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碩士學位 論文

# *Effects of TCDD on the expression of Matrix Metalloproteinase-2 and 9*

朝 鮮 大 學 校 大 學 院

生物新素材學科

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指導敎授　鄭　惠　光

이 論文을 理學 碩士學位신청 論文으로 제출함

2006年 11月

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生物新素材學科

崔　宰　濠

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# *List of Abbreviations*

| | |
|---|---|
| ***AP-1*** | Activator protein-1 |
| ***ECM*** | Extracellular matrix |
| ***EMSA*** | Electrophoretic mobility shift assay |
| ***Ets*** | E26 transformation specific |
| ***FBS*** | Fetal bovine serum |
| ***MMP*** | Matrix metalloproteinase |
| ***MT1-MMP*** | Membrane-type 1 matrix metalloproteinase |
| ***NF-κB*** | Nuclear factor κB |
| ***PMA*** | Phorbol 12-myristate 13-acetate |
| ***RT-PCR*** | Reverse transcription-polymerase chain reaction |
| ***TIMP*** | Tissue inhibitor of metalloproteinase |

# *ABSTRACT*

## *Effects of TCDD on the expression of Matrix Metalloproteinase-2 and 9*

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2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is one of the most toxic compounds in the endocrine disruptors. Exposure to the TCDD results in many pathogenic cases involved in carcinogenesis, immunotoxicity, reproductive and developmental toxicity. In this study, the effects of TCDD on the expression of metalloproteinase (MMP)-2 and MMP-9 which involved in tumor metastasis and invasion were investigated in human fibrosarcoma HT-1080 cells. TCDD induced the matrix MMP-2, MMP-9, membrane-type 1 matrix metalloproteinase (MT1-MMP), and E26 transformation specific (Ets) mRNA expression. TCDD also induced the protein expressions and activities of MMP-2 and MMP-9. Promoter analysis showed that TCDD increased levels of MMP-2 and MMP-9 mRNA. MMP-9 promoter region contains NF-kB, AP-1 and Ets transcription factor. TCDD increased level of MMP-9 mRNA through Ets but not NF-kB and AP-1. Treatment of the cells with TCDD induced the Ets protein expression and the DNA binding activity of

Ets complex. Ets siRNA decreased the MMP-2, MMP-9 and Ets expression. TCDD enhanced migration and invasion of HT-1080 cells. These results demonstrate that TCDD can inducement of tumor metastasis and invasion through up-regulation of MMP-2 and MMP-9.

# Ⅰ. *Introduction*

Tumor formation is a multiple step process that requires tumour cells to progress through many different stages, include initiation, growth, segregation, invasion, neovascularization, intravasation, extravasation and metastasis. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been known to endocrine disruptors in the environment and a highly toxic agent. The potential carcinogenic risk to humans of xenobiotic compounds, especially related pesticides and substances which may act as endocrine disruptors. Exposure to the TCDD results in many pathological cases involved in matrix and tissue remodeling, including prostate, mammary tubule morphogenesis, immunotoxicity, tumorigenecity, reproductive and developmental toxicities (Brown et al., 1995; Hiroyuki et al., 2003; Lisa et al, 2000; Roman et al., 1998; Takagi et al., 2000). These pathological lesions is an important matrix remodeling by the expression of MMPs.

The MMP family is composed of at least 20 extracellular endopeptidases that include four classes: the collagenases, gelatinases, stromelysins, and membrane type MMPs (MT-MMPs). Common features of the MMP family include: 1) the presence of zinc in the active site of the catalytic domain, 2) synthesis of the MMPs as preproenzymes that are secreted in an inactive form, 3) activation of the latent zymogen in the extracellular space, 4) recognition and cleavage of the ECM by the catalytic domain of the enzyme, and 5) inhibition of enzyme action by both serum-borne and tissue-derived metalloproteinase inhibitors in the extracellular environment (Thomas et al.,, 2003). MMPs contained signal peptide, propeptide, catalytic domain with the conserved zinc binding motif and hemopexin domain. The MMPs suggest that is a highly conserved cysteine residue in the proenzyme domain. This cysteine-switch mechanism allows the condensation of activation of MMPs (Sajal et al., 2003). Attachment of cells to extracellular matrix (ECM) molecules is mediated by the integrin family of

ECM receptors. Integrins are a large family of heterodimeric proteins that transduce a variety of signals from the ECM. Through integrin and matrix interactions, many of the genes, which are critical for cell migration, survival, proliferation, differentiation, and ECM degradation, are activated. In the majority of metastasizing tumors, cellular interactions with the ECM, which promote adhesion and migration, are thought to be required for primary tumor invasion, migration, and metastasis (Banerji et al., 2004).

MMP-2 (72 kDa gelatinase A, type Ⅳ collagenase) and MMP-9 (92 kDa gelatinase B, type Ⅳ collagenase) have been implicated as playing an important role in cancer invasion and metastasis (Chung et al., 2004). Both MMP-2 and MMP-9 are expressed in various malignant tumor and contribute to invasion and meastasis of tumors (Cha et al., 1998). These two gelatinases (MMP-2, MMP-9) contain a fibronectin-like sequence within their catalytic domain, which results in a potent ability for these MMP-2 and MMP-9 to bind to and cleave gelatin. MT-MMPs were proteinases anchors to the plasma membrane. The trimolecular complex of MT1-MMP/TIMP-2/proMMP-2 is located to the cell surface. MT1-MMP cleave the prodomain of MMP-2 and activate the gelatinase. Thus TIMP-2, in conjunction with the MT1-MMP, acts to control the specific site of MMP-2 activation. High or absence concentration of TIMP-2 decreases MMP-2 activation. TIMP-2 (21 kDa) has a high affinity for MMP-2 and TIMP-1 (28.5 kDa) preferentially binds to MMP-9. MMPs enhance metastasis by degrading structural proteins of the extracellular matrix and basement membranes, it is becoming apparent that these enzymes have a more complex role in tumor formation, development and progression specifically through regulation of cell growth and angiogenesis.

The Ets family is associated with malignant tumor. Especial Ets is important regulator of ECM remodeling and multiple development functions. Ets transcription factors are helix-turn-helix proteins that share a modular domain structure characterized by a highly conserved Ets domain. Ets transcription

factors have been found in all inducible MMP promoters. During carcinogenesis, degradation of the ECM occurs in the process of metastasis of malignant tumor. Metastasis formation is a multiple step that requires tumor cells to progress through many different stages, governed by successive changes in the expression of certain genes or alterations of gene structures and encoded products (Villano et al., 2005).

The human Ets oncogene family consists of greater than 20 sequence-specific nuclear transcription factors that have a conserved DNA-binding domain known as the Ets domain (Macleod et al., 1992). The name "Ets" stems from a sequence that was detected in an avian erythroblastosis virus, E26, where it formed a transforming gene together with Agag and c-myb (Blair et al., 2000; Nunn et al., 1983). The newly discovered sequence was called E26 transformation specific aequence or Ets. Later, a cellular homologue to the viral ets (v-ets) gene, c-etsl, was found suggesting that v-ets derived from c-etsl (Ghysdael et al., 1986; Watson et al., 1985). The binding of Ets proteins to this sequence can either activate transcription of their target genes. Ets binding sites are highly conserved in the MMP-9 promoter. Ets related protein have been identified as a target for a positive regulation of MMP-9.

This study investigated that the induction of MMP-2 and MMP-9 mRNA and protein expression, zymogen activity by TCDD is largely due to induced transactivation. TCDD induced the Ets protein expression and the DNA binding activity of Ets complex. Ets siRNA decreased the MMP-2, MMP-9 and Ets expression. TCDD enhanced migration and invasion of HT-1080 cells. These results demonstrate that TCDD is able to induce tumor metastasis and invasion in human fibrosarcoma HT-1080 cells.

# Ⅱ. *Materials & Methods*

## Ⅱ-*1. Chemicals and Materials*

Chemicals and cell culture materials were obtained from the following sources: 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), Triton X-100, Gelatin, Brij ®35 and Crystal Violet from Sigma Co. Ets-1 siRNA from Santa Cruz Biotechnology, Inc. Trans-well purchased from Coring Costar. Matrigel from BD Biosciences. MMPs, TIMPs, MT1-MMP PCR primer from Bioneer. MMP-2 and MMP-9 antibody, anti-mouse and anti-rabbit IgG, HRP-linked antibody from Cell Signaling, RPMI 1640 and fetal bovine serum (FBS), LipofectAMINE Plus, penicillin-streptomycin solution and trypsin from invitrogen™. Luciferase assay system from Promega. Centriplus YM-30 from Amicon Inc., WEST-ZOL™ plus, western blotting detection system from iNtRON biotechnology.

## Ⅱ-*2. Cell culture*

Human fibrosarcoma HT-1080 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown in RPMI 1640 supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 2 mM L-Glutamine, 100 units/㎖ penicillin and 100㎍/㎖ streptomycin at 37 ℃ in a 5 % $CO_2$ humidified incubator.

## Ⅱ-*3. Isolation of RNA and reverse transcription-polymerase chain reaction (RT-PCR)*

The cells were subcultured a 60 ㎜ plate in RPMI 1640 supplemented with 10 % FBS 24 hours before RNA extract. The cells were treated with or without various concentration of TCDD in serum-free media for 24 hours and then cellular RNA were harvested. Total cellular RNA was isolated using an acidic phenol extraction procedure. Total RNA were reverse-transcribed and then used

for PCR as template as described previously. For the human MMP-2 primer, PCR consisted of initial denaturation at 94 ℃ for 5 min each cycle consisted of 94 ℃ for 2 min, 58 ℃ for 1 min and 72 ℃ for 1 min. After 26 cycles, another 72 ℃ cycle was run for 7 min. For the MMP-9 primer, each cycle consisted of 94 ℃ for 30 sec, 64 ℃ for 30 sec and 72 ℃ for 30 sec. After 37 cycles, another 72 ℃ cycle was run for 7 min. For the MT1-MMP primers, each cycle consisted of 94 ℃ for 1 min, 60 ℃ for 1 min and 72 ℃ for 1 min and 28 cycles were performed. For the TIMP-1 and TIMP-2 primers, each cycle consisted of 94 ℃ for 1 min, 58 ℃ for 1 min and 72 ℃ for 1 min and 29 cycles were performed. For the Ets primers, each cycle consisted of 94 ℃ for 1 min, 62 ℃ for 30 sec and 72 ℃ for 1 min and 32 cycles were performed. For the GAPDH primers, 1 cycle consisted of 94 ℃ for 1 min, 54 ℃ for 1 min and 72 ℃ for 1 min and a total of 24 cycles were performed. PCR products were visualized by ethidium bromide staining of 1 % agarose gels. Integrity of RNA and cDNA synthesis were monitored by amplification of GAPDH mRNA.

### Ⅱ-*4. Western blotting*

The cells were subcultured a 100 ㎜ plate in RPMI 1640 supplemented with 10 % FBS 24 hours before western blotting. Exponential culture of cells were incubated for 24 or 48 hours with or without various concentrations of TCDD in serum free media. The whole cell lysates were isolated and were lysis by lysis buffer (150 mM NaCl, 100 mM Tris (pH 8.0), 1 % Tween 20, 50mM diethyldithiocarbamate (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), cocktail solution, 10 ㎍/㎖ aprotinin and 10 ㎍/㎖ leupeptin) or these conditional media were concentrated by centrifugation on Centriplus YM-30. The protein concentration were measured using the bradford assay. SDS-PAGE was performed under inducing conditions on 8 or 10 % polyacrylamide gels. The resolved proteins were transferred on to the nitrocellulose membrane. After blocking with 5 % skim milk, the blots were

incubated with MMP-2 and MMP-9 polyclonal antibodies and Ets polyclonal antibody or β-actin monoclonal antibody. And then, the blots incubates horseradish peroxidase conjugated secondary antibodies. The blots were probed with the West-Zol western blotting detection system according to the manufacturer's instruction.

### Ⅱ-5. *Gelatin zymography*

The cells were subcultured a 100 ㎜ plate in RPMI 1640 supplemented with 10 % FBS 24 hours before zymogen activities. Exponential culture of cells were incubated for 48 hours with or without various concentrations of TCDD in serum free media. These conditional media were concentrated by centrifugation on Centriplus YM-30. Volumes for cells were adjusted to 5 ㎍ protein as determined by bradford assay. Equal protein amounts of conditioned media were mixed with zymography sample buffer without reducing agent and separated on 8 % SDS-PAGE co-polymerized with gelatin (1 mg/ml). The gels were washed with 2.5 % Triton X-100 for 30 min two times, washed with DW for 20 min three times, equilibrated the gel for 30 minutes at room temperature with gentle agitation then replace with fresh developing buffer at 37℃ for 18 hours. The gels were stained with 0.25 % Coomassie Brilliant Blue G250 in 50 % methanol, 10 % acetic acid and 50 % DW and then destained in 50 % methanol, 10 % acetic acid and 40 % DW. Clear bands were observed against the blue background to determine the proteolytic activities of MMP-2 and MMP-9.

### Ⅱ-6. *Luciferase and β-galactosidase assay*

The cells were subcultured a 48 well plate in RPMI 1640 supplemented with 10 % FBS 24 hours before transfection. The cells were co-transfected with 1 $\mu$g of the wild type MMP-2 and MMP-9, point mutated type NF-$\kappa$B, AP-1, Ets and Ets/AP-1 regulated luciferase reporter gene and 0.5 $\mu$g of pCMV-$\beta$-gal reporter plasmid using LipofectAMINE Plus in antibiotics-free media. Four

hours after transfection, a fresh media containing 10 % FBS was added to the cells, which were treated with or without various concentration of TCDD. Following 24 hours exposure, the cells were washed once with PBS and lysed. The lysed cell preparations were then centrifuged, and the supernatants were assayed for luciferase and β-galactosidase activity. Luciferase activity was determined using the luciferase assay system and used according to the manufacturer's instructions using a luminometer. The β-galactosidase assay was carried out in 250 ㎕ of assay buffer containing 0.12 M $Na_2HPO_4$, 0.08 M $NaH_2PO_4$, 0.02 M KCl, 0.002 M $MgCl_2$, 0.1 M β-mercaptoethanol, 50 ㎍ of o-nitrophenyl-β-galactoside, and 100 ㎍ of the cell extract. The luciferase activity was normalized using the β-galactosidase activity and is expressed as a proportion of the activity detected with the vehicle control.

### Ⅱ-7. *Electrophoretic mobility shift analysis (EMSA)*

The cells were subcultured a 100 ㎜ plate in RPMI 1640 supplemented with 10 % FBS 24 hours before EMSA. Exponential culture of cells were incubated for 90 minutes with or without various concentrations of TCDD in serum free media. Nuclear extracts were prepared : Following a rinse with cold PBS, cell lysis with lysis buffer on ice bath, lysed cell were centrifuged, suspension with K buffer on ice, suspended cell were centrifuged and then aliquot for eppendorf tubes. Ets oligomer labeling : The oligomer were added r-$^{32}$P-dATP, T4 kinase and 10x kinase buffer and then incubation on 37 ℃ water bath for 1 hour, stopped enzyme reaction on ice bath, centrifuged with column and the oligomer were end-labeled with r-$^{32}$P-dATP. The nuclear extracts (50 ㎍) were incubated with 1 ㎕ of poly (dI-dC) and the r-$^{32}$P-dATP-labeled DNA probe in a binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10 % Glycerol, 1 mM DTT, 1mM PMSF, and 1 ㎍/㎖ aprotinin and leupeptin) on ice bath for 20 minutes. The specificity of binding was examined by competition with the unlabeled oligonucleotide. After incubating for an additional 20 min, the

DNA protein complex was separated on a 5% non-denaturing polyacrylamide gel. Following electrophoresis, the gel was vacuum dried and exposed at -80 ℃ to X-film.

### Ⅱ-*8. Chromatin Immunoprecipitation (ChIP) Assay*

HT-1080 cells were treated TCDD with or without various concentrations of TCDD for 24 hours. Cells were resuspended in 1x TE buffer (10 mM Tris-Cl, and 0.1 mM EDTA, pH 8.0) with 2 mM PMSF, 2 g/㎖ pepstatin A, 2 g/㎖ aprotinin, and 2 g/㎖ leupeptin for sonication. RNase A as well as proteinase K were added to the samples after cross-links were reversed. Antibodies used in the immunoprecipitation were Ets (Santa Cruz Biotechnology). Immunoprecipitated samples were resuspended in 50 ㎕ of $H_2O$ and analyzed by PCR. After 40 cycles of amplification, PCR products were analyzed on 1 % agarose gel with ethidium bromide.

### Ⅱ-*9. Small interfering RNA transfection*

The cells were subcultured a 60 ㎜ plate in RPMI 1640 supplemented with 10 % FBS 24 hours before siRNA transfection. The cells were transfected with the diluted siRNA and siRNA transfection reagent complex in antibiotics-free media. Forty-eight hours after transfection, a fresh media containing 10 % FBS was added to the cells.

### Ⅱ-*10. Migration assay*

The cells were subcultured a 48 well plate in RPMI 1640 supplemented with 10 % FBS 24 hours before migration assay. Migration assay was performed on the confluent cell monolayer of 90 % in 48 well plate. It was wounded by manually scraping the cells with a tip with 2 ㎜ in width. Following a rinse with PBS, the cells were treated with or without various concentrations of TCDD in replaced serum-free media. Cell migration was measured the distance

of the moved cells from the start point to the migrated point was monitored by microscopy at various times.

### Ⅱ-*11. Matrigel invasion assay*

Invasion assay was performed using Transwell plate (Corning, Corning, NY) with polycarbonate filters containing 8 μm pores as previously described. Matrigel (BD Biosciences Discovery Labware, San Jose, CA) was thawed in an ice bath. An equal amount of cold RPMI 1640 media was mixed with Matrigel and used to coat the polycarbonate membrane of each Transwell plate. Matrigel polymerization was done by incubating the coated transwell plate at 37 ℃ $CO_2$ incubator for 1 hour. The 350 $\mu\ell$ of $1\times10^5$ cells in serum-free media was plated on the matrigel-coated transwell with or without various concentrations of TCDD. The media in the lower chambers contained 10 % FBS. The transwell plate was incubated at 37 ℃ for 24 hours. The invaded cells were fixed with methanol, Matrigel coated on transwell plate was removed and the transwell plate was stained with crystal violet. The invasive phenotypes were determined by counting the cells that migrated to the lower side of the filter with microscopy at x 200.

### Ⅱ-*12. Statistical analysis*

All experiments were repeated at least three times to ensure reproducibility. The results are reported as means ± SD. ANOVA was used to evaluate differences between multiple groups.

*Table 1. Primers and reaction conditions for RT-PCR of human MMP-2, MMP-9, MT1-MMP, TIMP-1, TIMP-2 and Ets.*

| Gene | Primer sequence | size(bp) |
|---|---|---|
| MMP-2 | (F) : 5′-TTTTCTCGAATCCATGATGG-3′<br>(R) : 5′-CTGGTGCAGCTCTCATATTT-3′ | 620 |
| MMP-9 | (F) : 5′-AGACCTGAGAACCAATCTCAC-3′<br>(R) : 5′-GGCACTGAGGAATGATCTAA-5′ | 312 |
| MT1-MMP | (F) : 5′-CGCTACGCCATCCAGGGTCTCAAA-3′<br>(R) : 5′-CGGTCATCATCGGGCAGCACAAAA-3′ | 481 |
| TIMP-1 | (F) : 5′-CAATTCCGACCTCGTCATCA-3′<br>(R) : 5′-TCAGAGCCTTGGAGGAGCT-3′ | 429 |
| TIMP-2 | (F) : 5′-AGATGTAGTGATCAGGGCCA-3′<br>(R) : 5′-AGCACCCCTCACTTCTCTT-3′ | 503 |
| Ets | (F) : 5′-TGGAGTCAACCCAGCCTATC-3′<br>(R) : 5′-TCTGCAAGGTGTCTGTCTGG-3′ | 233 |
| GAPDH | (F) : 5′-GATGAATTCTGAAGGTCGGAGTCAAC GGATTTGGT-3′, (R) : 5′-GATAAGCTTCATG TGGGCCATGAGGTCCACCAC-3′ | 983 |

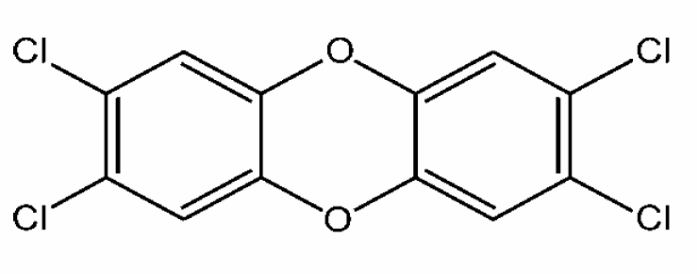



***Figure 1.*** Chemical structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

# Ⅲ. *Result*

### Ⅲ-*1. Effects of TCDD on MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 mRNA expression.*

To examine whether TCDD affects the steady-state levels of MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 mRNAs, RT-PCR analysis of RNA from HT-1080 cells was performed. As shown in Fig. 2, the amounts of transcripts for MMP-2, MMP-9, and MT1-MMP were induced with increasing TCDD concentration in a dose dependent manner, but TIMP-1, TIMP-2 were not changed.

### Ⅲ-*2. Effects of TCDD on MMP-2 and MMP-9 protein expression.*

To determine the effects of TCDD on MMP-2 and MMP-9 expression, HT-1080 cells were treated with TCDD (10 and 50 nM) for 48 hours, and MMP-2, MMP-9 expression in the conditioned media was assayed using western blotting. As shown in Fig. 3, TCDD increased MMP-2 and MMP-9 protein expression in HT-1080 cells, respectively.

### Ⅲ-*3. Effects of TCDD on MMP-2 and MMP-9 activities.*

A shown in Fig. 4, TCDD treatment of HT-1080 cells results in increased activities of MMP-2 and MMP-9. Cells were treated with TCDD (10 and 50 nM) for 48 hours, and MMP-2, MMP-9 activity in the conditioned media was assayed using zymography. The effects of TCDD on MMP-2 and MMP-9 activities paralleled those on MMP-2 and MMP-9 protein expression.

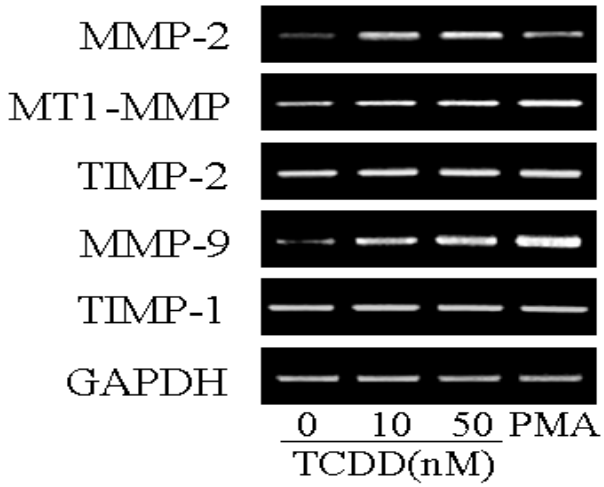



***Figure 2.*** Effects of TCDD on the MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 mRNA expression in HT-1080 cells. Cells were treated with TCDD (10 and 50 nM) for 24 hours. The cells were lysed and the total RNA was analyzed by RT-PCR. PCR amplification was performed for each sample using primers specific for human MMPs, TIMPs, and GAPDH. The PCR amplification products were electrophoresed in 1% agarose gel and stained with ethidium bromide. These figures are representative of three independent experiments carried out in duplicate, each of which demonstrates similar results.

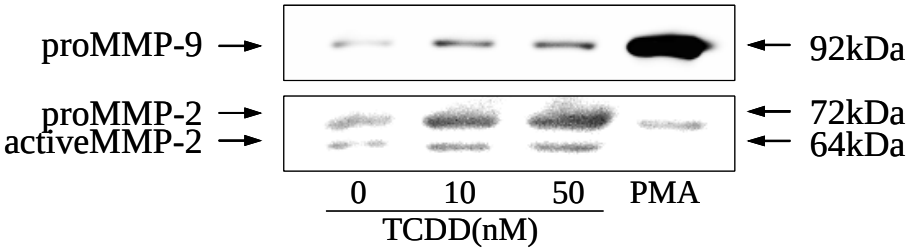



***Figure 3.*** Effects of TCDD on the MMP-2 and MMP-9 protein expression in HT-1080 cells. Cells were treated with TCDD (10 and 50 nM) for 48 hours. MMP-2 and MMP-9 protein expression in the conditioned medium was detected using western blot analysis. These figures are representative of three independent experiments carried out in duplicate, each of which demonstrates similar results.

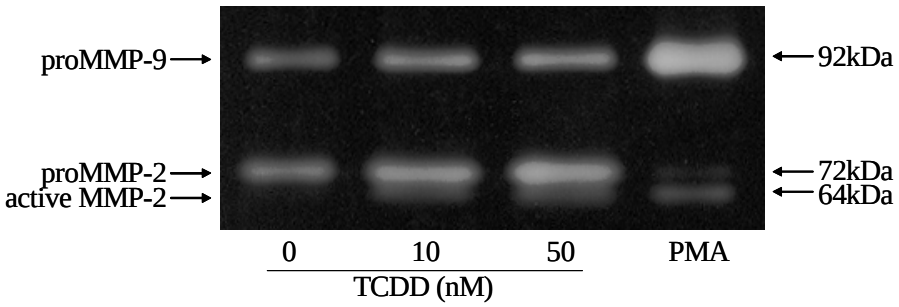



***Figure 4.*** Effects of TCDD on the MMP-2 and MMP-9 activities in HT-1080 cells. Increasing volumes of media were electrophoresed on a SDS-PAGE containing 1 mg/ml gelatin. MMP activity was determined by the ability of proteases in the media to degrade the gelatin. These figures are representative of three independent experiments carried out in duplicate, each of which demonstrates similar results.

### Ⅲ-*4. Effects of TCDD on MMP-2 and MMP-9 transcriptional activities.*

Because of the observed alterations in MMP-2 and MMP-9 expression induced by TCDD. I next sought to determine the effects of TCDD on MMP-2 and MMP-9 transcriptional activities using a luciferase-based promoter assay. As shown in Fig. 5 and 6, TCDD increased MMP-2 and MMP-9 promoter activities in HT-1080 cells, respectively.

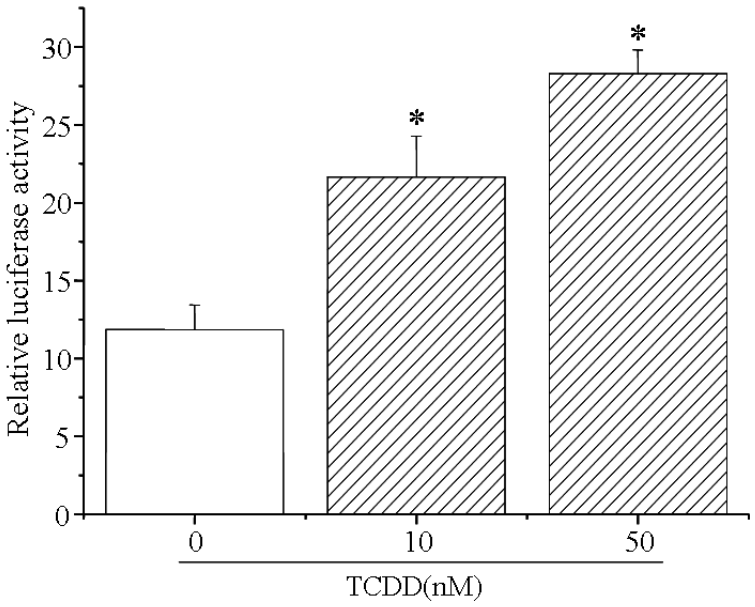



***Figure 5.*** Effects of TCDD on the MMP-2 dependent transcriptional activity. HT-1080 cells were transiently co-transfected with pCMV-β-gal and MMP-2 regulated luciferase reporter gene, and the luciferase activity in cell lysates was assayed at 24 hours. Each bar shows the mean±S.D. of three independent experiments, performed in triplicate. $*P < 0.05$, significantly different from control(NA).

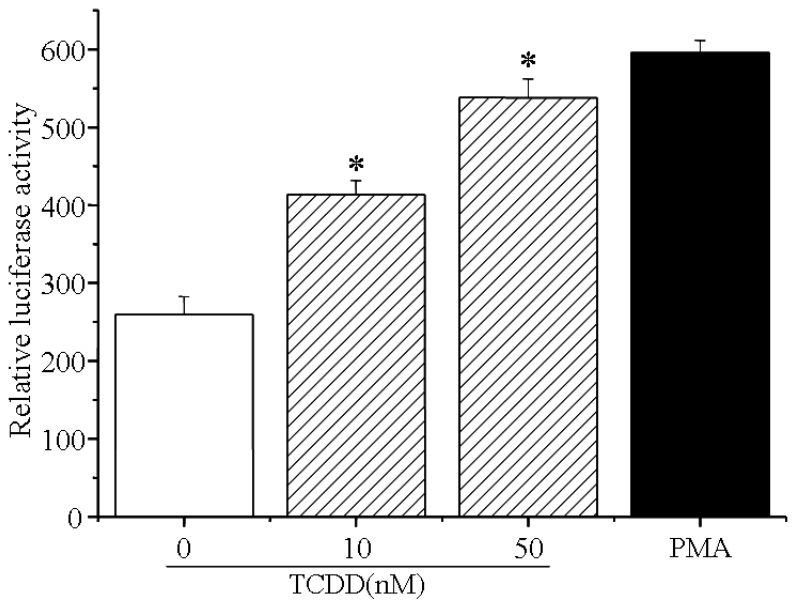



***Figure 6.*** Effects of TCDD on the MMP-9 dependent transcriptional activity. HT-1080 cells were transiently co-transfected with pCMV-β-gal and MMP-9 regulated luciferase reporter gene, and the luciferase activity in cell lysates was assayed at 24 hours. Each bar shows the mean±S.D. of three independent experiments, performed in triplicate. $*P < 0.05$, significantly different from control(NA).

### Ⅲ-5. *Involvement of Ets binding sites in the activation of the MMP-9 gene promoter by TCDD.*

As shown in Fig. 7, disruption of the Ets and NF-kB site at within the longer pGL-MMP-9 construct reduced transactivation of MMP-9 by TCDD. In contrast, disruption of the AP-1 site at within the MMP-9 promoter fragment only moderately induced by TCDD. This result was implicated in the Ets binding sites in the activation of the MMP-9 promoter by TCDD.

### Ⅲ-6. *Effects of TCDD on Ets expression.*

To determine whether TCDD affects Ets expression, HT-1080 cells were exposed to TCDD for 24 hours. As shown in Fig. 8, TCDD increased Ets mRNA and protein expression in HT-1080 cells.

### Ⅲ-7. *Effects of TCDD on Ets DNA binding activity.*

Ets is a known transcriptional regulator of MMP-2 and MMP-9 expression. Because of the observation that TCDD upregulates MMP-2 and MMP-9 promoter activity, I sought to determine whether TCDD up-regulates Ets binding activity. As shown in Fig. 9, TCDD increased Ets binding activities in HT-1080 cells. These findings suggest that Ets may play a role in the TCDD-mediated increase in MMP-2 and MMP-9 transcription.

### Ⅲ-8. *TCDD induce Ets accumulation and DNA binding activity in nuclei.*

The interaction of Ets complex with MMP-9 promoter was investigated by chromatin immunoprecipitation (ChIP) using antibodies specific for Ets and as well as using pre-immune serum (IgG) as a negative control. The cross-linked chromatin from these two samples of cells was then immunoprecipitated with individual antibodies. The chromatin DNA was eluted after reversal of

cross-links and treatment by proteinase K and RNase A. The cross-linked samples without adding antibody was reserved as total input. The eluted DNA was PCR-amplified using primers specific for the MMP-9 promoter. As shown in Fig. 10, two antibodies specific for Ets immunoprecipitated contain any detectable amount of chromatin even when the amplification cycle of PCR. This data suggested that binding MMP-9 promoter to Ets transcription factor.

### Ⅲ-*9. Effects of Ets gene silencing on TCDD-induced increase in MMP-2 and MMP-9 expression.*

To determine whether TCDD-mediated MMP-2 and MMP-9 induction is Ets dependent, I suppressed Ets expression using RNA interference. At 48 hours after transfection of Ets siRNA and cell were treated with TCDD. MMP-2, MMP-9 and Ets expression were determined using RT-PCR analysis. As shown Fig. 11, Ets expression was significantly suppressed by transfection of siRNA in HT-1080 cells, as compared with their untransfected counterparts. Forty-eight hours after the transfection, cell were treated with TCDD and assayed MMP-2 and MMP-9 expression 24 hours subsequently. Ets silencing reduced baseline MMP-9 expression in HT-1080 cells (Fig. 11, 12). This finding suggests that Ets is critical for TCDD-mediated MMP-9 expression.

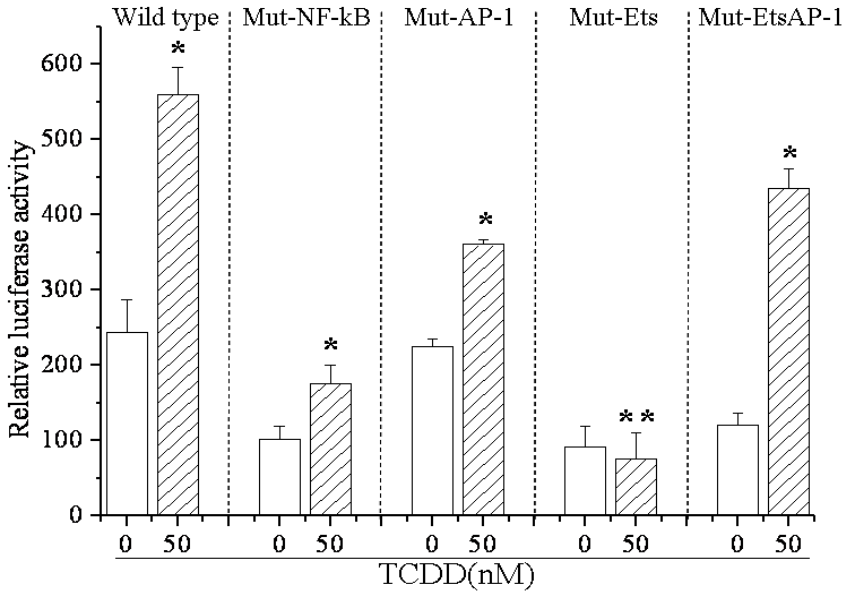



***Figure 7.*** Activation of wild-type and point-mutated MMP-9 promoter constructs by TCDD. HT-1080 cells were transiently co-transfected with pCMV-β-gal, wild type MMP-9 and point mutated MMP-9 luciferase reporter gene, and the luciferase activity in cell lysates was assayed at 24 hours. Each bar shows the mean±S.D. of three independent experiments, performed in triplicate. *$*P$, **$*P$ $< 0.05$ , significantly different from untreated control.

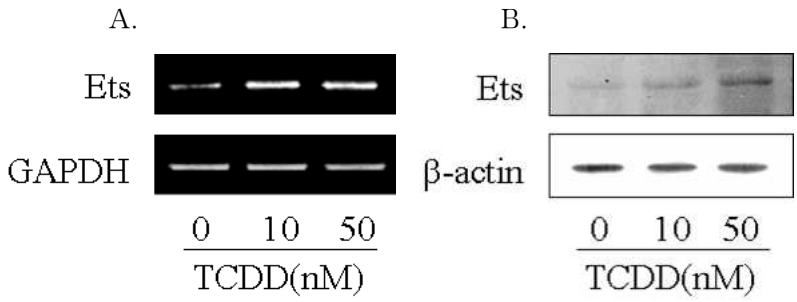



***Figure 8.*** Effects of TCDD on the Ets expression in HT-1080 cells. Cells were treated with TCDD (10 and 50 nM) for 24 hours. (A) The cells were lysed and the total RNA was analyzed by RT-PCR. PCR amplification was performed for each sample using primers specific for human Ets and GAPDH. The PCR amplification products were electrophoresed in 1 % agarose gel and stained with ethidium bromide. (B) The cells were lysed and the total cellular protein were analyzed by western blot. These figures are representative of three independent experiments carried out in duplicate, each of which demonstrates similar results.

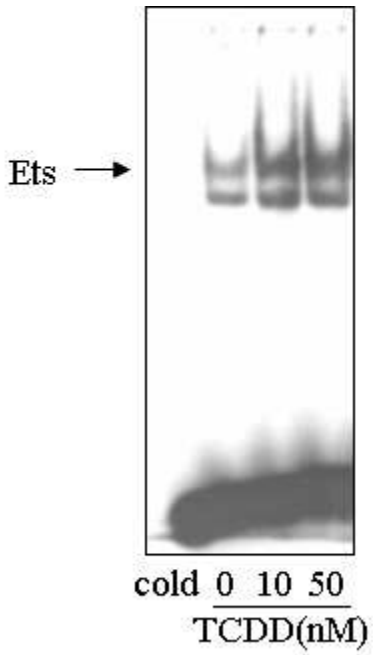



***Figure 9.*** Effect of TCDD on the Ets binding activity. HT-1080 cells were treated with TCDD (10 and 50 nM). Nuclear extracts were isolated and used in an electrophoretic mobility shift assay with $^{32}$P-labeled Ets oligonucleotide as a probe. The arrow indicates the Ets binding complex. Cold; 200-fold molar excess of non-labeled Ets probe. One of three representative experiments is shown.

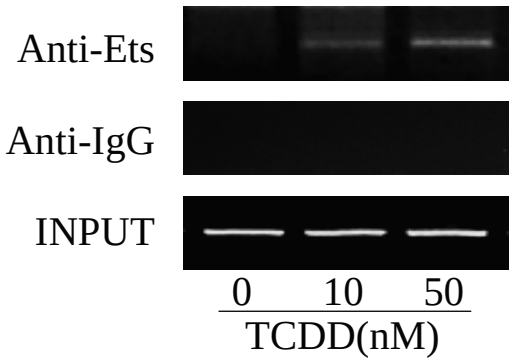



***Figure 10.*** TCDD induce Ets accumulation and DNA binding activity in nuclei from HT-1080 cells. HT-1080 cells were treated with various TCDD concentration for 20 hours. Equal amounts of cross-linked chromatin were incubated with pre-immune serum (IgG), anti Ets. Following DNA precipitation and dilution, samples were analyzed by PCR using primers specific to MMP-9 promoter. These figures are representative of three independent experiments carried out in duplicate, each of which demonstrates similar results.

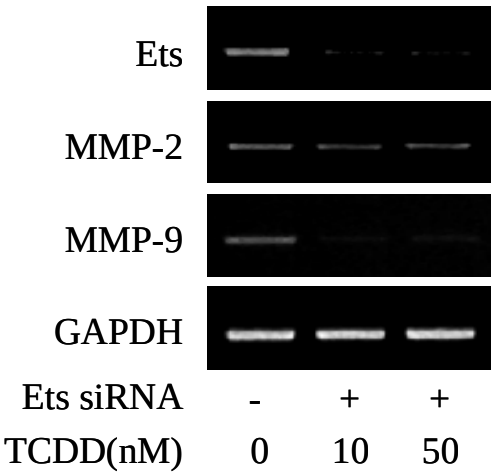



***Figure 11.*** Effects of Ets gene silencing on TCDD-mediated MMP-2 and MMP-9 expression in HT-1080 cells. The cells were transfected with Ets siRNA. Forty-eight hours after transfection, HT-1080 cells were treated with TCDD (10 and 50 nM). The total RNA was extracted from cells and analyzed using RT-PCR. The effect of Ets gene silencing on TCDD-mediated MMP-2, MMP-9 and Ets mRNA expression. These figures are representative of three independent experiments carried out in duplicate, each of which demonstrates similar results.

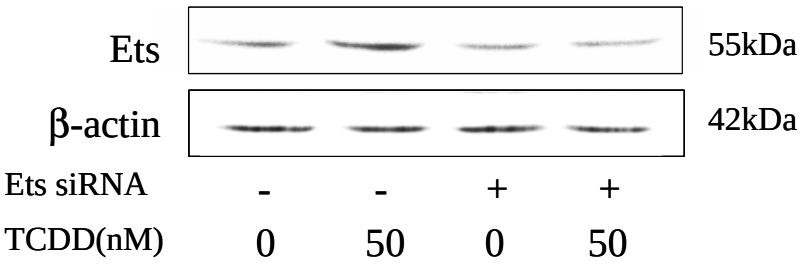



***Figure 12.*** siRNA-mediated down-regulation of the Ets expression in HT-1080 cells. The cells were transfected with Ets siRNA. Forty-eight hours after transfection, HT-1080 cells were treated with TCDD (10 and 50 nM). The total protein was extracted from cells and analyzed using western blot. These figures are representative of three independent experiments carried out in duplicate, each of which demonstrates similar results.

### Ⅲ-*10. Effects of TCDD on cellular migration and invasion.*

Previous study shown that MMP-2 and MMP-9 activity is associated with cancer invasive and migratory potential. Here I tested the effects of TCDD on cellular invasive and migratory potentials. As show in figure 13 and 14, TCDD induced cellular migration and invasion in HT-1080 cells, respectively. The results suggested that TCDD stimulated migration and invasion of tumor cell.

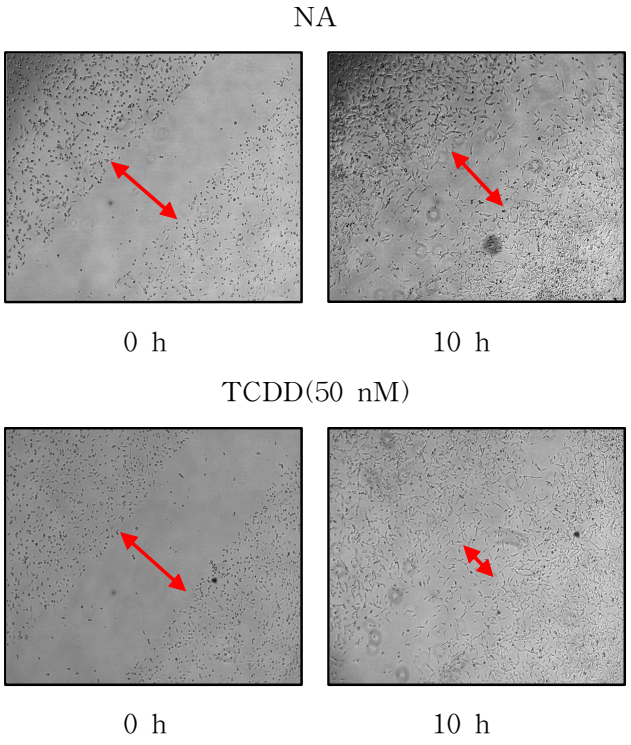
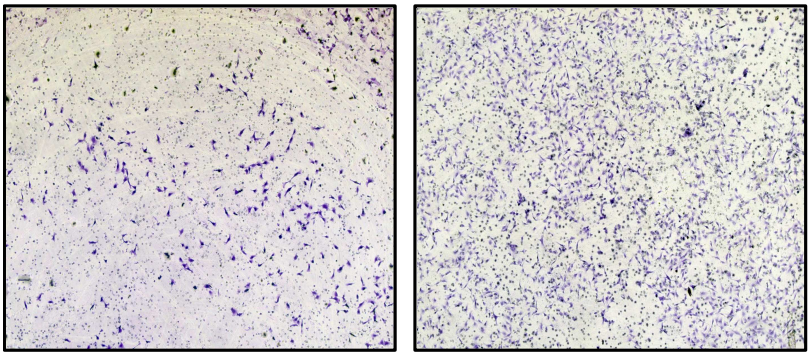



***Figure 13.*** Effect of TCDD on cellular migration in HT-1080 cells. Cells were treated with TCDD. After 10 hours, migrated cells were photographed under an microscope (200 x).



NA TCDD (50 nM)

***Figure 14.*** Effect of TCDD on cellular invasiveness in HT-1080 cells. HT-1080 cell seeded on a layer of transwell in the presence of TCDD. After 24 hours, migration had formed were photographed under an inverted microscope (200 x).

# Ⅳ. *Discussion*

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a prototype of many halogenated aromatic hydrocarbons, is a ubiquitous, persistent environmental contaminant and the most powerful tumor promoter in rodent bio assays. There is much evidence that implicates important roles for TCDD in carcinogenesis and progression for a wide range of malignancies, including pancreatic cancer. However, there are few data on the downstream targets by which TCDD mediate these processes. In this study, I identified Ets as an important mediator of TCDD-induced induction of MMP-9 expression. Therefore, TCDD has been demonstrated to be one of the strongest promoters for treatment of cancer and metastasis. Tumor invasion and metastasis are a stepwise and complicated process that includes cell division and proliferation, digestion of the ECM, cell migration through the basement membranes to range the circulation system, and the remigration and growth of tumors at metastatic sites. MMPs play a major function in promoting angiogenesis and tumor metastasis. Tumor-derived and stroma-derived MMPs may play important roles in tumor growth, the effect of MMP inducible activity is important for a experimental model. These study builds on these previously reported findings by potential mechanism by which TCDD mediates MMP-9 expression. I hypothesized that Ets-1 might play a role in TCDD-mediated MMP-9 induction.

Treatment of the cells with TCDD induced the matrix MMP-2, MMP-9, membrane-type 1 matrix metalloproteinase (MT1-MMP) and E26 transformation specific (Ets) mRNA expression. TCDD also induced the protein expressions of MMP-2 and MMP-9. I have confirmed the inducible effects of TCDD on MMP-2 and MMP-9 by the use of substrate and gelatin zymography. The gelatin zymography has been shown to the same result in enhanced expression

of MMP-2 and MMP-9. TCDD selectively induce MMP-2 and MMP-9 enzyme activities. The transcriptional activities were increased in the cells that have been transiently transfected with the wild type MMP-2 and MMP-9 promoter by treatment with various TCDD concentration. The promoter region of the MMP-9 has been shown to enhance the activation of transcription factors as Ets. These findings that TCDD induces Ets binding activity and MMP-9 promoter activity and that Ets silencing abolishes the effect of TCDD on MMP-2 expression clearly support my hypothesis. The Ets nuclear proteins cooperate with other transcription factors to activate or repress transcription in a variety of processes including cell proliferation, apoptosis, development, differentiation, angiogenesis and oncogenic transformation. Ets has been reported to be overexpressed in variety of malignancies, including pancreatic cancers, and to regulate the transcription of many MMPs, including MMP-9. A recently reported study localized an Ets binding site to the MMP-9 promoter. Ets, important regulators act as nuclear targets of signal transduction pathways, play important roles in various biological processes, cell proliferation, differentiation, apoptosis and immune response. TCDD treatment increased the amount of PCR product amplified from nucleic DNA derived from Ets immunocomplex over control cells. Gene silencing is a technique for down regulation the expression of a specific gene in mammalian cells by double stranded RNA (dsRNA) that is complementary to a interested target mRNA. It has been demonstrated 21 base pair RNA molecules can be a effective mediator of the RNA interference. MMP-2 expression is slightly reduced but MMP-9 expression is reduced in Ets siRNA transfected cells. Ets expression is reduced by treatment with or without TCDD in Ets siRNA transfected cells, too. The induction of attachment of the cells to the matrix by TCDD is an essential mechanism for the induction of invasion. After the tumor cell has become detached from neighboring cells by loosening its intercellular junctions, the ECM has to be proteolytic degradation in migration and invasion of the cells. So, matrix degrading proteinases are

importance for tumor cell metastasis. Many studied reveal that enhanced production of MMP-2 and MMP-9 correlates with the invasion, migration and metastasis of the tumors. In conclusion, these show that TCDD derivative TCDD: 1) increase the potential invasion through the induction of MMP-2 and MMP-9 gene transcriptional levels by inducing Ets function in the cells and 2) induce the enzymatic activities of MMP-2 and MMP-9 that plays an important role in cancer invasion, migration and metastasis. These suggest that the metastatic and tumorigenic effects of TCDD may be mediated through induction of MMP-2 and MMP-9 gene expression by the activation of Ets. Therefore, TCDD demonstrated to be one of the strongest promoters for treatment of cancer and metastasis.

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# (국문 초록)

## *Matrix Metalloproteinase (MMP)-2* 및 *MMP-9* 발현에 대한 *TCDD*의 영향

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2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)는 대표적인 내분비계 장애물질로 인체에 발암작용 및 생식과 발육장애, 면역체계와 호르몬 등에 심각한 영향을 주는 것으로 알려져 있다. 본 연구에서는 이러한 내분비계 장애물질의 암 전이에 대한 영향을 조사하기 위하여 human fibrosarcoma 세포인 HT-1080에 TCDD를 사용하였다. HT-1080 세포에 TCDD를 처리하여 RT-PCR, zymography, western blotting을 수행하여 암 전이에 관여하는 MMP-2와 MMP-9의 유전자 발현과 효소 활성도 및 단백질 발현이 증가됨을 확인하였다. MMP-2와 MMP-9의 luciferase reporter assay를 수행한 결과 MMP-2와 MMP-9의 promoter 활성도가 TCDD의 농도에 의존적으로 증가하였으며, MMP-9 promoter의 point mutated luciferase assay를 수행하여 확인한 결과 TCDD에 의한 MMP-9의 발현의 증가는 Ets 전사 조절인자가 관여함을 알 수 있었다. 또한 Ets EMSA, chip assay, siRNA transfection을 이용하여 TCDD에 의해 증가되는 MMP-9의 발현도 Ets에 의해 매개됨을 확인하였다. 마지막으로 TCDD에 의한 HT-1080 세포의 이동능과 침윤을 확인한 결과 TCDD에 의해 암 세포의 이동능 및 침윤이 증가하였다. 따라서 TCDD에 의한 암 전이에 관여하는 MMP-2와 MMP-9의 발현과 효소 활성, 암 세포의 이동능 및 침윤이 증가하므로, TCDD에 의해 암 전이가 촉진될 수 있을 것으로 사료되며, 본 연구를 통하여 내분비계 장애물질 중 TCDD에 의한 암세포의 전이에 대한 작용을 규명하였으며, 이는 발암 관련 기초 자료로 활용될 수 있을 것으로 기대된다.

# 감사의 글

어느 덧 12월이 되었습니다. 부족한 논문을 완성하는데 도움을 주신 분들에게 감사의 말씀을 드립니다. 이 작은 논문이 나오기까지 많은 사람들의 도움과 헌신이 있었기에 가능했습니다. 이제는 이 분들에게도 도움이 되는 사람으로 살기 위해 더욱 분발하리라는 소박한 다짐으로 미안한 마음을 대신하려고 합니다.

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마지막으로 세상에서 가장 사랑하는 우리 부모님...
어떤 말로도 또한 글로도 표현할 수 없는 큰 사랑을 저에게 주시기에 지금까지 올 수 있었습니다. 저에게 주신 큰 사랑과 은혜. 평생 보답할게요. 항상 건강하세요. 사랑합니다.

2006년 12월 어느 날
최재호

# 저작물 이용 허락서

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| 논문제목 | 한글 : Matrix Metalloproteinase (MMP)-2 및 MMP-9 발현에 대한 TCDD의 영향 | | | | |
| | 영어 : Effects of TCDD on the expression of Matrix Metalloproteinase-2 and 9 | | | | |

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

**동의여부 : 동의( ) 반대( ○ )**

2007 년 2 월

저작자: 최재호 (서명 또는 인)

## 조선대학교 총장 귀하