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Exposure to low dose cadmium enhances FL83B cells proliferation through down-regulation of caspase-8 by DNA hypermethylation

Bo Wang, [a, b] Kun Ji, [*, c] Yue Wang, [d] Yang Li, [d] Yufeng Tang, [e] Junlian Gu, [*, f] and Lu Cai, [g]

[a] Department of Pathology, The Second Clinical Medical School of Inner Mongolia University for the Nationalities (Inner Mongolia Forestry General Hospital), Hulun Buir, China

[b] Molecular Pathological Research Institute of Inner Mongolia University for Nationalities, Hulun Buir, China

c Department of Pathophysiology, Shenyang Medical College, Shenyang, China

d Department of Pathophysiology, Norman Bethune College of Medicine, Jilin University, Changchun, China

e Department of Orthopedic trauma, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan, China

f Department of Pathology, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan, China

g Departments of Pediatrics, Radiation Oncology and Pharmacology & Toxicology, University of Louisville, Louisville, Kentucky, USA

*Address correspondence to:

Dr. Kun Ji, Department of Pathophysiology, Shenyang Medical College, Shenyang, 110034, China, E-mail: jikun200907@163.com or Dr. Junlian Gu, Department of Pathology, Shandong Provincial Qianfoshan Hospital, Shandong University, JiNan, 250014, China, E-mail: mypopular@126.com

Running title: cadmium exposure enhances cells proliferation

Abstract

Cadmium (Cd) is classified as a human carcinogen probably associated with epigenetic change. However, it's underlying mechanism and role in epigenetic is still poorly understand. DNA methylation is one of epigenetic mechanisms by which cells control expression. Our previous *in vivo* experiment has shown that caspase-8 gene promoter of the rat liver tissue was hypermethylated in Cd at 20 nmol/kg for 4 weeks. In the present study, we would disclose whether DNA methylation is also involved in this Cd-stimulated FL83B cell proliferation. When the FL83B cells were exposed to Cd at a low dose (0.085 μM) for just only 14 days, cell proliferation and DNMT methyltransferase expression and activity were increased, while the mRNA and protein of tumor gene caspase-8 were remarkably decreased, along with a significant decrease in cell apoptosis and increase in cell invasion and metastasis. Furthermore, caspase-8 gene promoter in Cd-exposed Fl83B cells was hypermethylated, consistent with our *in vivo* experiment. A DNA methylation inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), prevented Cd-stimulated cell proliferation, invasion and metastasis associated with recovered caspase-8 expression. These results suggest that low dose Cd may induce caspase-8 gene promoter hypermethylation, resulting in down-regulation of e its expression, and consequently promoting cell proliferation, invasion and metastasis and decreasing apoptosis, both which would contribute to Cd-induced carcinogenesis.

Key words: Cadmium, Caspase-8, DNA hypermethylation, Malignant transformation, Apoptosis, Proliferation, Invasion, Metastasis

Introduction

Cadmium (Cd) is a highly persistent heavy metal, and the short- and long-term health effects of Cd exposure have been reported in the human population. Some of the long-term effects include cancers of the lung, kidney, prostate, and testis [1]. Both epidemiological and experimental evidence suggest that Cd can induce malignant transformation of normal cells, such as bladder urothelial, testicular leydig cells and prostate epithelial cells [2-5]. Based on the results of epidemiological and experimental studies, Cd was classified as human carcinogens in 1993 by the International Agency for Research on Cancer [6]. Thus, the carcinogenic potential of Cd is well established. However, the mechanism of Cd-induced carcinogenesis is still not clear. Current evidence suggests that exposure to Cd induces malignant transformation through complex and multifactorial mechanisms. Most importantly it seems to be Cd interaction with epigenetic changes that was associated with promoter DNA methylation mechanism at the molecular levels.

Aberrant DNA methylation has been recognized as one of the most common molecular alterations in human neoplasia. Hypermethylation of gene-promoter regions is being revealed as one of the most frequent events that cause loss of gene function. By silencing key regulatory genes such as tumor suppressor genes, DNA methylation can provide the epigenetic equivalent of mutation/deletion during oncogenesis. Previous experimental evidence suggests that Cd at low-dose, for environmental exposure relevant concentrations, induces hypomethylation and mutations by inducing oxidative DNA damage and that it decreases tumor suppress-gene stability by inhibiting the repair of endogenous and exogenous DNA lesions, which in turn increase the probability of mutations and consequently cancer initiation by this metal. In addition, Goyer et al. found that a long-term exposure to Cd could result in the emergence of aberrant DNA methylation and malignant phenotypes including resistance to apoptosis, cell proliferation increase, invasion or

metastasis enhancement, broad-based changes of gene expression, and epigenetic alterations [7-8], suggesting that Cd is a weak mutagen and may act as an epigenetic carcinogen to repress the tumor gene function [9]. Cd-induced Hypomethylation and hypermutability due to inhibition of tumor gene repair in yeast has also been reported [10-11]. These studies clearly indicate that Cd exposure may cause genomic instability and gene inactivity partly through methylation changes. Although it has been demonstrated that, after a long-term exposure of Cd, DNA methylation could be facilitated [3,12-14] and the cells proliferation was increased, as the same time apoptosis was decreased [15-17], the exact relationship between Cd-induced alterations of DNA methylation and the malignant phenotypes features remains to be unclear.

Caspase-8, a cysteine protease, is known as the initiator caspase of the extrinsic apoptosis pathway, which is triggered by several apoptosis signals that are related to death receptors. Absent or downregulation of Caspase-8 could cause resistance to apoptosis and is correlated with unfavorable disease outcome, such as childhood medulloblastoma and neuroblastoma[18-19]. The absence or downregulation of Caspase-8 may be due to epigenetic changes. Studies have also indicated that Caspase-8 function loss was previously attributed to DNA hypermethylation in as many as 61% of human neuroblastoma cases [20]. Furthermore, Caspase-8 expression could be down regulated by DNA methylation in many types of cancer cells such as HCC, lung carcinoma and haematological cancer [21-23]. Our previous study also found that rats were exposed to Cd at 20nmol/kg every other day for 4 weeks, and gene methylation was analyzed at the 48th week with methylated DNA immunoprecipitation-CpG island microarray, the liver displayed the aberrant Caspase-8 gene methylation and a persisitent increase in cell proliferation, steatosis and preneoplasia [24]. This suggested that hypermethylation of Caspase-8 may contribute to liver malignant transformation and eventual carcinogenesis via its inactivation in regulating apoptosis cell death. So the aim of the present study is to further verify the possibility whether the

hypermethylation of Caspase-8 could be involved in a low dose Cd-exposed enhanced FL83B mouse hepatocytes malignant transformation *in vitro*. If so, ongoing more investigations into the relationship of Caspase-8 gene silencing and its promoter hypermethylation in human liver malignancy are helping to prohibit cancer initiation and progression, and develop novel strategies for diagnosis, prognosis and treatment.

Materials and Methods

Cell culture and Chemicals

Mouse hepatocytes cell line (FL83B), was obtained from the American Type Cell Culture and cultured in F12 medium supplemented with 2% fetal bovine serum plus glutamine and antibiotics. Cadmium chloride (purity, 96.6%) and 5-aza-2'- deoxycytidine (5-aza-dC) were purchased from Sigma chemical company (Sigma–Aldrich, Shanghai, China).

Cell proliferation assay

Approximately 1×10^3 FL83B cells were treated for 14 days in Cd medium with or without 5-aza-dC to determine the effect of Cd on the growth of cells. After different treatments, both floating and adherent cells were collected and cell growth was evaluated using the 2, 3- Bis - (2-methoxy-4-nitro-5-sulfophenyl) - 2H - tetrazolium- 5 - carboxanilide (XTT) assay.

Real-time quantitative RT-PCR (qPCR) analysis

Total RNA was isolated from the FI83B cells with a different treatment using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Approximately, 2 μg of total RNA was converted to complementary cDNA using a commercially available RT-PCR kit (Promage, Madison, WI, USA). qPCR was performed using the FastStart Universal SYBR Green Master reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol with the ABI PRISM 7900HT Sequence Detection System. The primers used were as follows:

GAPDH sense: 5'- AGAAGGCTGGGGCTCATTTG - 3' and

antisense: 5'- AGGGGCCATCCACAGTCTTC- 3';

Caspase-8 sense: 5'- CCAGAGACTCCAGGAAAAGAGA- 3' and

antisense: 5'- GATAGAGCATGACCCTGTAGGC- 3'.

Western blot assay

To determine the changes in protein levels, western blot analysis was performed in FL83B cells with different treatments. The lysates were run on SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The following antibodies were used: monoclonal Caspase-8 (Cell Signaling Technology, Boston, MA, USA), PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (Santa Cruz), Cleaved caspase-3 (Cell Signaling Technology), TGF-β (Santa Cruz) and TNF-α (Cell Signaling Technology).

Bisulfite modification and Methylation Specific PCR (MSP)

Bisulfite conversion of genomic DNA was carried out using Zymo EZ DNA Methylation-Gold™ kit (D5005, Zymo Research Corp, Orange, CA) according to the manufacture's instructions. This process converts unmethylated cytosine residues to uracil, while methylated cytosine residues remain unchanged. Bisulfite modified DNA was used as a template, and then EpiTect Methyl PCR Assay (SABiosciences, Frederick, MD, USA) was employed to amplification. DNA methylation-sensitive restriction enzymes were used to obtain the product containing hypermethylated DNA sequences. The exact protocol was carried out as described previously [25]. The primer sequences for MSP are as follows:

Caspase-8 methylated forward：5'-TGTTGTTTGGGTAACGTATCGA- 3', methylated

reverse：5'-CCCTACTTAACTTAACCCTACTCGAC-3'；

and unmethylated forward：5'-TTGTTGTTTGGGTAATGTATTGA-3' ,

unmethylated reverse：5'-CAACCCTACTTAACTTAACCCTACTCA-3' .

The PCR products were electrophoresed in 1 % agarose gel containing ethidium bromide and

visualized by GIS Gelatumimagin system (Tanon, Shanghai, China).

Nuclear extraction and DNMT Activity Assay

Nuclear extracts were isolated using the EpiQuik Nuclear Extraction Kit (Epigentek Group Inc., USA). DNMT activity was calculated by using EpiQuikTM DNA Methyltransferase Activity/ Inhibition Assay Kit (Fluorometric, Epigentek Group Inc., USA). Nuclear extracts were incubated with methylation substrate for 1 h at 37□ and then exposed to the capture antibody for 60 min and the detection antibody for 30 min at room temperature. Absorbance was determined by using a microplate spectrophotometer at 450 nm and calculated according to the formula: (Sample OD-blank OD)/ (sample volume) ×1000.

Wound scratch assay

Scratch wound was made by scraping the cell monolayer across the cover glass with a sterile cell lifter (3008; Corning, NY, USA). We changed the culture medium immediately after wounding to prevent the medium from being conditioned with cell debris and factors released from the detached cells. Cells were then allowed to migrate for 14 days.

Transwell assay

Transwell analyses were carried out as described previously [26]. The FL83B cells with different treatment were then inoculated into the upper compartment of the Transwell chambers at 100 cells/ml while 1×10^4/well of F-12 supplemented with 2% FBS was added to the lower chamber of the Transwell chambers. The migrated cells remaining on the bottom surface were stained with 0.5% crystal violet for 3 min, and then examined by fluorescence microscopy. The stained insert was washed thoroughly, dissolved with 33% acetic acid, and the absorbance at 595 nm was measured.

TUNEL assay and immunohistochemical staining

Cell sections of different treatment were fixed in 4% formaldehyde in PBS for 25 minutes at 4°C. After washing with PBS, the samples were TUNEL stained according to the

manufacturer's instructions (Promega). Samples were analyzed by microscopy (Olympus, Japan). Cell sections of different treatment were processed for immunohistochemistry as described previously [26-28]

Statistical analysis

Results are expressed as mean values±standard deviation (SD). One-way ANOVA was performed among the groups. All experiments were repeated three times. All statistical analysis was performed using the SPSS19.0 statistical software (SPSS statistics, IBM, USA).

Results

Effects of different concentrations of Cd on FL83B cell proliferation

In order to know the cytotoxic action of low doses of Cd, we detected cell proliferation using the XTT assay. Results revealed that, when the cells were treated with 0～0.125 μM Cd, cell proliferation was enhanced in a concentration-related manner, and the maximal proliferation was induced by 0.125 μM Cd for just only 14 days **(Figure 1A)**. To further seek for the optimum concentration of Cd-induced cell proliferation, we finely segmented the concentration of around 0.125 μM Cd, and the results demonstrated that cell proliferation induced by 0.085 μM Cd was significantly increased than other concentrations **(Figure 1B)**. These data indicated that Cd could enhance cell proliferation at a series of low concentration; especially the cell proliferation induced by 0.085 μM was increased in maximum capacity for only just 14 days.

Effect of long-term Cd exposure on the expressions of Caspase-8 and its related protein in FL83B cells

From the above result, we chose low-dose Cd concentrations, mainly 0.085 and 0.145 μM for the following experiment of treatment. To investigate the effect of Cd exposure on the expression of Caspase-8, FL83B cells were continuously exposed to 0.085, 0. 145 and 0.165

μM Cd for 14 days and the expressions of Caspase-8 were examined by real time RT-PCR **(Figure 2A)** and Western blot **(Figure 2B)**. The results showed that the expression of Caspase-8 was significantly decreased in cells exposed to 0.085, 0.145 and 0.165 μM Cd for 14 days. Especially at 0.085 μM Cd exposure, the Caspase-8 mRNA and proteins levels were decreased by 33.1% and 59.2%, respectively.

From the above, we know the protein expression of Caspase-8 was decreased by Cd exposure. To further determine whether Cd exposure could affect the expression of Caspase-8 down-stream relative proteins, we detected the protein expression of Caspase-3, PCNA, TNF-a, TGF-β by Western blot **(Figure 2C)**. The results demonstrated that cell treated with 0.085 and 0.145 μM Cd significantly decreased the expression of Caspase-3, and increased the expression of PCNA and TNF-a. Unlike our previous *in vivo* experiments, the expression of TGF-β expression did not obviously changed. It may because the periods of Cd exposure is not long enough to react to the expression of TGF-β, which is an index of advanced fibrosis.

Effect of long-term Cd exposure on DNMTs levels and DNMT activity in FL83B cells

To investigate the effect of low-dose Cd exposure on the expression of different enzyme activity assay related to methylation, we performed DNMTs levels and activity by Epiquik assay kits. FL83B cells were continuously exposed to 0, 0.085 and 0.145 μM Cd for 14 days，then the levels as well as the activity of DNMTs were measured. As shown in **Figure 3,** the expressions of DNMT1, DNMT3a and DNMT3b were increased in cells exposed 0.085 and 0.145 μM Cd. At 0.085 μM Cd exposure, the DNMT1 **(Figure 3A)**, DNMT3a **(Figure 3B)** and DNMT3b **(Figure 3C)** protein levels were increased by 57.4%, 52.1% and 42.9%, respectively. Consistent with DNMTs levels, the total DNMTs activity **(Figure 3D)** was significantly enhanced by 0.085 and 0.145 μM Cd, and at 0.085 μM Cd exposure, the activity was increased in maximum.

Effects of the demethylating agent 5-aza-dC on the proliferation and expression of Caspase-8 in the Cd-exposed FL83B cells

To test whether the enhancement of Caspase-8 methylation contributes to the increase of cell proliferation, the Cd-exposed cells were further treated with 0.25 or 0.5 μM 5-aza-dC, an inhibitor of DNA methyltransferase. It was found that the Cd-stimulated cell proliferation was totally eliminated by this 5-aza-dC treatment for 3 days. And 0.5 μM 5-aza-dC had more strong inhibition effect than 0.25 μM 5-aza-dC **(Figure 4A)**. To determine whether DNA methylation of the Caspase-8 gene is involved in the decreases of expression in the 14-day low-dose Cd exposure cells, the effect of 5-aza-dC on the expression of Caspase-8 mRNA and protein were examined. Results showed that the mRNA levels of Caspase-8 in the cells exposed to any Cd concentration were markedly decreased than that in control cells, but it recovered to the normal level after the cells were treated with 0.5 μM 5-aza-dC **(Figure 4B).** Furthermore, the result of protein expression of Caspase-8 was consistent with Caspase-8 mRNA expression in the Cd-treated cells with or without 5-aza-dC treatment **(Figure 4C-F)**. These results demonstrated that the hypermethylation of Caspase-8 could decrease Caspase-8 mRNA and protein levels, and 5-aza-dC could recover to its normal levels, and improve over proliferation status of 14-day Cd-exposed FL83B cells.

Effect of 14 days Cd exposure on Caspase-8 DNA methylation

Since Caspase-8 promoter methylation in the FL83B cells exposure to low dose Cd was found to be associated with the significant increase in proliferation **(Figure 1 & 4)**, treating cells with low dose Cd for 14 days with or without 5-aza-dC treatment for 3 days, the results showed that Caspase-8 promoter methylation levels of FL83B cells were significantly high in the Cd-treated group, but not in the Cd/5-aza-dC-treated group compared to the control **(Figure 5)**. So the Caspase-8 promoter hypermethylation may be the reason for it's over proliferation.

Effect of Cd and 5-aza-dC on the apoptosis of FL83B cells

The proliferation of Cd was further determined by colony formation assay. Treatment with 0.085 or 0.145 μM Cd significantly promoted single cell colony formation compared with non-Cd treatment, and 5-aza-dC could recover this phenomenon **(Figure 6A&D)**. To further determine the function of low dose Cd in apoptosis of FL83B cells, we performed in situ nick end labeling (TUNEL) for apoptotic cell detection. In this part, we added 5 μM adriamycin (ADR) as a stimuli factor to induce apoptosis. The TUNEL assay also showed that Cd significantly decreases the apoptotic death, and 5-aza-dC could recover this phenomenon **(Figure 6B&E)**.

To determine the potential mechanism of cell proliferation and apoptosis, expression of proliferation-related protein and apoptosis proteins were examined. Cd-exposed cells significantly increased the expression of PCNA **(Figure 6C&F)**, which acts as a marker for cells in the early G1 and S phase of the cell cycle and proliferative indexes of tumor cells. 0.085 μM Cd exposures caused maximal enhancement of cell proliferation, because the number of PCNA positive cells was significantly increased compared with those of other groups. Furthermore, Cd-exposed cell significantly decreased the expression of Caspase-3 **(Figure 6F)**, which plays a central role in the execution-phase of cell apoptosis.

Effect of Cd and 5-aza-dC on the invasion and metastasis of FL83B cells

To examine whether Cd affects s cell invasion and metastasis *in vitro*, a wound-scratch healing model was used. A scratch that measured the average and directional migration of the cell populations was introduced into the confluent monolayer with different treatments. The results showed a significant increase in motility of the Cd exposure group compared with the control group, and 5-aza-dC could recover the cell motility to normal level **(Figure 7A&C)**.

To further investigate the effects of Cd exposure on cell invasion, we determined the ability of Cd treated cells to invade through the Transwell assay. As shown in **Figrue 7B and**

D, a significant increase in the number of invading cells was observed for Cd exposure group after 14 days compared with the control group, and 5-aza-dC also recover the cell invasion to normal level. To elucidate the mechanism of the enhancement of invasion after Cd exposure, we further examined the protein expression levels of MMP-2 and MMP-9. The Cd exposure group exhibited increased levels of MMP-2 and MMP-9 expression compared with control group **(Figure. 7E)**. Taken together, Cd exposure could cause maximal enhancement of invasion and migration, and related mechanisms, may be through up-regulation of MMP2 and MMP9 proteins**.**

Discussion

Cd is a widespread toxic pollutant of occupational and environmental concern because of its diverse toxic effects: extremely protracted biological half-life (approximately 20～30 years in humans), low rate of excretion from the body and storage predominantly in soft tissues (primarily, liver and kidneys). It was reported that Cd is a suspected human hepatic carcinogen and can induce hepatic tumors in rats [24,29], making the hepatic epithelium a suspect *in vivo* target of Cd. However, the underlying mechanisms involved in Cd-induced carcinogenesis remain unclear. Aberrant DNA methylation of promoter regions may represent one possible mechanism in Cd-induced carcinogenesis. DNA methylation can participate in long-range epigenetic silencing that targets many neighboring genes and has been shown to occur in several types of clinical cancers. It was reported that widespread hypomethylation accompanied by region-specific CpG island hypermethylation is frequently observed in many tumor suppressor genes such as P16, RB and BRCA1 [30-32], and can result in transcriptional down-regulation or silencing. Our previous study have identified a link between one important epigenetic modifications, DNA methylation, in regulation of hepatic gene expression and silencing. Those results suggested that Cd-induced global gene

hypermethylation, most likely tumor suppressor gene promoter hypermethylation down-regulated its expression, leading to the decreased hepatic apoptosis and increased preneoplastic lesions [24].

Considering the persistant nature and long residence time of Cd in organisms, the present study was to determine the effects of long-term low dose Cd exposure on FL83B cells, a mouse hepatocytes cell line. Through the experiment *in vitro* was used to verify the results *in vivo* and further explore its related mechanism. So there are two major implications of our *in vitro* study: (a) the malignant transformation of normal mouse hepatic cells is a compelling evidence that Cd indeed has the potential to be a human hepatic carcinogen; and (b) we can elucidate the genetic events involved in Cd-induced malignant transformation by doing some research on DNA methylation at the cellular and molecular levels and potentially develop a genetic "signature" for Cd-induced hepatic tumors. To address these questions, cells were given low-dose (0.085 and 0.145 μM) Cd treatment for 14 days. We found the 14-day exposure to noncytotoxic doses of Cd demonstrated a correlation with increased DNA methylation of the tumor suppressor gene Caspase-8. Our results also showed Cd exposure resulted in an increase of cell proliferation; decrease of cell apoptosis, as well as enhancement of invasion and metastasis in FL83B cells (Figure 6 and 7). It has been well known that resistance to apoptosis, cell proliferation increase, enhancement of invasion and metastasis and DNA methylation aberrance are the characteristic features of transformed cell and most types of cancer [33-35]. In fact, our previous reports have been shown that chronic Cd exposure could result in hepatic lesions and transformation. Therefore, the DNA methylation aberrance may be a potential mechanism of Cd-induced carcinogenesis in the hepatic system [24].

This study first showed that the enhancement of Caspase-8 gene methylation contributes to the increase of cell proliferation during 14-day low-dose Cd exposure in FL83B cells. It

was reported that Cd exposure could enhance excessive cell proliferation at low concentrations and promote cell apoptosis at high concentrations [3,17,36], and could also change the DNA methylation levels and silence some tumor suppressor genes [3,12-13,37]. Some researcher such as Dexiao-Yuan and Benbrahim-Tallaa have identified that chronic exposure Cd could induce malignant transformation associated with global DNA hypermethylation levels, enhancement of DMMTs levels and activity, and suppression of RASSF1a and P16 genes [13,30]. In general, inactivation of tumor suppressor gene is a result of the activation of DNMTs [38-39]. It was also reported that exposure of human lymphocyte HMy2 and lung fibroblast cells to Cd resulted in the increase in DNMTs levels and activity [12,30] and this phenomenon is further validated by our present study. Exposure of mouse FL83B cells to low-dose Cd for 14 days resulted in the increase in protein levels and activity of DNMT1, DNMT3a and DNMT3b (Figure 3). It is well known that DNMT1 is required for the maintenance of DNA methylation, while DNMT3a and DNMT3b are for de novo DNA methylation [40-42], suggesting that DNMTs plays an important role in both development and maintenance of DNA methylation. However, further studies are required to clarify whether hypermethylation of Caspase-8 is responsible for the over proliferation of Cd-exposed FL83B cells.

With MSP assays, we further validated that the putative CpG island of Caspase-8 gene in the low-dose and long-term Cd exposed cells was hypermethylated. But treatment of 5-aza-dC could result in the loss of methy-cytosine in the CpG island of Caspase-8 and the recovery of Caspase-8 expression, indicating that the effect of 5-aza-dC on the recovery of Caspase-8 expression is actually due to the demethylation of its CpG island (Figure 4 and 5). More importantly, as the same reason that it could also eliminate the Cd-stimulated cell over proliferation and resistance of apoptosis (Figure 6). These data demonstrated clearly that DNA hypermethylation of Caspase-8 gene, as a well-known tumor suppressor gene, is at

least partly responsible for the down-regulation of Caspase-8 expression and thus contributes to the over proliferation and apoptosis resistance of Cd-enhanced cells.

A role of Cd in enhancing cell malignant progression has been proposed recently based on both *in vitro* and *in vivo* studies [43-44]. In our present work, repeated Cd exposures clearly enhanced malignant progression of FL83B cells, as assessed by the rate of regional invasiveness and distant metastases. The elevated MMP-2 and MMP-9 levels observed in FL83B cells are also consistent with previous data from primary cultures derived from human prostate tumors with Cd treatment [45], which showed elevated expression of MMP-2 or MMP-9 has indicated the progression to a malignant state. Overall, the rapid development and pronounced invasiveness of tumors derived from FL83B cells provided persuasive evidence that Cd could enhance tumor progression (Figure 7). Furthermore, 5-aza-dC treatment could also remove the Cd-enhanced invasion and metastasis.

In summary, low-dose and chronic Cd exposure could induce malignant transformation of human hepatic cells *in vitro*, producing highly aggressive malignant progression of FL83B cells. This is the first report of Cd-induced malignant transformation of a mouse hepatocytes cells *in vitro*. In addition, this study provides compelling evidence that Cd has the potential to be a hepatic carcinogen and we demonstrated that the transcriptional silencing of Caspase-8 induced by Cd exposure is correlated with the hypermethylation of CpG islands in the Caspase-8 gene promoter. This study provides evidence that DNA hypermethylation of Caspase-8 contributes to malignant transformation. Further comparison between the transformed and control cells should lead to a better understanding of the DNA hypermethylation involved in Cd-induced carcinogenesis and, perhaps, a molecular "fingerprint" for the identification of Cd-induced hepatic malignancies.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Effect of Cd on the proliferation of FL83B cells. A, Cells were exposed to 0～5 μM Cd for 14 days to detect the cytotoxic action of Cd, and the non-toxic concentrations of 0～0.125 μM were chosen for 14 day Cd exposure. B, To further determine the optimal concentration of Cd on maximal proliferation of FL83B cells, the concentration was finely segmented between 0 and 0.25 μM to detect the cytotoxic action of Cd. **P* < 0.05 compared to the control without Cd exposure.

Figure 2. Effect of long-term Cd exposure on the expressions of Caspase-8 mRNA, protein and relative proteins in FL83B cells. Cells were exposed to 0, 0.085, 0.145 and 0.165 μM Cd for 14 days. A and B, the mRNA (A) and protein (B) expressions were measured by quantitative real-time RT-PCR and Western blot. C, The expression of Caspase-8 relative proteins (Cleaved caspase3, PCNA, TNFα and TGFβ) were detected by western blot. **P* < 0.05 compared to the control without Cd exposure.

Figure 3. Effect of long-term Cd exposure on DNMTs levels and DNMT activity in FL83B cells. Cells were exposed to 0, 0.085 and 0.145 μM Cd for 14 days. The levels of the DNMT1 (A), DNMT3A (B), DNMT3B (C) and DNMT activity (D) were measured. **P* < 0.05 compared to the control without Cd exposure.

Figure 4. Effects of the demethylating agent 5-aza-dC on the proliferation and expression of Caspase-8 in the Cd-exposed FL83B cells. Cells exposed to Cd for 14 days were grown in the presence or absence of 0.25 or 0.5 μM 5-aza-dC for 3 days. (A) Effect of 5-aza-dC on cell proliferation was examined by XTT, and 0.5 μM 5-aza-dC chosen as the following treatment dose. (B) Effect of 0.5 μM 5-aza-dC on the expression of Caspase-8 mRNA was

detected by qPCR. (C-F) Effect of 0.5 μM 5-aza-dC on the expression of Caspase-8 protein was detected by Western blot (C) and immunohistochemical staining (×200). (D), and semiquantitative analysis for Caspase-8 expression (E& F). Data shown are means ±S.D. of three separate experiments. *$P < 0.05$ compared to control cells without Cd exposure. $^{\#}P < 0.05$ compared to cells without 5-aza-dC treatment.

Figure 5. Effect of Cd on the status of Caspase-8 gene promoter methylation. FLB3B cells were treated with Cd at 0.085 and 0.145 μM for 14 days, and the Cd treated cells were given with or without the methylation inhibitor 5-aza-dC treatment for 3 days. After different treatments, the cells were collected for the analysis of Caspase-8 gene methylatiom status with EpiTect Methyl qPCR assay. (1~6, Methylation primer; 7~12, Unmethylation primer).

Figure 6. Effect of Cd and 5-aza-dC on the proliferation and apoptosis of FL83B cells. A, Effect of Cd exposure on cells proliferation was detected by cell colony forming assay. B, Effect of Cd exposure on cells apoptosis was detected by TUNEL assay (×200). C, The protein expression of PCNA was detected by immunohistochemical staining (×200). D, The results of the statistical analysis for the plate colony forming. E, the average data of TUNEL-positive cell are presented for the results after treatment. G, Semiquantitative analysis for the percentage of PCNA positive staining. Data shown are means±SD of three separate experiments. F, the protein expression of Cleaved Caspase-3 was detected by Western blot. *$P < 0.05$ compared to control cells without Cd exposure. $^{\#}P < 0.05$ compared to cells without 5-aza-dC treatment.

Figure 7. Effect of Cd and 5-aza-dC on the invasion and metastasis of FL83B cells. A, Migration abilities as determined by wound assays (×100). B, Invasion of cells through a

basement membrane (Matrigel) was detected in Transwell chamber assays (×100). C, The wound gaps were measured after 14 day's Cd exposure and the results were expressed as the average wound gap. The data represent the means±SD of triplicate experiments. D, the results were expressed as the numbers of migrated cells relative to untreated controls. The data represent the means±SD of three experiments. E, MMP-2 and MMP-9 protein expression were determined by Western blot assay and quantification of protein levels. The data are presented as the mean±SD of triplicate experiments. *$P < 0.05$ compared to control cells without Cd exposure. $^{\#}P < 0.05$ compared to cells without 5-aza-dC treatment.

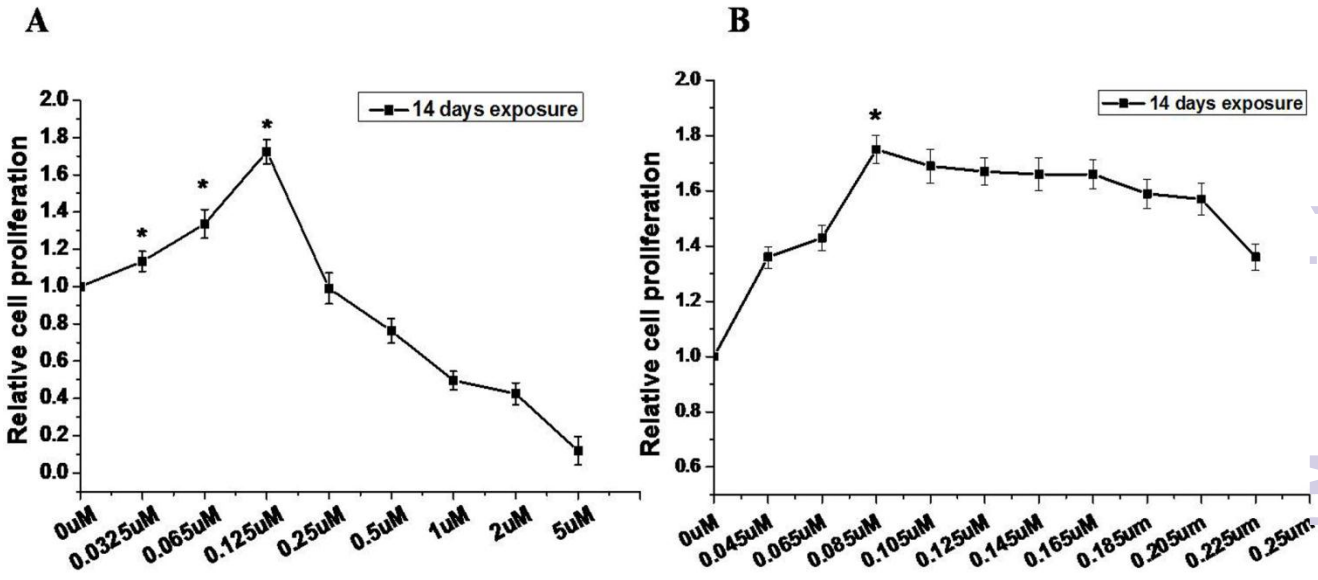
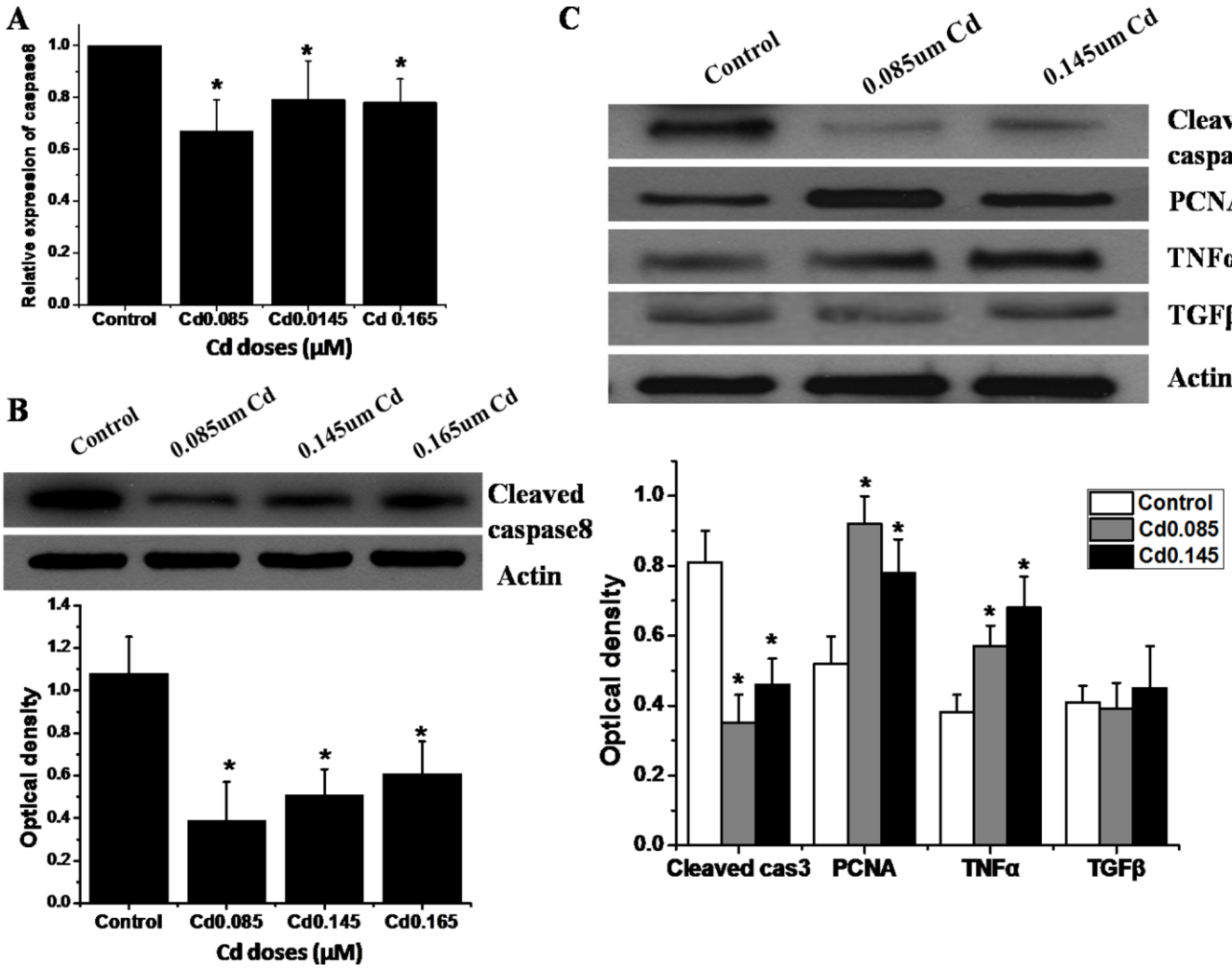


Figure 1.



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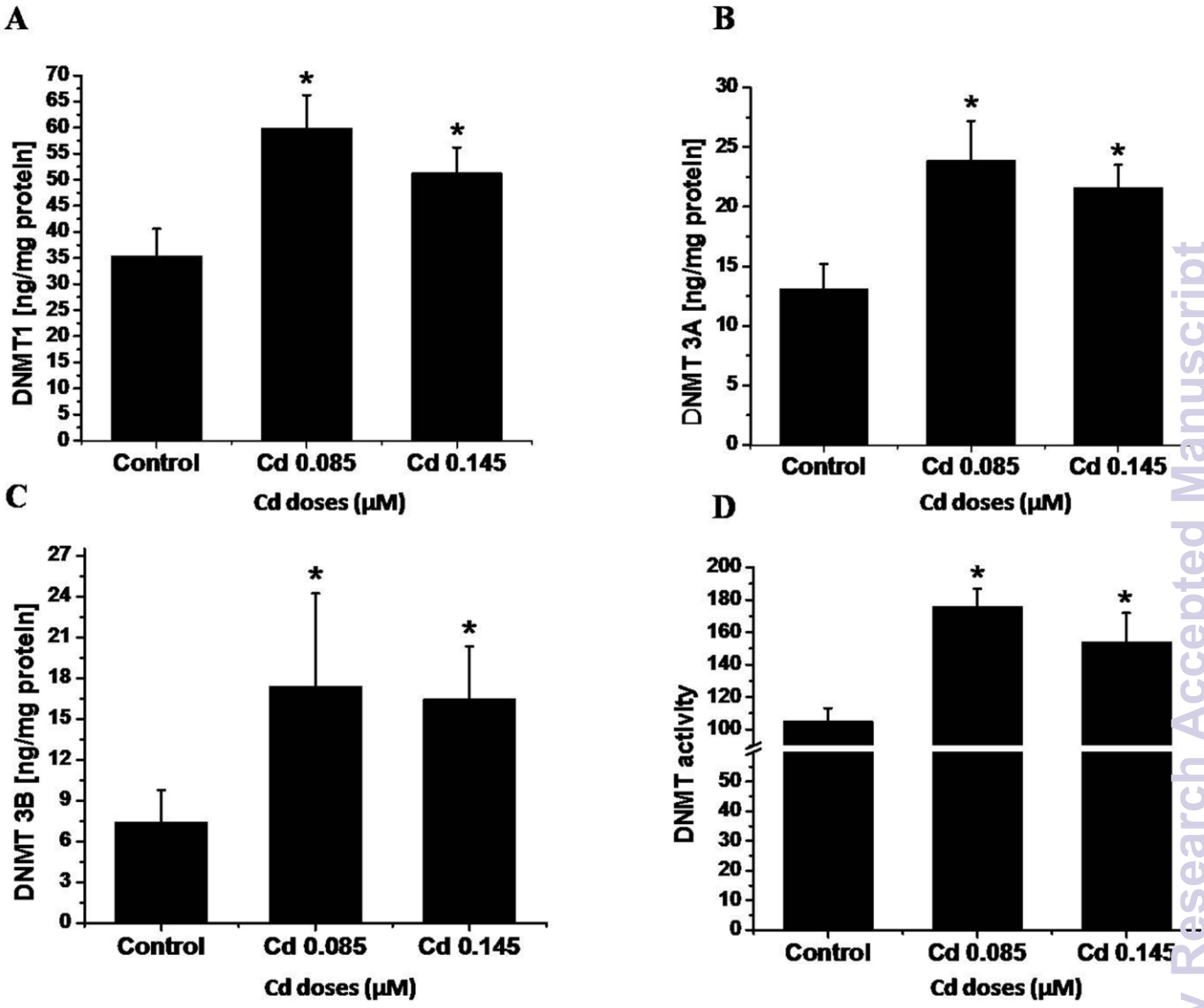

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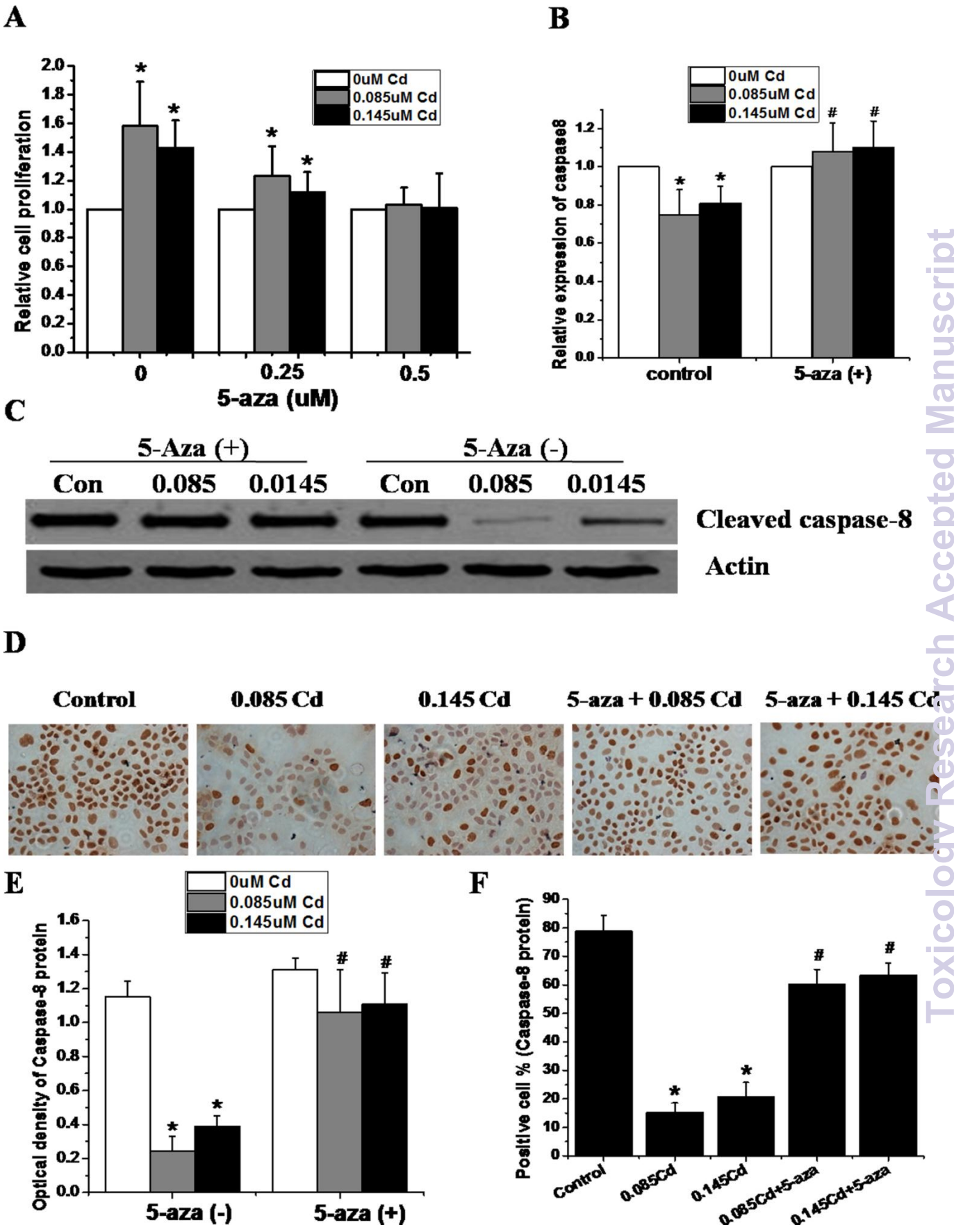

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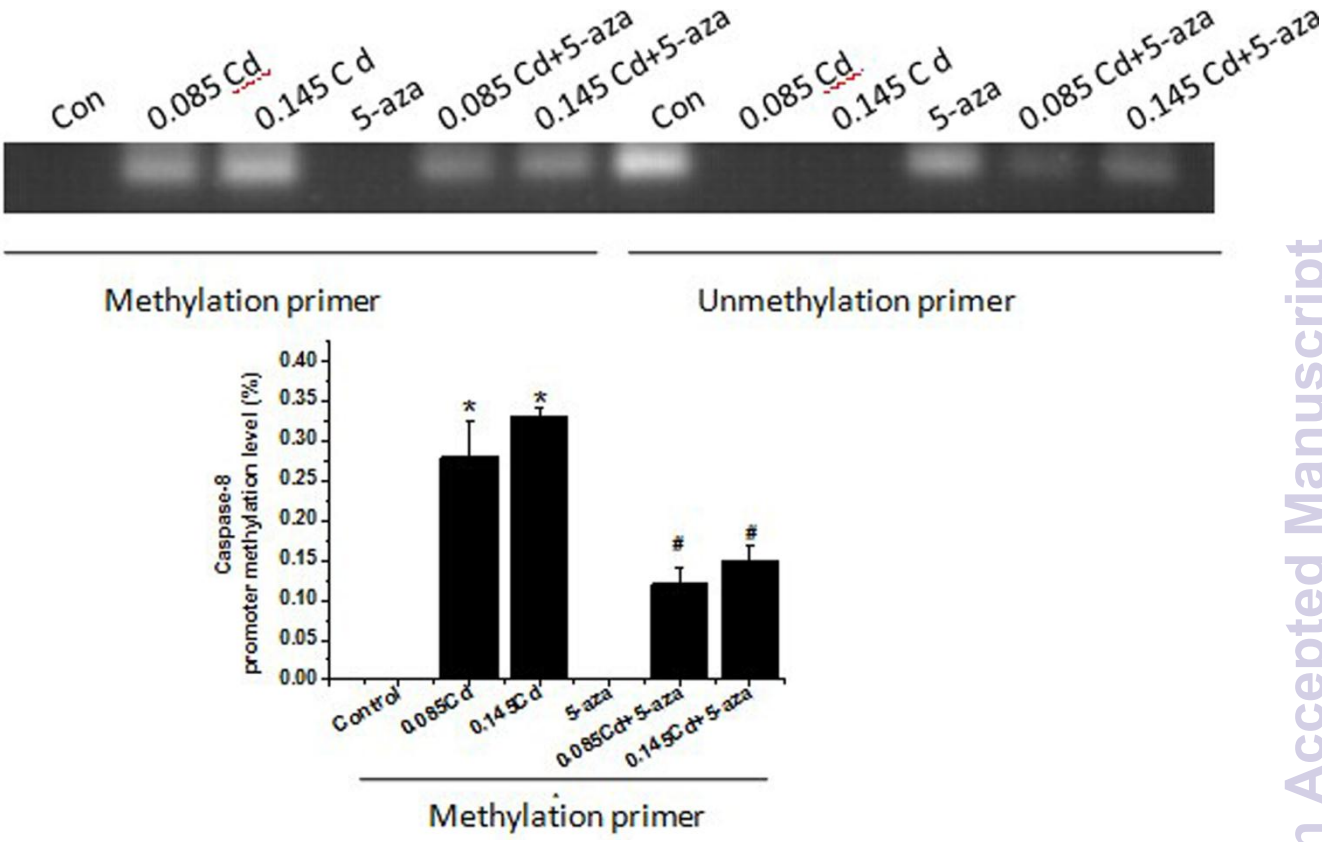

Figure 5.

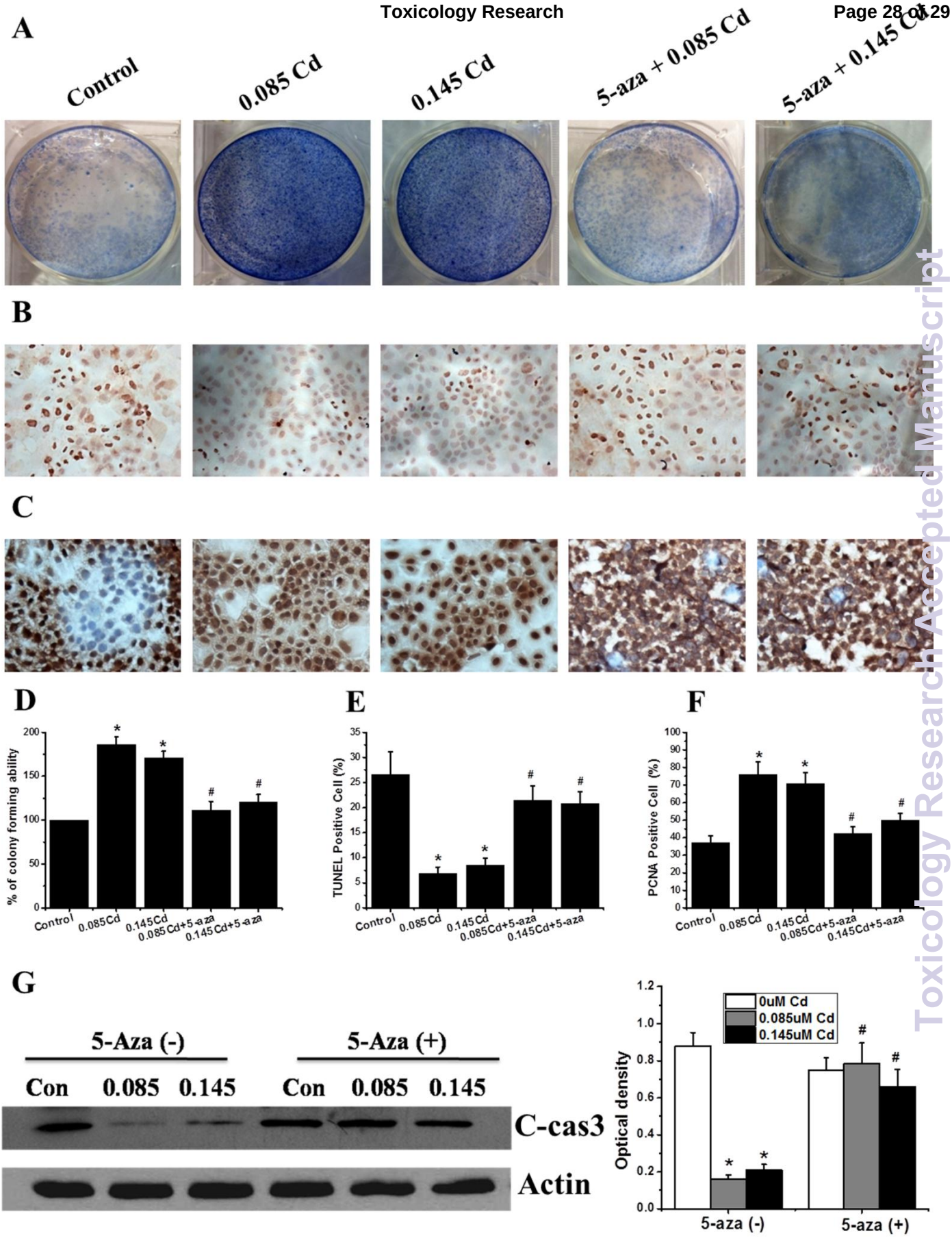

Figure 6.

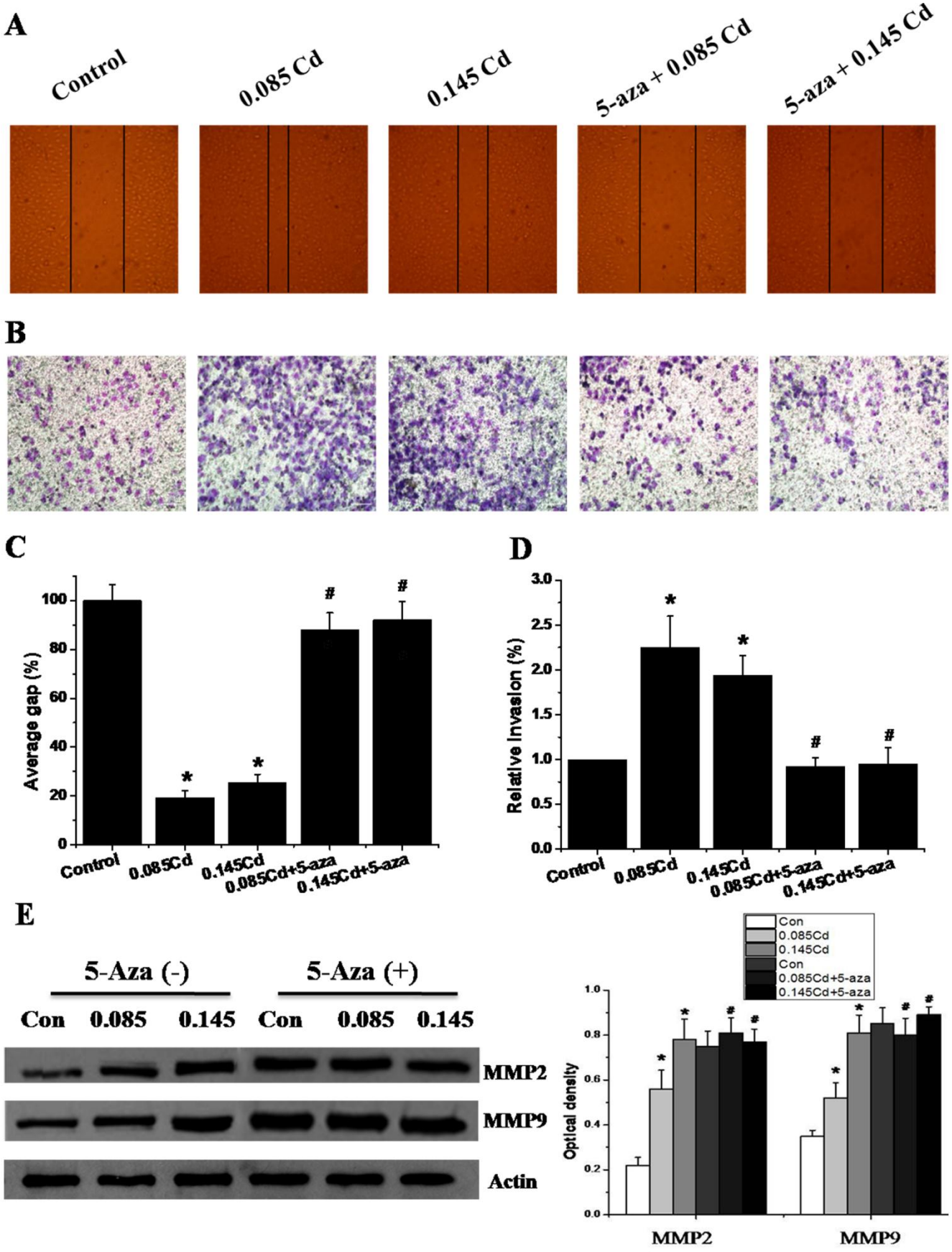

Figure 7.