

## Conjugated Linoleic Acid (CLA) Modulates Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) Signaling in Canine Mammary Cells

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**Abstract.** Background: Conjugated linoleic acid (CLA), a naturally occurring linoleic acid isomer found in ruminant-produced foods, has the potential to serve as an effective chemopreventive nutraceutical factor for breast cancer prevention based upon previous published studies. There are several CLA isomers in ruminant-produced food products, among which t10,c12-CLA and c9,t11-CLA are more potent. Expression of cyclooxygenase 2 (COX-2) in mammary tumors has been correlated with poor prognosis. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is a major COX-2 product in various cancers and, as in humans, PGE<sub>2</sub> concentrations in canine tumor tissues were frequently elevated. Moreover, a PGE<sub>2</sub> receptor subtype, EP2, is highly expressed in mammary tumors. Thus, various studies have implicated the important role of PGE<sub>2</sub> and EP2 in COX-2-regulated tumor development. Materials and Methods: Mammary tumor and normal mammary tissues were both collected from a female dog with mammary tumor. Both malignant and normal mammary tissues were subjected to isolation of epithelial and stromal cells. The effects of t10,c12-CLA and c9,t11-CLA on proliferation, as well as COX-2 and EP2 protein expression in canine mammary normal and cancerous cells, were detected by CellTiter 96™ AQueous assay and Western blot assay, respectively. Results: Both t10,c12-CLA and c9,t11-CLA not only suppressed malignant mammary cell growth, but also exerted inhibitory effects on tumor-associated non-malignant mammary cells. Similarly,

both t10,c12-CLA and c9,t11-CLA suppressed EP2 protein expression in both normal and malignant mammary cells. t10,c12-CLA was more effective in decreasing COX-2 protein expression in malignant mammary cells, while, in contrast, c9,t11-CLA down-regulated COX-2 protein expression in both normal and malignant mammary cells. Conclusion: The results indicate that the dietary component CLA regulates COX-2 and EP2 protein expression in both malignant mammary cells and cells from the tumor-associated stromal compartment. In turn, this may suppress PGE<sub>2</sub> signaling, leading to better prognosis. We further speculate that the knowledge obtained from canine studies may also be beneficial to study human breast cancer.

Conjugated linoleic acid (CLA) is produced by rumen fermentation of linoleic acid and is deposited in the subcutaneous fat and intramuscular fat layer in cattle, and is also present in milk fat (1). There are several CLA isomers in ruminant-produced foods, among which c9,t11-CLA and t10,c12-CLA are more potent (2). CLA has been found to possess anti-carcinogenic anti-diabetic, anti-atherogenic and anti-adipogenic activities in mouse and human cell lines, and in *in vivo* animal studies using mice (2). It was recently reported, in a large epidemiological study, that CLA dietary intake was associated with the regulation of estrogen receptor expression in breast cancer patients (3). CLA has been found to reduce the risk of having an estrogen receptor (ER)-negative tumor in premenopausal breast cancer. This may lead to a better therapeutic outcome for breast cancer patients as their cancers will probably respond to anti-estrogen therapy (3). CLA studies in our laboratory (4-7) also demonstrated the anti-mammary tumor effects of CLA on (i) anti-angiogenesis by suppressing the predominant vascular endothelial growth

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factor (VEGF) isomers, VEGF-A<sup>121</sup> and VEGF-A<sup>165</sup>, mRNA expression in a human breast cancer cell line, MCF-7; (ii) decreasing estrogenic agent-induced canine mammary cell and breast cancer cell proliferation; (iii) regulating ER in canine mammary tumors and human breast cancers; and (iv) up-regulating the estrogen-regulated tumor suppressor gene, protein tyrosine phosphatase  $\gamma$  (PTP $\gamma$ ), in human breast cells.

Mammary tumors are the most common tumors in middle aged (approximately 9 to 11 years old) female dogs. Typically these dogs are usually sexually intact, or spayed later in life (8, 9). Canine mammary tumors have epidemiological, clinical and morphological prognostic features similar to those of human breast cancer (10, 11). Evidence from genetic, pharmacological and epidemiological studies has indicated a critical role of cyclooxygenase 2 (COX-2) in tumorigenesis (12). Overexpression of COX-2 has been found in several epithelial tumors, including human breast cancer and malignant canine mammary tumors, and is associated with poor prognosis (13-17). Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is a major COX-2 product in various human cancers and PGE<sub>2</sub> concentrations in human and canine tumor tissues were frequently elevated (18, 19). PGE<sub>2</sub> binds to four cell surface G-protein-coupled receptors, EP1, EP2, EP3 and EP4, which direct different signal transduction pathways (12, 20). In normal human breast stromal cells, binding of PGE<sub>2</sub> to its receptors, EP1 and EP2, stimulates adenylyl cyclase activity which, in turn, increases cyclic AMP (cAMP) production. This signaling then stimulates aromatase gene expression through the cAMP response element-binding protein (CREB). Subsequently, estrogen production is enhanced by increased aromatase activity which converts androgens to estrogens. As a result, proliferation of estrogen responsive human breast cancer cells is increased (21, 22). Whereas PGE<sub>2</sub> decreases cAMP production by binding to EP3, indicating an inhibitory pathway (14, 21). Moreover, recently published studies have indicated the important roles of PGE<sub>2</sub> and EP2 in COX-2-mediated mammary and skin tumorigenesis (12, 14). Thus, significant efforts have been devoted to inhibit the COX-2 enzyme for the prevention and treatment of cancer (17). Some epidemiological studies have shown that utilization of non-steroidal anti-inflammatory drugs (NSAIDs), both COX-2-selective and non-selective inhibitors, is related to reduced cancer risk, including breast cancer, colorectal cancer, lung cancer and genitourinary cancers (17, 22). However, recently several studies have implicated the use of COX-2 inhibitors, especially Rofecoxib, in to enhanced cardiovascular adverse effects, which caused the withdrawal of Rofecoxib by the manufacturer MERCK and a warning concerning the use of Rofecoxib by the FDA (12, 17, 23-32). Although it is important to be concerned with the side-

effects of COX-2 inhibitors, it is likely that COX-2 plays a key role in tumorigenesis and that inhibition of COX-2 will have anti-tumorigenic effects. Thus, a secure and effective method may be to use COX-2 inhibitors as chemopreventive or chemotherapeutic agents. This idea guided us to naturally-existing compounds in food which possesses anticancer activity such as CLA. Since CLA is a natural by-product of rumen fermentation and is found in foods derived from ruminants, consumption of CLA from foods for the purposes of chemoprevention probably will be safe.

Although it is believed that mammary tumors originate from epithelial cells, the paracrine signal produced by the surrounding stromal cells plays crucial roles in modulating the cancer microenvironment (33-34). Interestingly, in mammary cancers, CLA has been suggested to be incorporated and stored in stromal cells and to affect cancer epithelial cell progression (35). Our present study utilized a canine mammary model to investigate the CLA-modulated canine mammary microenvironment capable of altering PGE<sub>2</sub> signaling. More importantly, the results from our canine mammary model may also be applicable to and beneficial in human breast cancer.

## Materials and Methods

*t*10,*c*12-CLA (+98% purity) was purchased from Matreya, Inc. (PA, USA) and the CLA stock solution was prepared based on the paper published by Dr. Ip's group (36). The main difference was that dextran-coated charcoal (DCC, Dextran T-70; Pharmacia; activated charcoal; Sigma)-treated fetal bovine serum (FBS, GibcoBRL, Bethesda, MD, USA), instead of FBS, was added to pure *t*10,*c*12-CLA and *c*9,*t*11-CLA to prepare the CLA stock.

### Cell culture and treatment

*Isolation of epithelial and stromal cells from canine mammary tissues.* Normal and cancerous canine mammary tissues were both collected from a female 4-year-old, intact, Cocker spaniel with mammary tumor by a veterinary surgeon at the Franklin County Dog Shelter in Columbus, Ohio, U.S.A. Sections of both normal and cancerous canine mammary tissues were sent to the Veterinary Clinical Pathology Laboratory, Veterinary Medical Teaching Hospital at The Ohio State University for diagnosis by pathologists. Canine mammary tissues were placed in DMEM/F12 and stored at 4°C during transportation. The isolation of epithelial and stromal cells from canine mammary tissues and the culture condition were described in detail previously (37). Briefly, the tissues were minced and digested in 0.1% collagenase I (GibcoBRL) supplemented with 5% FBS and antibiotic-antimycotic (100 unit/ml penicillin G sodium, 100 mg/ml amphotericin B) (GibcoBRL) in a 37°C humidified incubator (5% CO<sub>2</sub> : 95% air) overnight. The digested mixture was centrifuged at 200xg for 5 minutes at 25°C. The cell pellet was re-suspended and allowed to settle by gravity 3 times (20 minutes/time). Stromal cells (from both normal and cancerous mammary tissues) in the supernatant were then centrifuged at 200xg for 5 minutes at 25°C and the pelleted stromal cells were re-suspended in phenol red-free high-calcium DMEM/F12 (1.05 mM CaCl<sub>2</sub>) supplemented with 5% FBS. Epithelial cells (from either

normal or cancerous mammary tissues) in the initial sedimented part were re-suspended in low calcium DMEM/F12 (0.04 mM CaCl<sub>2</sub>) supplemented with Chelex-100 (Bio-Rad Laboratories, Richmond, CA, USA) - treated FBS (10%).

**Culture conditions.** All experiments were performed on epithelial cells and stromal cells not propagated beyond the third passage. The cells were allowed to grow to 80% confluence, before being passaged using 0.5% trypsin – 5.3 mM EDTA (GibcoBRL) with a 1/4 splitting ratio. Cell viability was greater than 95%, as determined by the trypan blue dye exclusion method (38).

Unless otherwise stated, cultures of cancer stromal cells (CASC), normal stromal cells (NSC), cancer epithelial cells (CAEC) and normal epithelial cells (NEC) were maintained as follows. Epithelial cells (from either normal or cancerous mammary tissues) were cultured in low-calcium DMEM/F12 (0.04 mM CaCl<sub>2</sub>) supplemented with Chelex-100-treated FBS (10%); stromal cells were cultured in phenol red-free high-calcium DMEM/F12 (1.05 mM CaCl<sub>2</sub>) supplemented with 5% FBS to allow the cells to attach on cell culture plates or dishes. After 1 day, all cell types were cultured in phenol red-free high-calcium DMEM/F12 supplemented with 5% DCC-treated FBS for 2 days.

**Cell proliferation assay.** CASC, NSC, CAEC and NEC were grown on 96-well plates (1x10<sup>3</sup> cells in 100 µl/well) and treated with 2 to 20 µM of *t10,c12-CLA*, or 2 to 20 µM of *c9,t11-CLA*, or 0.01% DCC-treated FBS as control, in phenol red-free high-calcium DMEM/F12 supplemented with 5% DCC-treated FBS for 3 days. At the end of the treatment, the cell proliferation rate was quantified using the CellTiter 96™ AQueous assay (Promega, Madison, WI, USA). Briefly, 20 µl of MTS:PMS (20 : 1) solution was added to each well. The plates were then incubated at 37°C for 1.5 hours and the color density was checked every 30 minutes. Finally, optical density was read at 490 nm (OD 490 nm) by an ELISA plate reader.

**Western blot analysis.** CASC, NSC, CAEC and NEC (5x10<sup>5</sup> cells) were cultured on flat-bottomed cell culture dishes and treated with 2 to 20 µM of *t10,c12-CLA*, or 2 to 20 µM of *c9,t11-CLA*, or 0.01% DCC-treated FBS as control, in phenol red-free high-calcium DMEM/F12 supplemented with 5% DCC-treated FBS for 3 days. At the end of the treatment, the cells were washed with ice-cold PBS and then lysed with extraction reagent (Pierce, Rockford, IL, USA) and protease inhibitor (Pierce) in ice. The cell lysates were separated by centrifugation at 15,000 rpm at 4°C for 30 minutes. An equivalent amount of protein (50 µg) from each supernatant with sample buffer (62.5 mM Tris-HCL, pH 6.8, 25% glycerol, 2% SDS, 0.004% bromophenol blue and 5% β-mercaptoethanol) was boiled for 5 minutes and resolved in a 4-15% SDS-polyacrylamide ready gel (Bio-Rad, Hercules, CA, USA). After electrophoreses, the proteins were transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA) by semi-dry transfer system (Bio-Rad) at room temperature for 1 hour. The transblotted membrane was washed twice with phosphate-buffered saline containing 0.1% Tween 20 (PBST). After blocking with PBST containing 10% non-fat milk for 1 hour, the membrane was incubated with diluted (1:500) COX-2 goat polyclonal antibody (Santa Cruz, CA, USA, SC-1745), with diluted (1:500) EP2 rabbit polyclonal antibody (Santa Cruz, SC-20675), or with the diluted (1:1000) β-actin goat polyclonal antibody (Santa Cruz, SC-1615) in

PBST, 5% non-fat milk at 4°C overnight. The membrane for COX-2 was then incubated with diluted (1:3000) donkey anti-goat secondary antibody (Amersham, Piscataway, NJ, USA), the membrane for EP2 was incubated with diluted (1:3000) anti-rabbit secondary antibody (Amersham), and the membrane for β-actin was incubated with diluted (1:5000) donkey anti-goat secondary antibody (Amersham) in PBST, 5% non-fat milk for 1 hour at room temperature. The immunoblots were enhanced by the ECL Plus™ Western Blotting Detection reagent (Amersham, Buckinghamshire, UK) and visualized by the Fuji image system (FUJIFILM Medical Systems U.S.A., Inc., Stamford, CT, USA).

**Statistics.** The data, from 3 replicated wells as one cell group, was presented as the mean ± standard deviation (SD) and was analyzed using StatView® ANOVA unpaired *t*-test. A *p*-value less than 0.05 was considered to be statistically significant.

## Results

***t10,c12-CLA* and *c9,t11-CLA* inhibited the growth of canine mammary cells.** The effect of CLA on the growth of canine mammary cells was examined. CLA's anti-mammary tumor properties have been examined using *in vitro* and *in vivo* mammary models and the results showed that the anti-tumor effects of CLA were exerted mainly by *c9,t11-CLA* and *t10,c12-CLA* (39-47). In the present study, 2 to 20 µM of *t10,c12-CLA* and *c9,t11-CLA* were used to examine CLA's anti-tumor effects in canine mammary cells.

We applied the identical method for isolating human breast epithelial and stromal cells to separate the canine mammary epithelial and stromal cells. The purity of the mammary stromal and epithelial cell preparations was examined by morphology and confirmed by immunohistochemistry staining, as previously reported from our laboratory (37). We were able to show that the stromal cells exhibited typical spindle-shaped morphology and the majority of stromal cells (>95%) were immunopositive for the presence of vimentin and no expression of cytokeratin was detected, which confirmed the fibroblastic nature of the stromal cells. The epithelial cells tended to grow in characteristic rounded shapes, and more than 95% of the cultured epithelial cells were positive for the presence of cytokeratin, but there was almost no expression of vimentin (37).

Although mammary malignancies are epithelial in origin, breast tumors actually involve other cell types surrounding the cancerous epithelial cells and influencing their progression (48). The influence of *t10,c12-CLA* and *c9,t11-CLA* on the proliferation of cell types isolated from both normal and cancerous mammary tissue was investigated. Cancer epithelial cells (CAEC), cancer stromal cells (CASC), normal epithelial cells (NEC) and normal stromal cells (NSC) were treated with 2 to 20 µM of *t10,c12-CLA* and *c9,t11-CLA* for 3 days and the cell proliferation rate was determined by the CellTiter 96™ AQueous assay. The experimental data showed that *t10,c12-CLA* and *c9,t11-CLA*

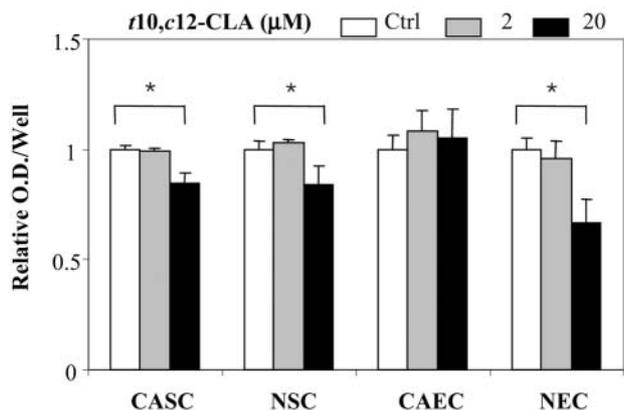


Figure 1. *t10,c12-CLA* inhibited canine mammary cell proliferation. CASC, NSC, CAEC and NEC refer to cancer stromal cells, normal stromal cells, cancer epithelial cells and normal epithelial cells, respectively. Each cell type was treated with 0, 2 or 20  $\mu\text{M}$  of *t10,c12-CLA* for 3 days, and the proliferative rate was quantified by using the CellTiter 96™ AQueous assay. Optical density was read at 490 nm (OD 490 nm) in 96-well plates by an ELISA plate reader for each group separately. Bars represent mean  $\pm$ SD, n=3. \* $p < 0.05$  stands for control versus *t10,c12-CLA*.

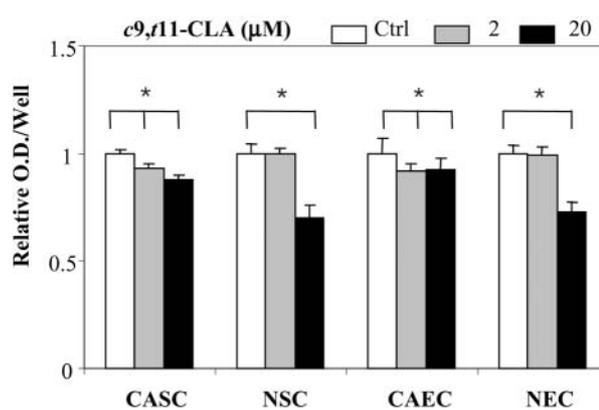


Figure 2. *c9,t11-CLA* inhibited canine mammary cell proliferation. CASC, NSC, CAEC and NEC refer to cancer stromal cells, normal stromal cells, cancer epithelial cells and normal epithelial cells, respectively. Each cell type was treated with 0, 2 or 20  $\mu\text{M}$  of *c9,t11-CLA* for 3 days, and the proliferative rate was quantified by using the CellTiter 96™ AQueous assay. Optical density was read at 490 nm (OD 490 nm) in 96-well plates by an ELISA plate reader for each group separately. Bars represent mean  $\pm$ SD, n=3. \* $p < 0.05$  stands for control versus *c9,t11-CLA*.

both suppressed CASC, NSC and NEC growth. However, only *c9,t11-CLA*, but not *t10,c12-CLA*, inhibited proliferation in CAEC (Figures 1-2). As little as 2  $\mu\text{M}$  of *c9,t11-CLA* significantly suppressed CASC and CAEC proliferation. Generally, 20  $\mu\text{M}$  of *t10,c12-CLA* and 20  $\mu\text{M}$  of *c9,t11-CLA* inhibited the proliferation of the canine mammary cells.

*t10,c12-CLA* and *c9,t11-CLA* suppressed COX-2 and EP2 expression in canine mammary cells. As in human mammary tumors, COX-2 was frequently expressed in the canine mammary carcinoma and usually not present in the normal mammary tissue, suggesting the potential role of COX-2 in canine mammary tumorigenesis. There are possible beneficial effects from blocking COX-2 in canine mammary tumor therapy (15, 16).

To compare the effects of *t10,c12-CLA* and *c9,t11-CLA* on COX-2 and EP2 protein expression in normal and cancerous canine mammary tissues, CAEC, CASC, NEC and NSC were treated with 2 to 20  $\mu\text{M}$  of *t10,c12-CLA* and *c9,t11-CLA* for 3 days. The COX-2 and EP2 protein levels were determined by Western blot analysis. The highest basal COX-2 levels were observed in CAEC (Figure 3). COX-2 could also be detected in NSC and NEC, but was almost undetectable in CASC; the highest EP2 basal levels were observed in CASC, while lower EP2 expression was detected in NSC, CAEC and NEC, suggesting a cell type-related specific expression pattern (Figure 3). *t10,c12-CLA* significantly decreased COX-2 protein in CASC and CAEC;

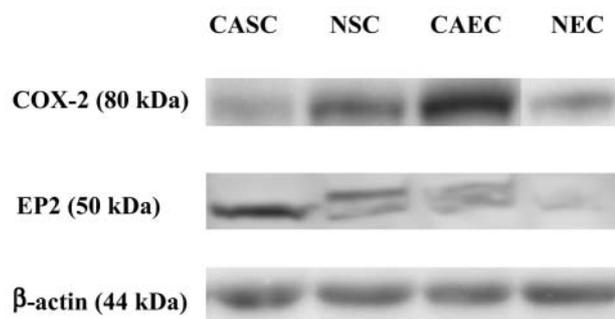


Figure 3. COX-2 and EP2 basal protein levels in canine mammary cells. CASC, NSC, CAEC and NEC refer to cancer stromal cells, normal stromal cells, cancer epithelial cells and normal epithelial cells, respectively. Whole cell lysate was isolated from each cell group separately and Western blot analysis of COX-2 and EP2 protein expression was performed.  $\beta$ -actin was used as the loading control.

however, under the tested doses, *t10,c12-CLA* did not induce significant change of COX-2 in NSC and NEC, suggesting the cell type-specific response pattern (Figure 4). Interestingly, although *c9,t11-CLA* did not significantly suppress COX-2 in CAEC, *c9,t11-CLA* appeared to be more effective on the surrounding other cell types, CASC, NSC and NEC (Figure 5).

After examining CLA's anti-COX-2 effects in different canine mammary cell types, we further investigated whether CLA possesses the ability to modulate PGE<sub>2</sub> downstream signaling. Although the role of EP2, a PGE<sub>2</sub> receptor

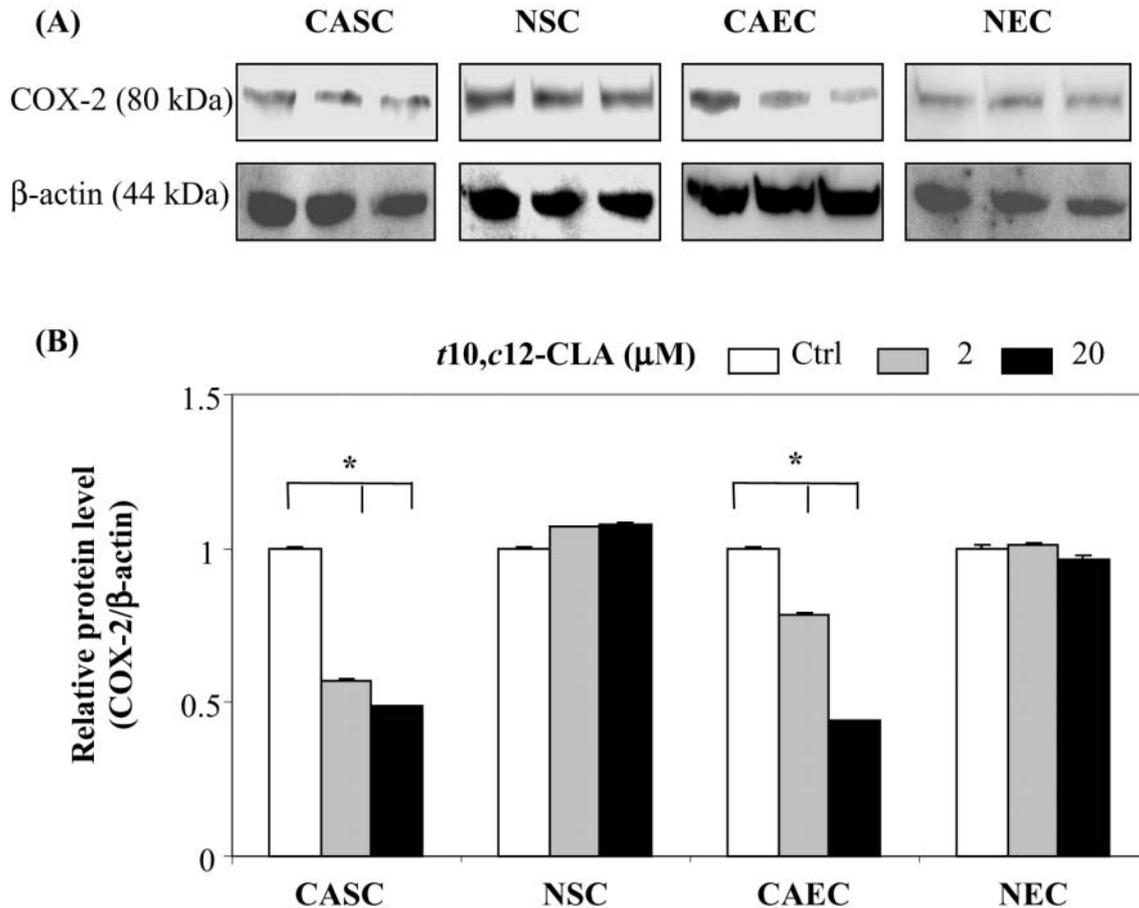


Figure 4. *t10,c12*-CLA decreased COX-2 protein expression in canine mammary cells. (A) Western blot analysis of *t10,c12*-CLA on COX-2 protein expression. CASC, NSC, CAEC and NEC refer to cancer stromal cells, normal stromal cells, cancer epithelial cells and normal epithelial cells, respectively. Each cell type was treated with 0, 2 or 20  $\mu$ M of *t10,c12*-CLA for 3 days and whole cell lysate was isolated from each group separately.  $\beta$ -actin was used as the loading control. (B) The protein ratio of COX-2 to  $\beta$ -actin was measured by densitometry. Bars represent mean  $\pm$  SD,  $n=3$ . \* $p < 0.05$  stands for control versus *t10,c12*-CLA.

subtype, in canine mammary tumor has not been established, pilot studies that applied genetic knock-out techniques in a mouse model have shown that EP2 is essential in mammary and skin tumor development (12, 14). Our experimental data demonstrated that 20  $\mu$ M of *t10,c12*-CLA induced >50% reduction of EP2 protein in CASC and NSC (Figure 6). *t10,c12*-CLA also significantly decreased EP2 protein in CAEC, however to a lesser extent (Figure 6). On the other hand, *c9,t11*-CLA exerted anti-EP2 effects mainly on the stromal cell types CASC and NSC (Figure 7). *c9,t11*-CLA did not induce a significant decrease of EP2 on the epithelial cell types CAEC and NEC (Figure 7). Together, our results showed that the isomers of CLA, *t10,c12*-CLA and *c9,t11*-CLA, down-regulated the COX-2 and EP2 protein levels in normal and cancerous canine mammary cells, suggesting that CLA might block PGE<sub>2</sub> signaling in canine mammary tumor.

## Discussion

PGE<sub>2</sub> signaling is exerted in both autocrine and paracrine mechanisms in canine mammary cells. Overexpression of COX-2 in tumors has been shown to be associated with increased angiogenesis; increased resistance to apoptosis; increased multidrug resistance; and increased estrogen production by enhancing aromatase activity. Thus it may explain the effective chemopreventive outcome of COX-2 inhibition on colorectal, breast cancer and many other malignant diseases (17, 22). A population-based case-control study examining the association between breast cancer risks, aspirin use and hormone receptor status by Terry *et al.* (49) showed that the use of aspirin, a COX-2 non-specific inhibitor, was related to the reduced breast cancer risk in women with hormone receptor-positive

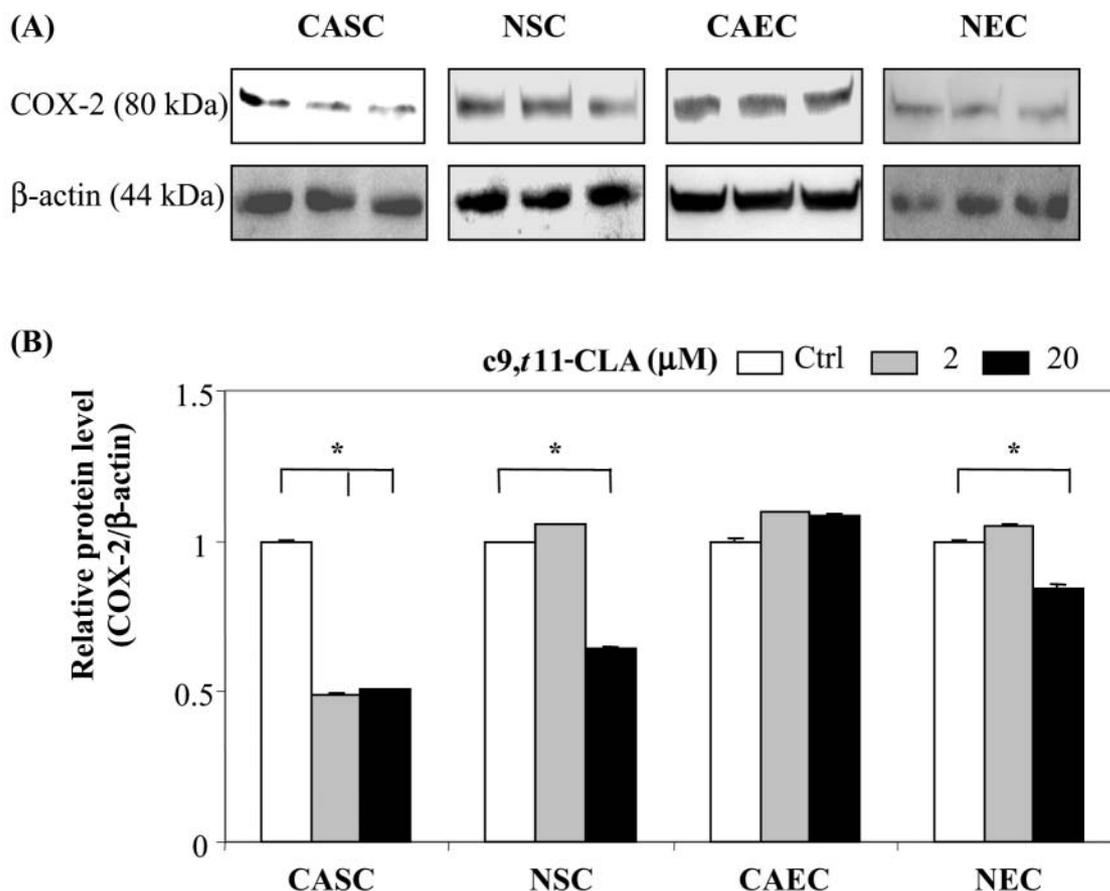


Figure 5. *c9,t11-CLA* decreased *COX-2* protein expression in canine mammary cells. (A) Western blot analysis of *c9,t11-CLA* on *COX-2* protein expression. CASC, NSC, CAEC and NEC refer to cancer stromal cells, normal stromal cells, cancer epithelial cells and normal epithelial cells, respectively. Each cell type was treated with 0, 2 or 20  $\mu$ M of *c9,t11-CLA* for 3 days and the whole cell lysate was isolated from each group separately.  $\beta$ -actin was used as the loading control. (B) The protein ratio of *COX-2* to  $\beta$ -actin was measured by densitometry. Bars represent mean  $\pm$  SD,  $n=3$ . \* $p<0.05$  stands for control versus *c9,t11-CLA*.

tumor, but not in those with hormone receptor-negative tumors. Use of aspirin may decrease estrogen biosynthesis by blocking *COX-2*, thus suppressing hormone responsive tumor development (22, 49). In human breast cancers, aromatase, stimulated by  $PGE_2$ , is expressed not only in tumor epithelial cells, but also in tumor stromal cells, endothelial cells and adipocytes (50). These observations may suggest that, in the tumor microenvironment,  $PGE_2$  produced by *COX-2* plays an important role in stimulating estrogen production in both tumors and surrounding tissues (50). Our findings showed that *COX-2* was mainly detected in tumor epithelial cells (CAEC), but was also expressed in the normal section of the mammary tissues (NSC and NEC) (Figure 3). Although it has been suggested that *COX-2* is usually absent in normal mammary tissue (15, 16), in our system, there was low expression of *COX-2* in the normal specimen of mammary tissue from a dog with mammary tumor. This indicates that normal tissues might be under the

influence of tumor tissues, causing normal tissue to start to undergo the process of tumorigenesis. In a solid tumor, there are not only malignant cells, but also many other non-malignant cell types (51). Evidence also shows that tumors can trigger tumor-associated macrophages to promote malignant progression and further metastasis (51). This suggests an essential role of the tumor microenvironment in the full development of tumors and also implies the potential benefit for targeting non-malignant cells for cancer therapy (51). Paracrine signaling between breast cancer epithelial and stromal cells has been proposed to explain the mechanism behind  $PGE_2$ -stimulated estrogen production (22).  $PGE_2$  synthesis is increased by overexpressed *COX-2* in human breast cancer epithelial cells.  $PGE_2$  then binds to its receptor EP on stromal cells, activating a signaling cascade that stimulates aromatase activity. This leads to increased estrogen production in the stromal cells which, in turn, causes increased growth of

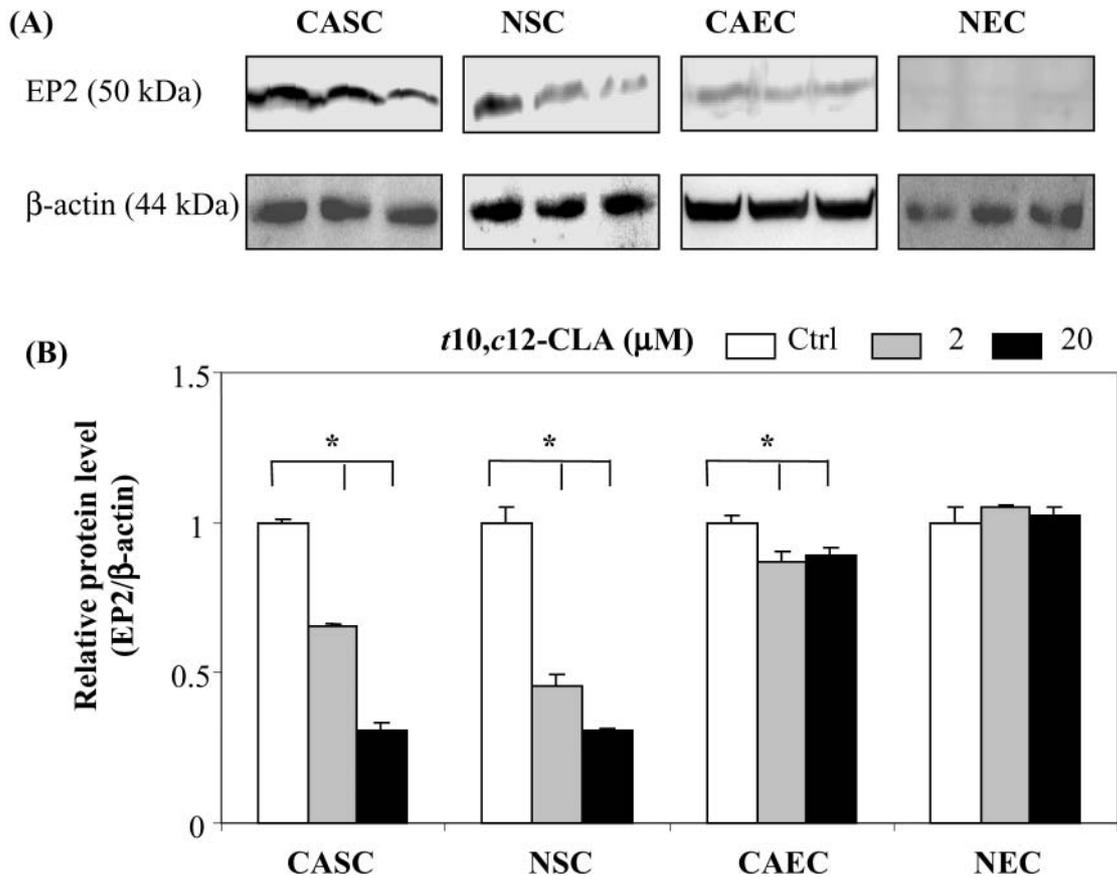


Figure 6.  $t_{10,c12}$ -CLA decreased EP2 protein expression in canine mammary cells. (A) Western blot analysis of  $t_{10,c12}$ -CLA on EP2 protein expression. CASC, NSC, CAEC and NEC refer to cancer stromal cells, normal stromal cells, cancer epithelial cells and normal epithelial cells, respectively. Each cell type was treated with 0, 2 or 20  $\mu$ M of  $t_{10,c12}$ -CLA for 3 days and the whole cell lysate was isolated from each group separately.  $\beta$ -actin was used as the loading control. (B) The protein ratio of EP2 to  $\beta$ -actin was measured by densitometry. Bars represent mean  $\pm$  SD,  $n=3$ . \* $p < 0.05$  stands for control versus  $t_{10,c12}$ -CLA.

breast cancer epithelial cells (22). Our findings showed that EP2 is mainly expressed in the stromal cell types, CASC and NSC, (Figure 3) supporting this paracrine signaling between breast cancer epithelial cells and stromal cells. However, EP2 can be detected in the epithelial cell types, CAEC and NEC, as well, possibly suggesting autocrine signaling. Furthermore, we observed that COX-2 and EP2 protein were not only expressed in mammary tumor, but also in the normal part of the mammary gland in this subject (Figure 3). Therefore, in our system, our results suggested that complicated PGE<sub>2</sub> signaling, in both paracrine and autocrine manners, may influence the mammary tumor microenvironment and contribute to tumor development.

*t<sub>10,c12</sub>-CLA and c<sub>9,t11</sub>-CLA suppress canine mammary cell growth and decrease COX-2 and EP2 protein expression.* A high proliferative rate of tumors has been positively correlated with metastases, death from neoplasia, low disease-free

survival rates and low overall survival rate in human and canine cancer models (52, 53).  $t_{10,c12}$ -CLA and  $c_{9,t11}$ -CLA suppressed the growth of both tumor cells and cells from the surrounding tissue (Figures 1-2), suggesting the ability of CLA to modulate the tumor microenvironment.

The effect of CLA on PGE<sub>2</sub> signaling has been examined by researchers in different tumor models. A CLA-enriched mixture of  $t_{10,c12}$ -CLA or  $c_{9,t11}$ -CLA decreased PGE<sub>2</sub> synthesis in human breast cancer cells (MDA-MB-231 (54) and MCF-7 (55)) and colon cancer cells (SW480) (55). Work from Dr. Yamasaki's group (56) demonstrated, by transplanting hepatoma dRLh-84 cells into rat livers and feeding rats a diet containing CLA, that CLA was able to depress PGE<sub>2</sub> and COX-2 mRNA in tumor tissues. Another study (57) used 1,2-dimethylhydrazine to induce colon tumor in rats, then feeding the rats with a CLA-containing diet showed that the COX-2 levels were not affected, but the PGE<sub>2</sub> levels were decreased. Other tumor models have

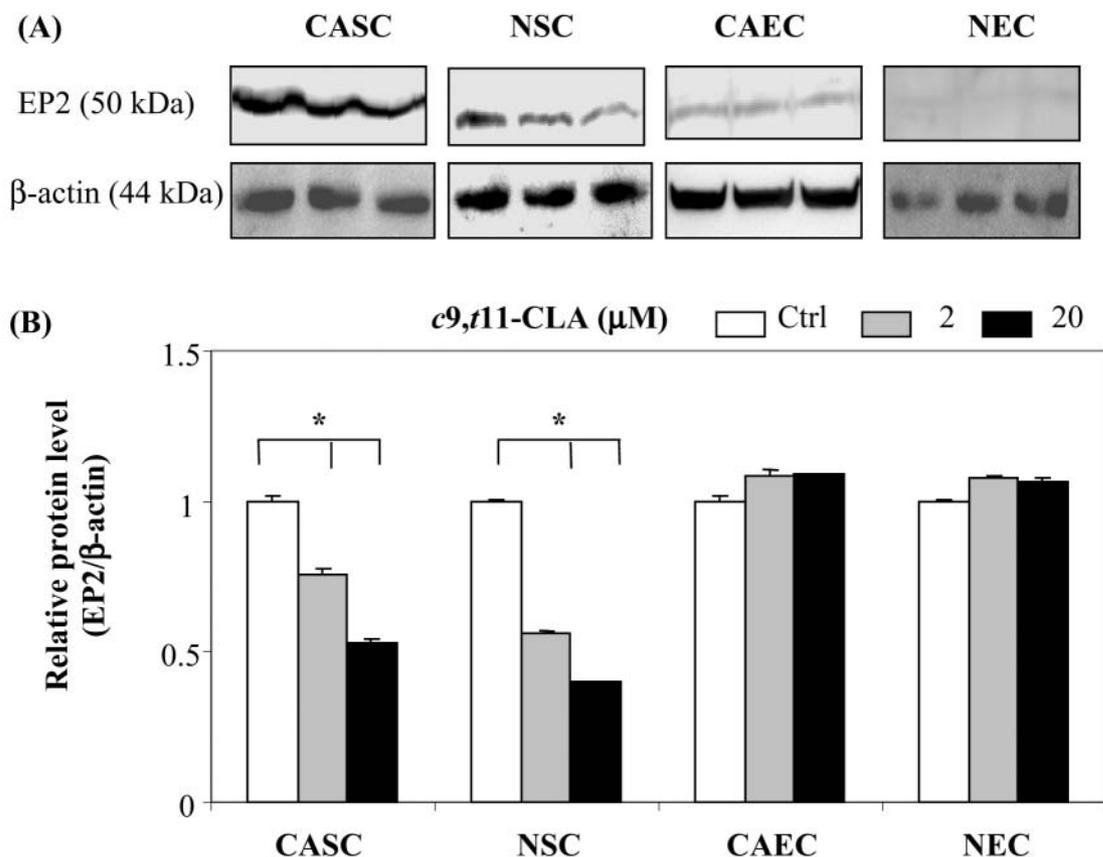


Figure 7.  $c9,t11$ -CLA decreased EP2 protein expression in canine mammary cells. (A) Western blot analysis of  $c9,t11$ -CLA on EP2 protein expression. CASC, NSC, CAEC and NEC refer to cancer stromal cells, normal stromal cells, cancer epithelial cells and normal epithelial cells, respectively. Each cell type was treated with 0, 2 or 20  $\mu$ M of  $c9,t11$ -CLA for 3 days and the whole cell lysate was isolated from each group separately.  $\beta$ -actin was used as the loading control. (B) The protein ratio of EP2 to  $\beta$ -actin was measured by densitometry. Bars represent mean  $\pm$  SD,  $n=3$ . \* $p < 0.05$  stands for control versus  $c9,t11$ -CLA.

shown that CLA diminishes the expression levels of COX-2 and PGE<sub>2</sub> in macrophages (58-60). We investigated CLA's anti-PGE<sub>2</sub> signaling effect on epithelial and stromal cells isolated from normal and malignant portions of mammary tissues from a canine mammary tumor. Our findings showed that  $t10,c12$ -CLA and  $c9,t11$ -CLA appeared to possess different potencies on different cell types, suggesting an isomer-specific effect on modulating COX-2 and EP2 expressions (Figures 4-7). We further speculate that, although  $t10,c12$ -CLA and  $c9,t11$ -CLA suppressed COX-2 and EP2 proteins in specific cell types, ruminant-produced foods containing both  $t10,c12$ -CLA and  $c9,t11$ -CLA and other trace CLA isomers would present synergistic effects on suppressing PGE<sub>2</sub> signaling.

To date, CLA's anti-PGE<sub>2</sub> signaling studies in mammary tumor models have not focused on other cell types surrounding the tumor. Our work showed that both  $t10,c12$ -CLA and  $c9,t11$ -CLA appeared to decrease COX-2 and EP2 protein expressions in both canine mammary tumor epithelial cells

and in other cell types from the tumor-associated stromal compartment. These results implicate that CLA interrupts autocrine and paracrine signaling of PGE<sub>2</sub> in the canine mammary tumor microenvironment. In conclusion, our findings add to the growing evidence that the consumption of ruminant dairy and beef products containing CLA might have chemo-preventive or/and chemo-therapeutic potentials (19). Further studies investigating CLA regulation of PGE<sub>2</sub> and estrogen production in mammary tumor cells and in cell types surrounding the tumor are in progress in our laboratory.

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