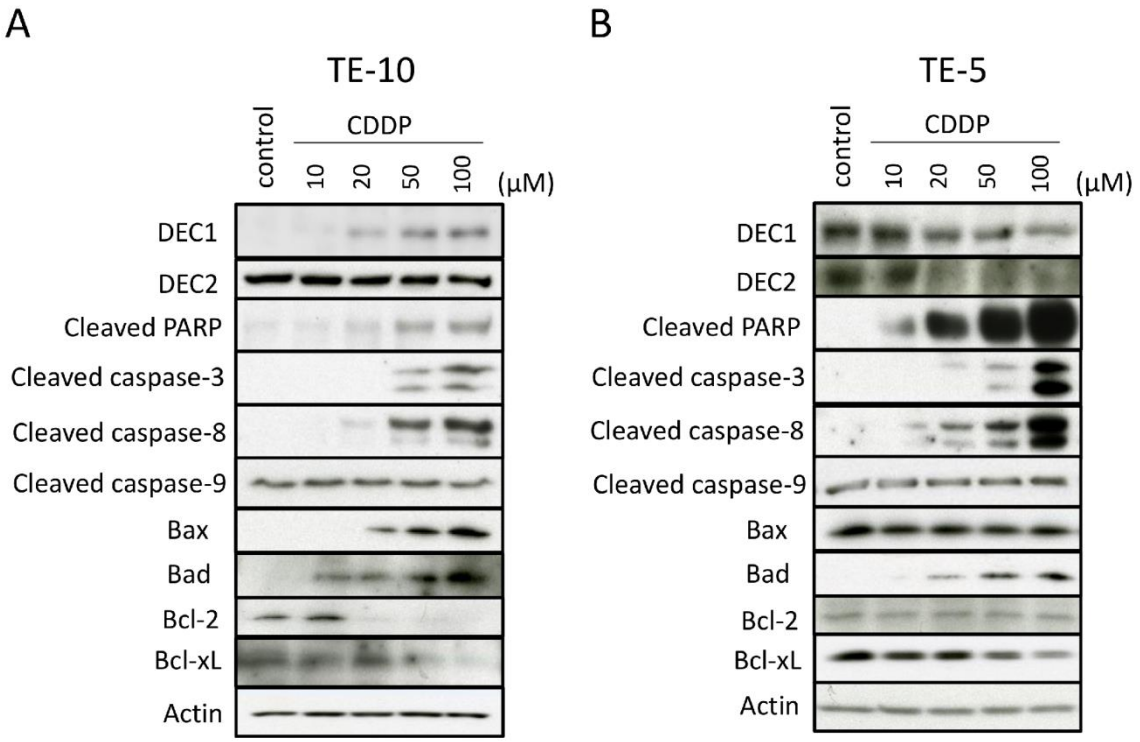
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Figure 2

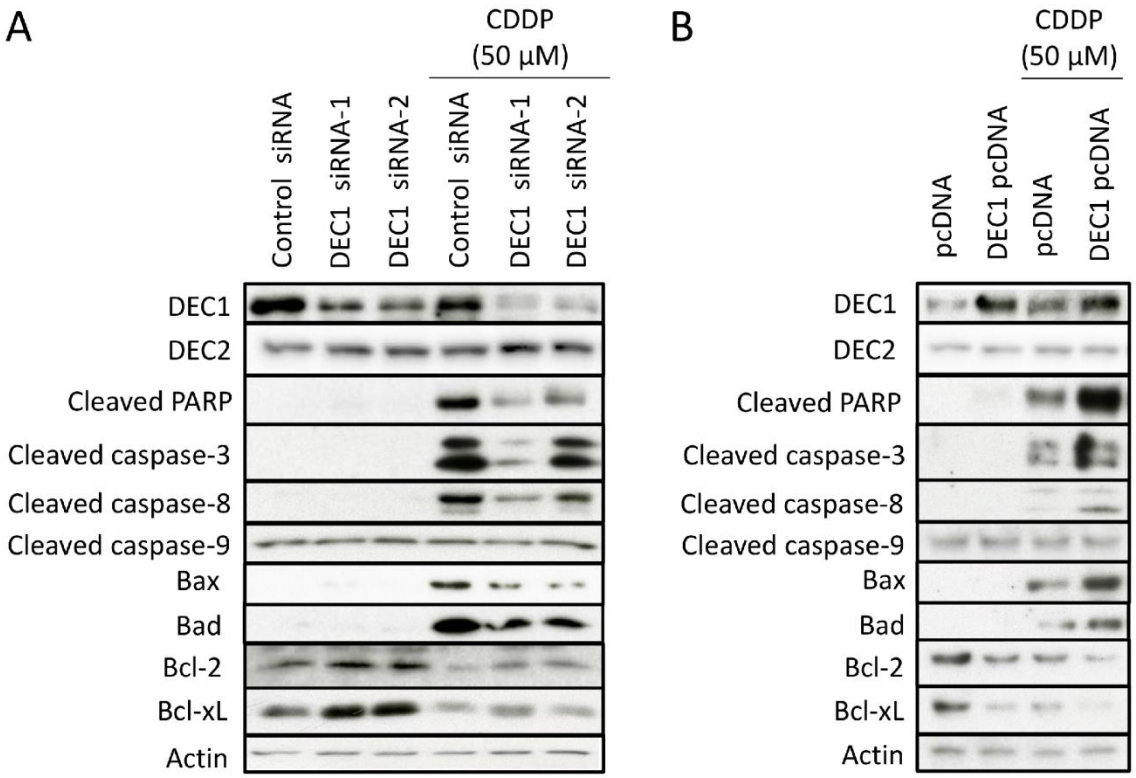
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Figure 3

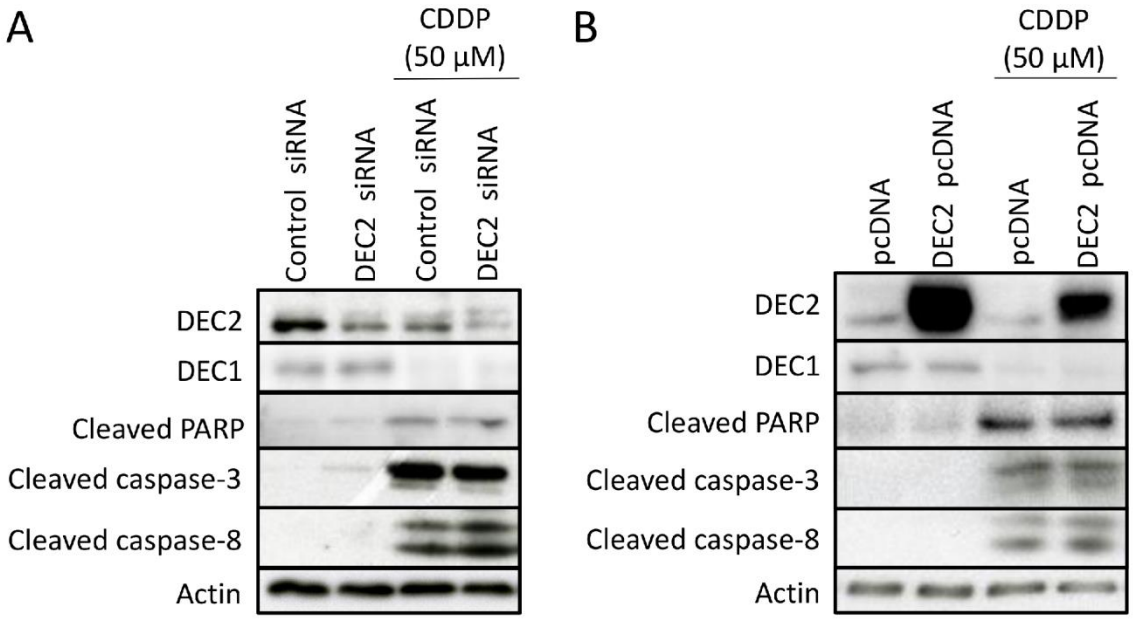
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Figure 4

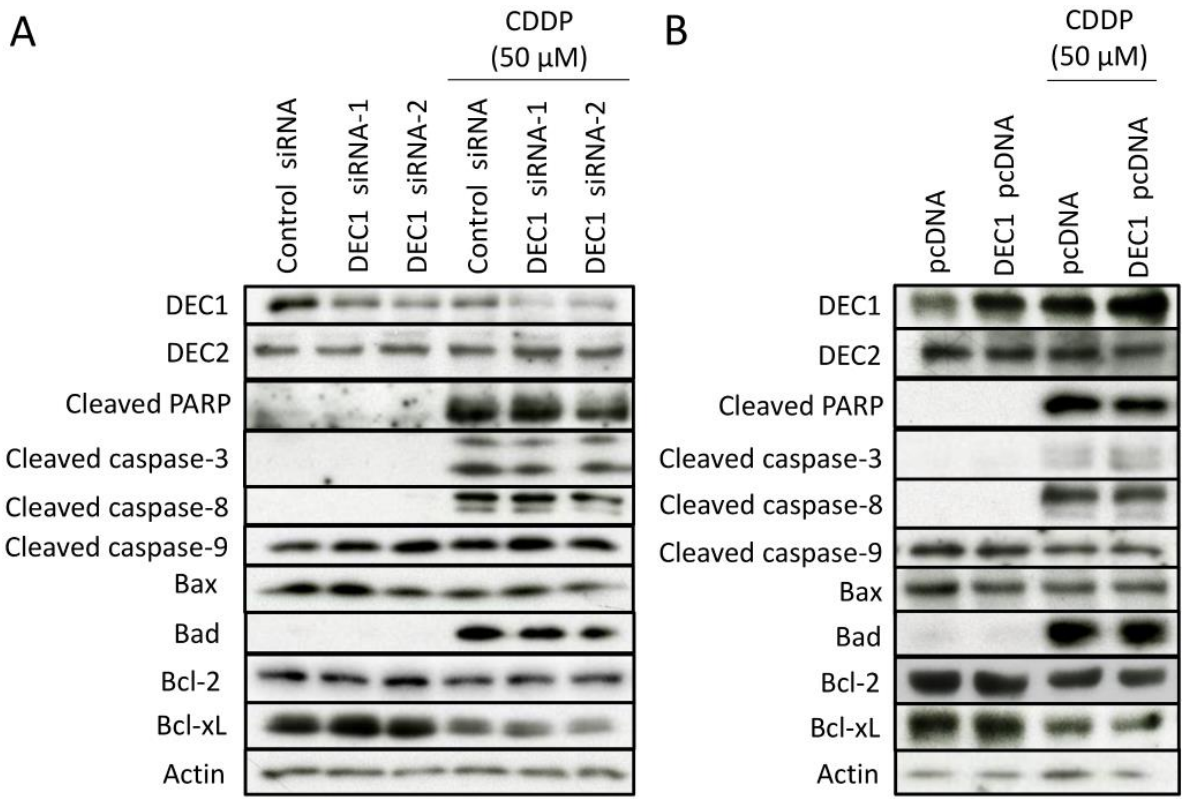
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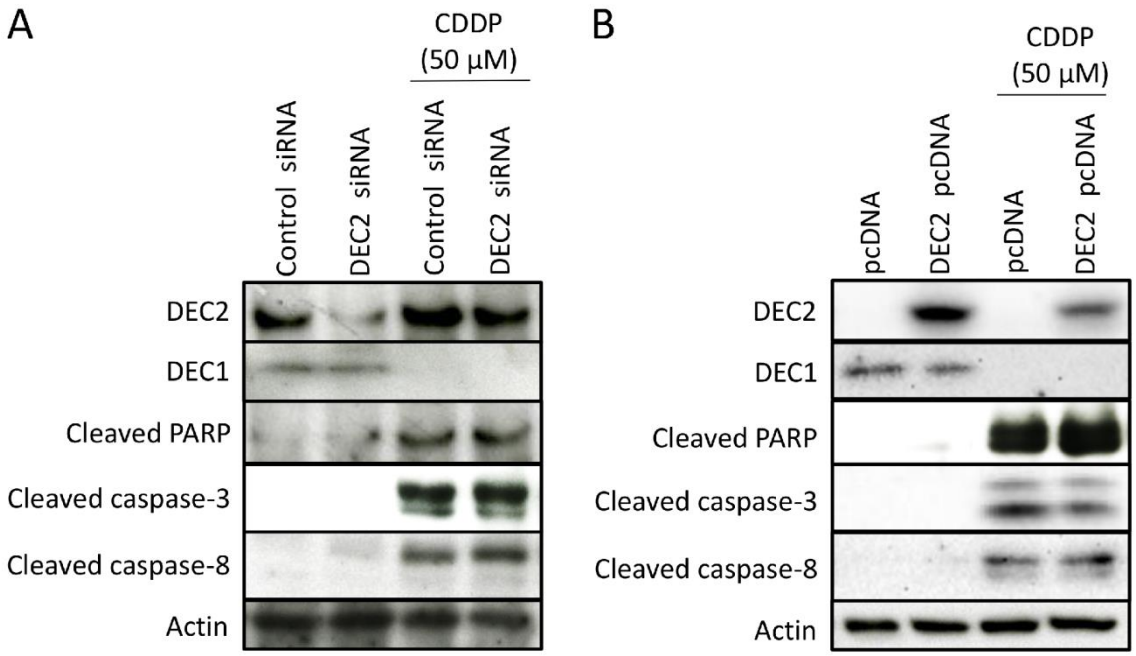
Figure 5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Actin
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(50 μM)
pcDNA
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pcDNA
DEC2 pcDNA
DEC2
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Cleaved PARP
Cleaved caspase-3
Cleaved caspase-8
Actin

Figure 6