

**In Vivo Effects of Cyclooxygenase-2 (COX-2) Deletion  
on Cellular Signaling in HCC Xenografts in Nude Mice**

**(Running Title: COX-2 Deletion and HCC Cellular Signaling)**

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**The abbreviations.** COX-2, cyclooxygenase-2; HCC, hepatocellular carcinoma; C2D, cyclooxygenase-2 deletion; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; AFP, alpha fetoprotein; CD1, cyclin D1; HDAC2, histone deacetylase-2; AC-H3 and AC-H4, acetylation of histone H3 and H4; PTEN, phosphatase and tensin homolog deleted on chromosome ten; MAPK, mitogen-activated protein kinase; p-ERK, phosphorylated extracellular-regulated kinase; IP, immunoprecipitation; IB, immunoblotting.

## **Abstract**

**AIM:** It is known that cyclooxygenase-2 (COX-2) overexpression is associated with growth of hepatocellular carcinoma (HCC) cells, but its in vivo mechanisms remains to be determined. **METHODS:** In the present study, the parental HuH7, a human HCC cell line, and COX-2 deleted-HuH7 cells were inoculated to nude mice to assess the in vivo effects of COX-2 deletion (C2D) on cellular signaling in HCC xenografts. **RESULTS:** We found that C2D significantly inhibited Ki-67 and increased peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) expression, and reduced plasma prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and alpha fetoprotein (AFP) levels in association with reduced Rb phosphorylation and cyclin D1 (CD1)/CDK4 complex, and increased p21/CDK4 complex, indicating COX-2 overexpression promotes G1-S transition of the cell cycle in human HCC xenografts via a series of complicated signaling. C2D also resulted in significantly reduced phosphorylation of Akt, indicating a potential role of PTEN/PI3K/Akt pathway in COX-2 mediated alteration of HCC growth. We also demonstrated that C2D significantly inhibited histone deacetylase-2 (HDAC-2) expression, but increased acetylation of histone-3 (AC-H3) and AC-H4 expression, indicating that altered histone acetylation by HDACs may be involved in COX-2 mediated HCC cell proliferation. In contrast to previous in vitro reports, we found that C2D did not significantly affect apoptosis and formation of activated caspase-3 and 9 in HCC xenografts. **CONCLUSION:** Our results revealed that C2D results in a series of in vivo alteration of cellular signaling in HCC xenografts that demonstrated important pathogenic mechanisms of COX-2 overexpression in HCC growth and provided valuable information on searching novel approach to HCC chemoprevention.

**Key Words:** Hepatocellular carcinoma, HCC xenografts, cyclooxygenase-2 (COX-2), cell cycle, apoptosis, histone acetylation, and signal transductions

## Introduction

Hepatocellular carcinoma (HCC) accounts for approximately 6% of all human cancers worldwide and its incidence remains in rising (1-4). COX-2 overexpression has been associated with hepatocarcinogenesis (5-11). We have reported that celecoxib, a selective COX-2 inhibitor, results in potent growth inhibition of human HCC cells that is associated with altered HCC cell proliferation, cell cycle progression, and apoptosis (9). We also found that celecoxib results in a comparable growth inhibition of PLC/PRF/5, a low COX-2 expressing hepatoma cell line, and HuH7, a high COX-2 expressing hepatoma cell line (9). Although these suggest COX-2 independent mechanism mediated by celecoxib, a direct comparison of HCC cells derived from a single line with different level of COX-2 expression will be needed to confirm our previous findings.

Phosphatase and tensin homolog deleted on chromosome ten (PTEN), phosphatidylinositol 3'-kinase (PI3K) and Akt (PTEN/PI3K/Akt) is associated with carcinogenesis (12). Activated PI3K-Akt signaling promotes carcinogenesis (13, 14). PTEN is a negative regulator of PI3K-Akt signaling and frequently inactivated in malignancies (15, 16). The mitogen-activated protein kinase (MAPK) pathway is a mitogenic signaling cascade. The phosphorylated extracellular-regulated kinase (ERK), the final intermediate in the MAPK signaling cascade, promotes cell proliferation, cell cycle progression, and oncogenesis (17-19). It remains unknown whether PTEN/PI3K/Akt and MAPK pathways are involved in COX-2-induced HCC growth.

Histone acetylation enhances access of the transcriptional factors to DNA by modifying nucleosome structure that leads to DNA relaxation and reduces the affinity of histone complexes with DNA. Accumulating evidence has indicated that aberrant regulation of histone deacetylases (HDACs) is associated with carcinogenesis, such as gastric cancer, by hypo-acetylation of histone (20-23). However, it remains unknown whether COX-2-induced HCC growth is associated with altered HDAC-2 expression.

Anti-sense RNA has been used to inhibit COX-2 expression (10). However, this approach only results in a partial and short-term reduction of COX-2 expression. Recently, we developed COX-2 deleted HCC cells (i.e. C2D-HuH7) from a high COX-2 expressing human HCC line, HuH7 cells, and demonstrated C2D significantly reduced growth of C2D-HuH7 cells

(11). This provided us with a unique opportunity to use a single cell line with different level of COX-2 expression to assess in vivo pathogenic roles of COX-2 overexpression in HCC growth.

In the present study, both the parental HuH7 and C2D-HuH7 cells were used to assess the in vivo effects of C2D on cellular signaling of HCC xenografts in nude mice. Our results indicate that C2D significantly inhibited Ki-67 and increased PPAR $\gamma$  expression in association with decreased plasma AFP and PGE<sub>2</sub> levels, and G1-S transition in C2D-HuH7 xenograft tissue as indicated by reduced Rb phosphorylation, CD1/CDK4 complex, and increased p21/CDK4 complex. C2D also resulted in significantly reduced phosphorylation of Akt. In addition, C2D significantly inhibited HDAC-2, but increased AC-H3 and AC-H4 expression.

## **Materials and Methods**

**Reagents.** The reagents for cell culture were the same, as previously reported (9). Antibody against human COX-2 was from Cayman Chemical Company (Ann Arbor, MI). Antibody against activated caspase-3 was from Sigma Chemical Co. (St. Louis, MO). Antibodies against human cyclin D1 (CD1), CDK4, phosphorylated-retinoblastoma (p-Rb), p21, p27, PPAR $\gamma$ , activated caspase-9, Akt, p-Akt, p-ERK (i.e. p-ERK1, 2), PTEN, Ki-67, HDAC-2, AC-H3 and H4 and  $\beta$ -actin were from Santa Cruz Biotechnology (Santa Cruz, CA). An EIA kit for AFP quantification was from Monobind, Inc (Costa Mesa, CA). Real-time PCR (RT-PCR) kit was from Applied Biosynthesis Company (Foster city, CA). An EIA kit for cell death detection was from Roche Applies Science (Indianapolis, IN) (9).

**Cell Culture.** HuH7 cells, a human HCC cell line expressing a high level of COX-2 (9), were used as control and the parental cells to generate a pool of C2D-HuH7 cells. The cells were cultured (9) and used to establish HCC xenografts in nude mice, as previously reported (11) and described below.

**Construction of Plasmid pCRCOX2NEO and Generation of C2D-HuH7 Cells.** C2D-HuH7 cells were established by transfecting HuH7 cells with plasmid pCRCOX2NEO and clone selection as previously reported (11, 24). Briefly, a human COX-2 DNA fragment of 2,570 bp (nt 11-2580, GeneBank Accession#: U04636) was obtained through in vitro PCR amplification of HuH7 DNA. The PCR product was cloned into plasmid pCR2.1 TOPO (Invitrogen Co., Carlsbad, CA) through TA cloning to generate plasmid pCRCOX2.5. The sequences between the Mfe I (nt 832) and Hpa I (nt 1572) sites of the human COX-2 fragment in the plasmid

pCRCOX2.5 was replaced by a DNA fragment containing SV40 promoter, neomycin resistant gene, and SV40 poly A sequences obtained from plasmid pcDNA3.1 to generate plasmid pCRCOX2NEO.

The plasmid pCRCOX2NEO and restriction enzymes ApaI, BsrGI and BglII were employed to recover the fragment containing COX-2 DNA sequences on both terminals and insert of SV40 promoter, neomycin resistant gene and SV40 poly A sequences in the middle. HuH7 cells in 70% confluence were then transfected with the above DNA fragment using SuperFect transfection reagent (Qiagen, Valencia, CA). Eight hours after transfection, G418 (Promega, Madison, WI) at the concentration of 800 mg/L was added to the medium to select the transfected cells. Survived cells were serially diluted and continuously cultured with fresh medium supplemented with G418. After more than 5-times passage, a pool of C2D-HuH7 cells was established. Deletion of COX-2 expression was confirmed by Real time-PCR, Western blot, and PGE<sub>2</sub> quantification.

To exclude the effect of the plasmid vector, HuH7 cells were transfected with pCR2.1 TOPO, the parental plasmid for pCRCOX2NEO. Compared to non-transfected HuH7 cells, transfection of pCR2.1 TOPO did not affect cell proliferation and apoptosis (data not shown). Non-transfected HuH7 and C2D-HuH7 cells were then used for the following studies.

**Preparation of HCC Xenograft Tissue.** After obtaining approval of the Institutional Animal Care and Use Committee, 4-weeks old male NCRNU-M nude mice (Taconic Farms, Inc.) were used to prepare HCC xenograft tissue as previously described (9). The study included 20 mice which were divided into two groups with 10 mice in each group. After subcutaneous inoculation of  $5 \times 10^6/0.25$  ml of HuH7 cells (n=10) or C2D-HuH7 cells (n=10), mice were maintained with standard rodent chow and water *ad libitum* for 5 weeks, as previously reported (9). Three mice from each group with HCC xenograft volume close to the group mean were used to collect xenograft tissue specimens. The plasma specimens were simultaneously collected to quantify PGE<sub>2</sub> and AFP. Approximately 300 mg of tumor tissues were homogenized and the supernatants of xenograft lysate were stored in -80°C (9) and used for experiments described below.

**Quantification of Plasma PGE<sub>2</sub> and AFP Levels.** The plasma PGE<sub>2</sub> level was quantified using an EIA kit, as previously reported (9). The plasma AFP level was quantified using 10 µl plasma sample and an EIA kit according to manufacturer's instruction. The results were recorded as the absorbance at 450 nm (using a reference wavelength of 620 nm) using

ELX800 Universal Microplate Reader (9). The standard curve was used to calculate plasma AFP level.

**Apoptosis Assays.** Apoptosis was quantified using an EIA kit, as previously reported (9). The degree of apoptosis was compared between the two groups.

**Immunoprecipitation (IP) and Immunoblotting (IB) Assays.** The supernatants of xenograft lysate isolated as described above were used to detect Ki-67, PPAR $\gamma$ , p21, E2F1, CD1, CDK4, p-Rb, activated caspase-3, caspase-9, Akt, p-Akt, p-ERK, HDAC-2 and AC-H3 and AC-H4 expression. The IP assays were same as previously reported (9). The IB of  $\beta$ -actin was used as internal controls. The relative amount of each protein was quantified by digitally scanning its hybridizing bands, as previously reported (9).

**Statistical analysis.** The descriptive statistics was provided with mean  $\pm$  SD. An independent sample t-test was used to assess the effect (i.e. mean differences) on PGE<sub>2</sub> and AFP production, apoptosis, as well as the scanning data of Western blot, a P < 0.05 was considered statistically significant.

## Results

**Establishment of C2D-HuH7 Cells from HuH7 Cells.** Successful deletion of COX-2 expression in C2D-HuH7 cells was confirmed by (1) absence of COX-2 mRNA expression in the cells assessed by a real time-PCR (Figure 1A), (2) deletion of COX-2 protein expression determined by IB (Figure 1B), and (3) absence of PGE<sub>2</sub> production by an EIA assay (data not shown).

**C2D Reduced Ki-67 and Increased PPAR $\gamma$  Expression, and Decreased Plasma AFP and PGE<sub>2</sub> Levels.** C2D reduced mean xenograft weight in the nude mice bearing C2D-HuH7 xenografts, but this was not statistically significantly. This may be due to limited sample size. As showed in Figure 2 A, C2D significantly reduced Ki-67 expression in the xenograft tissue (29% less than HuH7, p<0.05), indicated decreased HCC cell proliferation. We then examined whether COX-2 is involved in controlling PPAR $\gamma$  expression. As shown in Figure 2B, C2D significantly increased PPAR $\gamma$  expression compared to HuH7 xenografts (1.9-folds, p<0.05). Consistent with reduced Ki-67 and increased PPAR $\gamma$  expression, both plasma AFP and PGE<sub>2</sub> levels were reduced in C2D-HuH7 group (30% and 39% respectively, p<0.05, Figures 2C-D) compared to COX-2 expressing HuH7 group.

**C2D Inhibited HCC Cell Cycle Progression.** To study the effects of COX-2 on cell cycle

progression, we assessed expression of a group of cell cycle modulators. As showed in Figures 3A-C, p-Rb expression was 40% lower in C2D-HuH7 than in HuH7 xenografts ( $p < 0.05$ ). Additionally, C2D significantly reduced CD1/CDK4 complex (24%,  $p < 0.05$ ), but increased p21/CDK4 complex (79%,  $p < 0.05$ ). However, p27/CDK4 complex was not significantly different between the two groups (data not shown). These suggested C2D down regulates cell proliferation by altered cell cycle progression in G1-S transition.

**C2D Did not Significantly Alter Apoptosis and the Production of Activated Caspase 3 and 9.** Using a quantitative EIA kit detecting cytoplasmic histone-associated DNA fragment (9), we found that compared with HuH7 xenografts, C2D did not result in significant change in cell apoptosis (Figure 4A,  $p > 0.05$ ) in C2D-HuH7 xenografts. Consistent with this, C2D did not significantly affect the production of activated caspase-3 and 9 as shown in Figures 4B and C ( $p > 0.05$ ). These suggested that COX-2-induced in vivo HCC growth appears not mediated by altered HCC cell apoptosis.

**C2D Reduced HDAC-2, but Increased AC-H3 and AC-H4 Expression.** To determine HDACs potential role in COX-2 mediated HCC growth, we examined whether C2D alters expression of HDACs in HCC xenografts. Compared with HuH7 xenografts, C2D resulted in inhibited HDAC-2 expression, and increased AC-H3 and AC-H4 (Figures 5A-C,  $p < 0.05$ ) in C2D-HuH7 xenografts. These suggested that COX-2 expression up-regulates HDAC-2 expression, and down-regulates AC-H3 and AC-H4 expression in HCC xenografts, a possible novel pathway that may be associated with COX-2 mediated hepatocarcinogenesis.

**C2D Inhibited Akt, but Not ERK Phosphorylation.** To examine the role of PTEN/PI3K/Akt and MAPK pathways in COX-2-induced HCC growth, we assessed whether C2D alters phosphorylation of Akt and ERK. Our results revealed that C2D did not affect total Akt expression, but significantly inhibited Akt phosphorylation (Figure 6A-B). C2D did not affect ERK phosphorylation in C2D-HuH7 xenografts (data not shown).

## Discussion

Aberrant COX-2 expression has been associated with carcinogenesis, including HCC (5-7). Understanding COX-2-mediated mechanism of HCC growth will help in developing an effective chemoprevention. Using a standard gene knockout technique (24), we established C2D-HuH7 cells (11). Both real time-PCR and IB confirmed undetectable COX-2 mRNA and protein in C2D-HuH7 cells that was associated with undetectable PGE<sub>2</sub> in C2D-HuH7 cells and the culture medium. These confirmed successful deletion of COX-2 gene expression in C2D-HuH7 cells. The establishment of C2D-HuH7 cell line provided us a unique way to determine the in vivo mechanisms of COX-2-mediated HCC growth.

Compared with HuH7 xenografts, C2D resulted in a significantly inhibited Ki-67 expression, an important biomarker of cell proliferation, in C2D-HuH7 xenografts. These findings extended the previous in vitro study results (9), and indicated that COX-2 overexpression promotes in vivo HCC cell proliferation. PPAR $\gamma$  is a member of the nuclear hormone receptors. Increased PPAR $\gamma$  expression inhibits cell growth of human liver cancer cells (25, 26). In the present study, we found that C2D significantly increased PPAR $\gamma$  expression in C2D-HuH7 xenografts, suggesting that COX-2 overexpression may inhibit PPAR $\gamma$  expression that might be associated with COX-2-promoted HCC growth.

PGE<sub>2</sub> has been shown to stimulate cell proliferation, angiogenesis and cell invasion. As a widely used biomarker, increased level of PEG<sub>2</sub> has been reported in multiple malignancies (27). To further determine the effect of COX-2 on PGE<sub>2</sub> production, we assessed plasma levels of PGE<sub>2</sub> in the two groups of nude mice containing HCC xenografts with or without COX-2 expression. We found the significantly lower plasma PGE<sub>2</sub> was associated with reduced Ki-67 and increased PPAR $\gamma$  expression in C2D-HuH7 group, indicating that increasing PGE<sub>2</sub> production is another important medium of COX-2-induced HCC proliferation.

AFP has been widely used for diagnosing HCC, assessing HCC differentiation, and treatment effects of HCC in humans (28-30). Our results showed that deletion of COX-2 expression reduced plasma AFP level in nude mice bearing C2D-HuH7 xenografts, suggesting that COX-2 overexpression may stimulate AFP production and alter HCC cell differentiation. The association of decreased plasma AFP with reduced Ki-67 and increased PPAR $\gamma$  expression in the nude mice bearing C2D-HuH7 xenografts further confirmed in vivo pathogenic role of COX-2 in HCC proliferation.

Enhanced cell cycle progression featured by increase in p-Rb and CD1/CDK4 complex is an important way to promote tumor growth (31). Our results demonstrated that C2D significantly inhibited formation of p-Rb, CD1/CDK4 complex in C2D-HuH7 xenografts. It is known by forming complex with CDKs, p21 inhibits the activity of CDKs and cell cycle progression (32). We also demonstrated that deletion of COX-2 significantly increased formation of p21/CDK4 complex in C2D-HuH7 xenografts. Taken together, our results support the notion that COX-2 overexpression promotes *in vivo* HCC cell cycle progression by stimulating G1-S transition, as previously reported *in vitro* results (9).

Inhibition of cell apoptosis promotes tumor growth (6, 33). Our results indicated that compared with HuH7 xenografts, C2D had no significant effects on apoptosis in C2D-HuH7 xenografts. Consistent with this, we found that C2D did not significantly affect production of the activated caspase-3 and 9 in C2D-HuH7 xenografts, the two biomarkers of apoptotic activity. These results indicated that the previously reported *in vitro* results may not reliably reflect the *in vivo* role of COX-2 in apoptosis.

HDACs inhibit histone acetylation that promotes cell proliferation, differentiation, and cell cycle regulation (20-22). Dysregulation of the histone acetylation in the cells is associated with carcinogenesis (34). Overexpression of HDAC-2, one of the HDACs isoforms (35), has been associated with human gastric cancer (23). For the first time, we demonstrated that C2D significantly inhibited HDAC-2, but increased AC-H3 and AC-H4 expression in C2D-HuH7 xenografts, suggesting that COX-2-induced HCC growth may also be mediated by altered histone acetylation. Because inhibition of HDACs enhances p21 expression (36, 37), altered HDACs expression by C2D might be associated with enhanced formation of p21/CDK4 complex. If convinced, HDAC-2 may serve as a potential target for HCC chemoprevention.

Studies have indicated important roles of PTEN/PI3K/Akt and ERK signaling in carcinogenesis and cancer progression (38-46). Akt is composed of an N-terminal pleckstrin homology domain and a C-terminal kinase catalytic domain. Phosphorylation at Thr<sup>308</sup> and Ser<sup>407</sup> results in Akt activation, that dissociates from the cell membrane and enters the cytoplasm and nucleus, where it phosphorylates several key proteins resulting in promoting cell cycle progression(8). We found that C2D did not affect total Akt expression, but significantly reduced p-Akt production in C2D-HuH7 xenografts, suggesting COX-2 overexpression promotes p-Akt formation that may be involved in COX-2-mediated HCC growth. Increased p-ERK activates

transcription of the mitogenic and cell regulatory genes and promotes oncogenesis (38, 45). Increased ERK was reported in HCC (46), suggesting its involvement in HCC development. In the present study, we found that p-ERK production was not significantly affected by C2D, suggesting that COX-2 expressing status may not significantly affect p-ERK production.

In conclusions, the present study demonstrated that C2D in HuH7 xenografts results in decreased HCC cell proliferation and cell cycle progression via a complicated cell cycle modulation, PTEN/PI3K/Akt and HDAC signaling. Our findings provide important insights on pathogenic mechanisms of COX-2 overexpression in HCC growth and potential targets for HCC chemoprevention.

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## Figure Legends

**Figure 1. COX-2 Expression in HuH7 and C2D-HuH7 Cells.** **A.** Real time-PCR showed COX-2 mRNA was detectable in HuH7 cells, but not in C2D-HuH7 cells. **B.** IB assay showed COX2 was detectable in the standard control and HuH7 cells, but not in C2D-HuH7 cells. **C.**  $\beta$  actin was used as internal control.

**Figure 2. Effects of C2D on Ki-67, PPAR $\gamma$ , AFP, and PGE<sub>2</sub>.** **A.** Ki-67 expression was significantly decreased in C2D-HuH7 cells. **B.** PPAR $\gamma$  expression was significantly increased in C2D-HuH7 cells by a semi-quantified IB assay. **C.** Plasma AFP (ng/ml) was significantly decreased in C2D-HuH7 cells quantified by an EIA assay. **D.** Plasma PGE<sub>2</sub> (pg/ml) was significantly decreased in C2D-HuH7 cells quantified by an EIA assay. “#” indicates  $p < 0.05$  between two groups.

**Figure 3. Effects of C2D on the Cell Cycle Modulators.** **A.** p-Rb and **B.** CD1/CDK4 complex were significantly decreased in C2D-HuH7 cells by IP and IB assay. **C.** p21/CDK4 complex was significantly increased in C2D-HuH7 cells by IP and IB assay. “#” indicates  $p < 0.05$  between two groups.

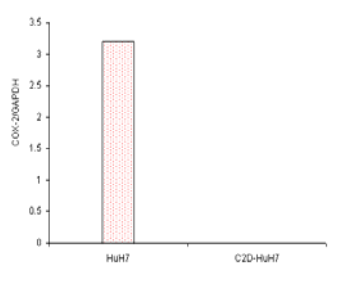
**Figure 4. Effects of C2D on Apoptosis, Activated Caspase-3 and 9.** **A.** the change of HCC cell apoptosis was no significant in C2D-HuH7 cells quantified by an EIA assay. **B.** Activated caspase-3, and **C.** Active caspase-9 expression were not significantly changed in C2D-HuH7 cells by IB assay.

**Figure 5. Effect C2D on Expression of HDAC2, AC-H3 and AC-H4** **A.** HDAC2 was significantly decreased in C2D-HuH7 cells quantified by IB assay **B.** AC-H3, and **C.** AC-H4 were significantly increased in C2D-HuH7 cells quantified by IB assay. “#” indicates  $p < 0.05$  as compared between two groups.

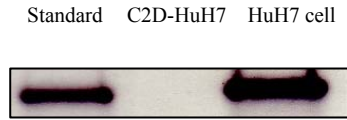
**Figure 6. Effects of C2D on Total Akt and p-Akt.** **A.** Total Akt expression was not significantly changed in C2D-HuH7 cells. **B.** p-Akt expression was significantly decreased in C2D-HuH7 cells quantified by IB assay. “#” indicates  $p < 0.05$  between two groups.

**Figure 1.**

**A.**



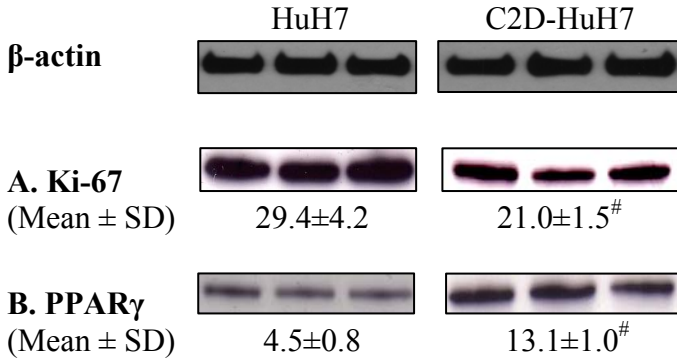
**B. COX-2**



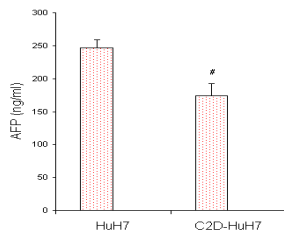
**C. β-actin**



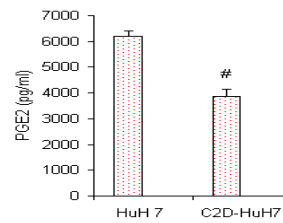
**Figure 2.**



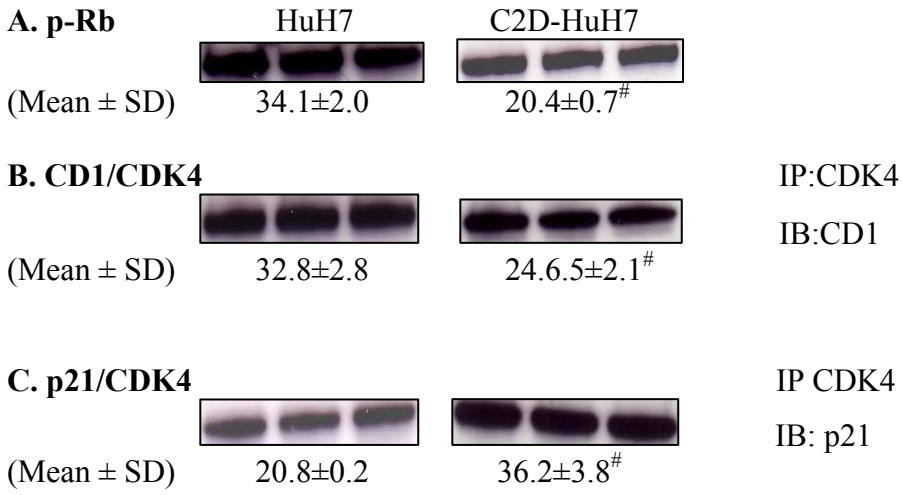
**C. Plasma AFP**



**D. Plasma PGE<sub>2</sub>**

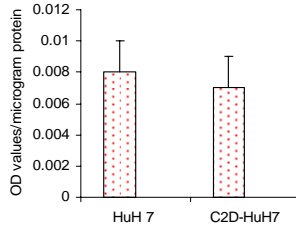


**Figure 3.**

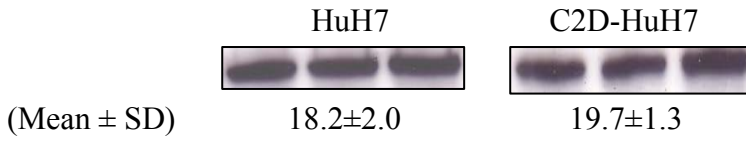


**Figure 4.**

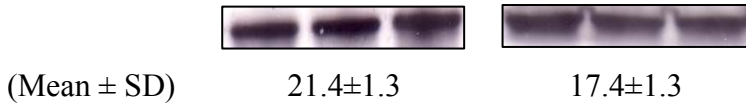
**A. Apoptosis**



**B. Active caspase-3**

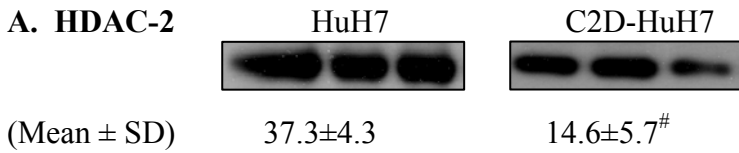


**C. Active caspase-9**

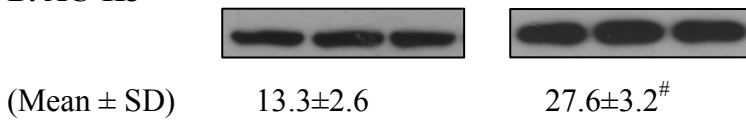


**Figure 5.**

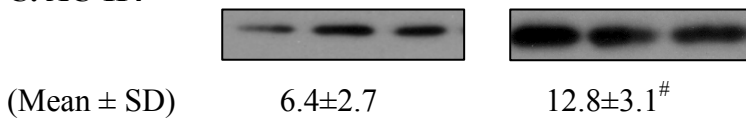
**A. HDAC-2**



**B. AC-H3**

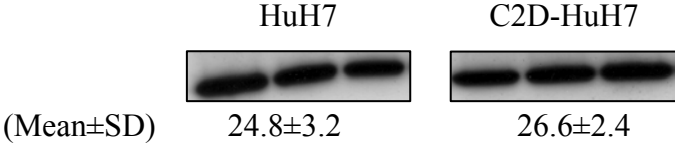


**C. AC-H4**



**Figure 6**

**A. Total Akt**



**B. p-Akt**

