RESEARCH ARTICLE

Silencing of COX-2 by RNAi Modulates Epithelial-Mesenchymal Transition in Breast Cancer Cells Partially Dependent on the PGE_2 Cascade

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Abstract

In order to prove whether downregulation of COX-2 (Cyclooxygenase-2) could modulate the epithelial-mesenchymal transition (EMT) of breast cancer, celecoxib and siRNA were respectively used to inhibit COX-2 function and expression in MDA-MB-231 cells. The EMT reversal effect in the RNAi treated group was better than that of the celecoxib group while there were no obvious differences in the medium PGE_2 levels between the two groups. The results show that COX-2 pathways may contribute considerably to EMT of breast cancer cells, partially dependent on the PGE2 cascade. Akt2, ZEB2 and Snail were measured to clarify the underlying mechanisms of COX-2 on EMT; COX-2 may modulate EMT of breast cancer by regulating these factors. This finding may be helpful to elucidate the mechanisms of selective COX-2 inhibitor action in EMT modulation in breast cancer.

Keywords: COX-2 - snail - ZEB2 - PGE_2 - breast cancer

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Introduction

Cyclooxygenase (COX) is the rate-limiting enzyme that regulates the synthesis of prostaglandins and thromboxanes from free arachidonic acid (Williams et al., 1999). Two isoforms of the COX enzyme exist, referred to as COX-1 and COX-2. COX-2 is overexpressed in breast, lung, stomach, pancreatic and (Harris, 2009; Thiel et al., 2011); its overexpression is a characteristic feature of many malignant neoplasms and promotes cancer progression and metastasis (Harris, 2007; Cheng and Fan, 2013). COX-2 is also overexpressed in breast cancer tissues and often associated with poorer prognosis (Kulkarni et al., 2008; Hoellen et al., 2011); COX-2 selective inhibitors significantly delay the incidence of mammary tumors (Falandry et al., 2009; Gonzalez-Villasana et al., 2013; Jendrossek, 2013).

Epithelial-mesenchymal transition (EMT) refers to the transdifferentiation of epithelial cells to a more mesenchymal state characterized by the loss of cell-to-cell junctions and the acquisition of spindle-shaped morphology (Huber et al., 2005). As carcinomas progress, these tumors may lose epithelial morphology and acquire mesenchymal characteristics which contribute to metastatic potential. A series of phenotypic and molecular changes occur in cancer cells during EMT, and leads to adverse prognosis (Thompson et al., 2005; Kalluri and Weinberg, 2009), EMT is often associated with a poor prognosis in women with breast cancer (Moody et al., 2005; Ansieau, 2013; Roxanis, 2013).

Due to both overexpression of COX-2 and EMT are correlated directly with highly aggressive and metastatic breast cancer, so there may have close relationship between them (Ogunwobi et al., 2012). In order to clarify whether COX-2 could modulate EMT and its underlying mechanisms of action in breast cancer, in the present study, MDA-MB-231 cells with high expression level of COX-2, which is also base-like phenotype and EMT breast cancer cell line, were selected (Lester et al., 2007; Li and Mattingly, 2008). Celecoxib and small interfering RNA were used to inhibit the function and the expression of COX-2 respectively; the EMT condition of the MDA-MB-231 cells was detected by expression change of E-cadherin, vimentin, Snail, ZEB2. Moreover, many signaling pathway are involved in the process of EMT. Akt is a well-known key pathway for many basic cellular processes, including cell cycle progression, cell proliferation, cell survival, metabolism and EMT. For this reason, we concentrate on the regulation of COX-2 on the activation and expression of AKT.

Materials and Methods

Cell line and cell culture

The human breast cancer MDA-MB-231 cells were cultured in DMEM (Hyclone, Logan, UT, USA) with 10% FBS (Gbico, Grand Island, NY, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Sigma, St Louis, MO, USA). The cells were incubated at 37°C in humidified air with 5% CO_2. For the celecoxib treated group, cells

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cultured in medium containing 60 μmol/l celecoxib (Pfizer Inc, USA) with dimethyl sulfoxide (DMSO, the vehicle in which celecoxib was dissolved).

Transfection with expression vectors and small interfering RNA

Cells were transfected with a vector expressing ZEB2 (pCI-neo-RL-ZEB2, from addgene.org) using Lipofectamine 2000 or electroporation (Invitrogen, Carlsbad, CA). To inhibit the expression of COX-2 in MDA-MB-231 cells, cells were transfected with negative control small interfering RNA (siRNA) or siRNA specific to COX-2 by lipofectamine 2000 for 48 hours, the siRNA target sequences for COX-2 (GenBank accession number NM_000963) was selected according to Park et al. (2006), the targeting sequence is 5'-GTCCCTGAGCATCTACGGT-3', homologous to nt of 792-810.

Prostaglandin E_2 production

Cells were treated with 60 μmol/l celecoxib or RNAi targeting for COX-2 in the medium supplemented with 5% FCS for 72h. Level of PGE_2 released into the media was measured using a PGE_2 enzyme immunoassay kit from Cayman chemical Co. (Ann Arbor, MI, USA). Medium was sampled, centrifuged to remove floating cells and frozen immediately at -70 °C until assay. The PGE_2 assay was performed in accordance with the manufacture's instructions, following dilution to ensure that readings were within the limits of accurate detection by the assay. The results were expressed as pg/ml±standard deviation.

Immunofluorescence staining of E-Cadherin, vimentin, and immunohistochemistry for COX-2 and snail

MDA-MB-231 cells were seeded onto round gelatin coated glass cover slips placed in 24-well plates, fixed using 4% paraformaldehyde for 30 min at 4°C, permeabilized with 0.2% Triton for 3 min and blocked in 10% goat serum for 1h at room temperature, and then incubated with primary Ab against E-cadherin (diluted 1:500; mouse monoclonal, clone C20820; BD-Biosciences, San Jose, CA), vimentin (diluted 1:250; mouse monoclonal, Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min at 25°C. Cells were then incubated with fluorescein-conjugated secondary Ab (diluted 1:500; ICN Pharmaceuticals, Inc., Aurora, OH, USA) for 30 min at 25°C. Hoechst 33258 dye (1μg/ml; Sigma, St Louis, MO, USA) was subsequently used to stain nuclei; the samples were visualized by fluorescence microscopy.

For the detection of COX-2 and Snail, immunohistochemistry was used, after being incubated with primary Ab against COX-2 (diluted 1:250; Santa Cruz Biotechnology, Santa Cruz, CA) and Snail (diluted 1:250; Sigma, St Louis, MO, USA) for 60 min at 25°C. Negative control was incubated with 1% bovine serum albumin in PBS. Next, the slides were incubated with the biotinylated secondary antibody (diluted 1:500, ABC Vectastain Elite Kit, Burlingame, USA) for 1h at room temperature. After this, the slides incubated in preformed avidinbiotinylated peroxidase complex (ABC Vectastain Elite Kit, Burlingame, USA) for 1h. The color was developed with diaminobenzidine tetrahydrocloride (DAB) (Sigma, St. Louis, MO, USA).

Quantitative RT-PCR for snail and COX-2

Quantitative real time reverse transcription-PCR (qRT-PCR) analyses for Snail (NM_005985.2), and COX-2 (NM_000963) were performed by using the ABI Prism 7700 Sequence Detection System (P.E. Applied Biosystems, Foster City, CA), house keeping genes, GAPDH and ACTB, were detected to make measurement accurately. The oligonucleotide primers designed for Snail, COX-2, GAPDH and ACTB were as follows: Snail: 5'-ACCACTATGCCGCGCTCTT-3' (sense) and 5'-GGTCGTAGGGCTGCTGGAA-3' (antisense); COX-2: 5'-CAAGTCCCTGAGCATCTACG-3' (sense) and 5-'CATTCCTACCACCAGCAACC-3' (antisense); GAPDH: 5'-ACGATCCCGAGACTCTGCTTC-3' (sense) and 5'-GCACGGCTACTGGCACACT-3' (antisense); ACTB : 5'-CTGGAACGGTGAAGGTGACA-3' (sense) and 5'-AAGGGACTTCCTGTAACAATGCA-3' (antisense) (Sangon & Bio Basic Inc. China).

Total RNA was extracted and purified from MDA-MB-231 cells using Trizol Reagent (Invitrogen Corporation, Carlsbad, CA, USA). Reverse transcriptase reactions were manipulated using Superscript One step RT PCR kit (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions, the constituents of each PCR were 25 μl 2×Reaction Mix, 1μl RT/ Platinum Taq Mix, 2× (forward and reverse) 1 μl of primer 10 μM each, ddH_2O 20μl, 1μl SYBR Green (P.E. Applied Biosystems, Foster City, CA). The PCR conditions were an initial denaturation step of 30 min at 50°C and 2 min at 95°C, followed by 40 cycles consisting of 15s at 95°C for denaturation, 45s at 58°C for annealing, and 45s at 72°C for extension. Relative mRNA expression of treated groups to nontreated ones was calculated as a multiplicative factor by subtracting the number of cycles for treated group from that of control (Vandesompele et al., 2002).

Invasion assay

Cell invasion through a three-dimensional ECM was assessed using BD Matrigel invasion chambers (BD, Biocoat, Bedford, MA, USA) with 8.0-μm filter membranes. Where indicated, Transwell membranes were coated on both surfaces with 50 μg/ml human cellular FN (Sigma Aldrich) or MMP-9-degreaded FN by immersion overnight at 4°C, before aspiration and 3 washes in PBS. Cells (5×10^4) were resuspended and plated into the chamber inserts. After 24 h, cells invading the lower surface of the filters were fixed, stained, and counted. The percentage change in invasion was determined by counting the number of cells that migrated to the lower surface of the filters. At least three separate microscopic fields were counted per membrane.

Western blotting

1×10^4 cells were seeded in 25 cm^2 cell culture bottles, cellular protein extracts were prepared. Samples of 30 μg cellular proteins diluted with sample buffer were equally loaded on 12% SDS-polyacrylamide gel,

separated proteins were transferred to PVDF membrane. To avoid unspecific binding, filters were incubated in 5% nonfat dry milk, 0.05% Tween-20 in TBS for 2h, then the membranes was incubated with antibodies (diluted 1:2000 for COX-2 and 1:1000 for Snail, Calbiochem, San Diego, CA) for 2h, followed by horseradish peroxidase (HRP)-linked secondary antibodies (Fuzhou, Maxim Biotechnology, China), detection was performed using ECL chemoluminescence (ECL system, Amersham International, Little Chalfont, UK). As controls for equivalent protein loading, filters were simultaneously incubated with a mouse MAb directed against β-actin (diluted 1:1000; Chemicon, Temecula, CA). The membranes were scanned and the intensity of the bands was analyzed using Smart view density software. The ratio of object gene and β-actin was considered as the gene expression result.

Statistical analysis

For each condition, the data were presented as mean±standard deviation (SD) and analyzed using the SPSS13.0. Analysis of variance was conducted, followed by independent-samples t test. P value less than 0.05 was considered significant.

Results

Celecoxib and RNAi targeting for COX-2 inhibits expression of COX-2 and PGE_2

COX-2 converts arachidonic acid to bioactive prostanoids, and COX-2 derived PGE_2 is the major prostaglandin produced by breast cancer cells. In order to determine whether COX-2 activity was affected by celecoxib and RNAi treatment, the expression of COX-2 mRNA and protein were determined by RT-PCR and Western blotting; β-actin was selected as a loading control. The relative expression of COX-2 mRNA in 60 μmol/l celecoxib and RNAi treated group were 0.97, 0.22 respectively. Celecoxib cannot inhibit the expression of COX-2 mRNA, however, the expression COX-2 mRNA level in RNAi treated group decreased obviously compared with celecoxib group (p<0.01) (Figure 1A). Results of COX-2 protein expression detected by inmmunochemistry and Western blotting were coincidence with COX-2 mRNA level (Figure 1B, 1C).

PGE_2 production was also measured in conditioned medium collected from the breast cancer lines after celecoxib and RNAi treatment for 72h. PGE_2 level was measured using a commercially PGE_2 specific enzyme linked immunosorbent assay. Celecoxib and RNAi targeting for COX-2 could significantly reduced PGE_2 secretion, indicating that celecoxib and RNAi could inhibit COX-2 induced PGE_2; and the PGE_2 levels have no obvious difference between the celecoxib and RNAi treated groups (Figure 1D).

Table 1. Celecoxib and RNAi Targeting for COX-2 Inhibited Prostaglandin E_2 Production of MDA-MB-231 Cells

Treatment	prostaglandin E_2	P value
control	382.6±54.6	
DMSO	357.2±67.3	
celecoxib	46.8±6.49	*p<0.01
pAd-Shuttle	349.8±7.5.6	
PAd-RNAi	54.2±8.45	*p<0.01

Compared to control group, culture medium prostaglandin E_2 of the celecoxib and RNAi treated group decreased obviously (compared with control group); however, there has no difference between elecoxib and RNAi treated group

Inhibition of COX-2 up-regulates E-cadherin and down-regulates vimentin

The EMT conditions of the MDA-MB-231 cells were detected by the expression change of E-cadherin, vimentin after celecoxib and RNAi treatment. Results of immunohistochemistry (Figure 2A) and western blotting analysis (Figure 2B) show that MDA-MB-231 cells treated with 60 μmol/l celecoxib for 72 h expressed more E-cadherin and less vimentin than RNAi treated group; so MDA-MB-231 cells treated with small interfering RNA for 72h acquired more epithelial characteristics and less mesenchymal structures than treated by celecoxib. We further determined whether silence of COX-2 would restraint tumor cell invasion. Transient transfection of a siRNA COX-2 plasmid into MCF-7 cells significantly induced invasion (Figure 2C, 2D).

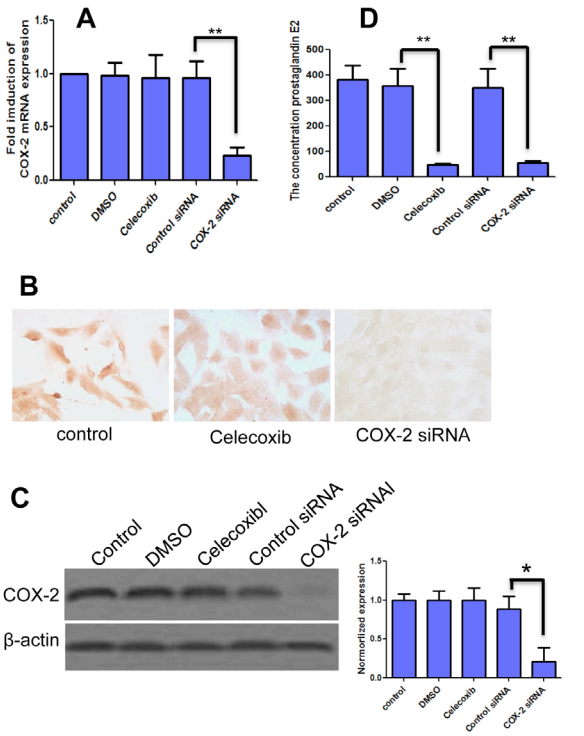

Figure 1. (A) The expression COX-2 mRNA in RNAi treated group decreased obviously compared with celecoxib group; (B) COX-2 expression in the MDA-MB-231 cells following celecoxib and RNAi treatment by immunochemistry; (C) COX-2 expression in the MDA-MB-231 cells following celecoxib and RNAi treatment by western blotting; (D) Celecoxib and RNAi targeting for COX-2 inhibited PEG2 production of MDA-MB-231 cells, compared to control group, culture medium PGE_2 of the celecoxib and RNAi treated group decreased obviously however, there has no difference between celecoxib and RNAi treated group. *p<0.05 *vs* control group; **p<0.01

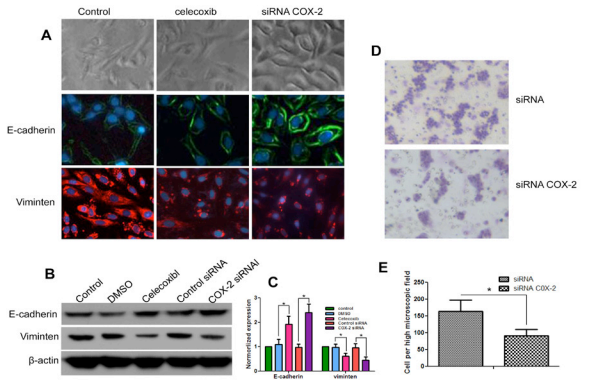

Figure 2. (A) Morphological change, expression E-cadherin and vimentin of MDA-MB-231 cells in RNAi treated group, MDA-MB-231 cells appearance to more epithelial state than that of celecoxib group. The expression of E-cadherin is enhanced gradually in celecoxib treated and RNAi group; and on the contrary, the expression of vimentin is decreased gradually; (B, C) Upregulation of E-cadherin and downregulation of vimentin in MDA-MB-231 cells treated with RNAi COX-2 by western blotting; (D, E) Invasion profiles of COX-2 silence cells were significantly lower than that of control cells. *p<0.05; **p<0.01

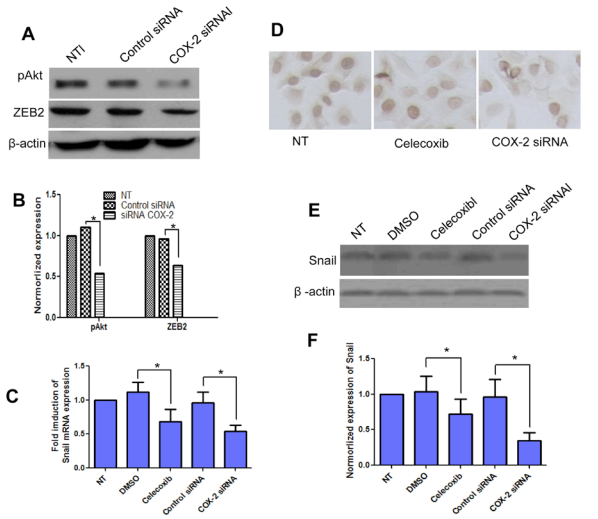

Figure 3. (A, B) Expression of pAkt 2 Ser473 and ZEB2 in COX-2 silence cells was lower than that in parent cells transfected with control siRNA plasmids indicated by western blotting; (C) The relative expression of Snail mRNA in celecoxib and RNAi treated group; (D) Immunohistochemistry showed that Snail was mainly located at the nuclear of MDA-MB-231 cells, with low expression in celecoxib and RNAi treated group. (E, F) Snail protein expression was decreased in siRNA treated group is more obvious than that of celecoxib treated group as shown by western blotting. *p<0.05

Inhibition of COX-2 leads to inactivation of Akt2

AKT was initially described as an oncogene. AKT is frequently upregulated and activated in ovarian, breast and pancreatic tumors (Bellacosa et al., 2004). Akt is involved in many basic cellular processes, including cell cycle progression, cell proliferation, cell survival, metabolism and EMT. The EMT induced by activated Akt involves: loss of cell-cell adhesion, morphological changes, and loss of apico-basolateral cell polarization, induction of cell motility, decrease in cell-matrix adhesion, and changes in the production or distribution of specific proteins (Arboleda et al., 2003). Concurrently, Akt activation increased indicated by an increase in the phosphorylation of Akt at Ser473. To assess the mechanism of COX-2 during EMT of breast cancer, in this experiment, Akt2 was valued after silence of COX-2. We found that expression of pAkt 2 Ser473 but not pAkt 1 Ser473 in COX-2 silence cells was higher than that in parent cells transfected with control siRNA plasmids. (Figure 3A, 3B).

Down-regulation of COX-2 reduce the expression of ZEB2

ZEB2/SIP1 is a member of the delta EF-1 family of two-handed zinc-finger factors and is expressed in gastric, ovarian and squamous cell carcinomas. ZEB2 specifically binds to CACCT (G) sequences in the E-cadherin promoter during EMT. Besides E-cadherin, ZEB2 is also implicated in the transcriptional upregulation of vimentin that occurs during EMT.

To confirm that COX-2 regulates ZEB2 expression, we examined the effect of suppressing COX-2 on ZEB2 expression. Transient transfection of COX-2 siRNA in MDS-MB-231 cells, which normally display endogenous COX-2 expression, significantly increased E-cadherin protein and decreased vimentin protein (Figure 2A, B), which was accompanied by a moderate reduction in ZEB2 (Figure 3A, 3B).

RNAi targeting for COX-2 deter snail expression

The most common biochemical change associated with EMT is the loss of E-cadherin expression. E-cadherin transcriptional repressors, such as Snail, have traditionally been implicated in promoting EMT in tumor progression, so the expression of Snail mRNA and protein was detected by RT-PCR and Western blotting. The relative Snail mRNA expression of blank control group was regarded as 1, the relative expression of Snail mRNA in celecoxib and RNAi treated group were 0.68 and 0.54 respectively (Figure 3C). Immunohistochemistry showed that Snail was mainly located at the nuclear of MDA-MB-231 cells (Figure 3D); Snail protein expression was decreased obviously following celecoxib and siRNA treatment, and the suppression in siRNA treated group is more obvious than that of celecoxib treated group (Figure 3E, 3F).

Discussion

Coherent scientific evidence from molecular, animal, and human investigations supports the hypothesis that aberrant induction of COX-2 and upregulation of the prostaglandin cascade play a significant role in carcinogenesis. Many studies have shown that overexpression of COX-2 in cancer is associated with resistance to programmed cell death as well as stimulation of cell migration, cell proliferation, and angiogenesis in breast cancer (Xin et al., 2012; Gonzalez-Villasana et al., 2013).

Epithelial mesenchymal transition (EMT) has been implicated in the progression toward an advanced cancer phenotype (Kudo-Saito et al., 2009). The occurrence of

EMT during tumor progression allows tumor cells to acquire the capacity to infiltrate surrounding tissue and metastasize to distant sites ultimately (Ksiazkiewicz et al., 2012; Zhu et al., 2013). Several transcription factors, including theSnail/Slug family, Twist, ZEB1, SIP1/ZEB2 and Twist respond to microenvironmental stimuli and function as molecular switches for the EMT program (Wang et al., 2013). EMT is often associated with recurrence and poor prognosis in women with breast cancer (Moody et al., 2005; Ansieau, 2013; Roxanis, 2013). Inhibition of the EMT might be a useful therapeutic tool in controlling cancer dissemination and metastasis in cancer patients.

Recently, data have showed that selective COX-2 inhibitors, such as celecoxib have antiancer effects (Sooriakumaran et al., 2009; Patel et al., 2013), treatment with celecoxib maximally affected growth of MDA-MB-231 cells (Bocca et al., 2011); RNAi targeting for COX-2 was also used to block the growth of breast cancer cells. In this study, we tested the ability of celecoxib on EMT modulation; results show that celecoxib could change the cell appearance to epithelial state slightly. Beside the morphological change, results of immunofluorescence and Western blotting showed this morphological transition was accompanied by upregulation of E-cadherin with increase membrane localization in the MDA-MB-231 cells. Moreover, the expression of vimentin was down-regulated in celecoxib treated MDA-MB-231 cells, whereas its expression was abundant in the MDA-MB-231 cells.

A variety of signal transduction pathways impinge on the regulation of E-cadherin levels or subcellular distribution during EMT (Wang et al., 2013). Very recently, the oncogenic serine/threonine kinase AKT, a downstream effector of the phosphatidylinositol 3' kinase (PI3K), has been shown to repress transcription of the E-cadherin gene. PI3K/Akt is a well-known key pathway for cell invasion (Hennessy et al., 2005); this transcriptional repression induces cellular responses leading to the conversion of epithelial cells into invasive mesenchymal cells. For this reason, we concentrate on the regulation of COX-2 on the activation and expression of AKT. In this experiment, we found that down-regulation of pAkt Ser473 in siRNA/COX-2 treated cell was higher than that in parent cells transfected with siRNA plasmids. Therefore COX-2 could downregulate Akt2 activation by a decrease in the phosphorylation of Akt2 at Ser473.

Several transcription factors have been characterized as transcriptional repressors of E-cadherin and are implicated in promoting EMT in tumor progression: the Snail superfamily of zinc-finger factors (Snail and Slug), the ZEB family (ZEB1 and ZEB2) and basic helix-loop-helix factors (Twist, E47 and TCF4) (Li et al., 2011; Nam et al., 2012). The mechanisms through which these repressors downregulate E-cadherin and other epithelial genes during EMT are fairly well established. To investigate whether ZEB2 and Snail are regulated by COX-2, we used siRNA to suppress COX-2 expression in MDA-MB-231cells. Real-time qPCR showed that knockdown of COX-2 significantly reduced the expression of ZEB2 and Snail. Immunoblotting also showed that COX-2 is required for the modulation of ZEB2 and Snail in MDA-MB-231. When the MDA-MB-231 cells cultured with the celecoxib, the expression of Snail mRNA and protein level in MDA-MB-231 cells decreased slightly compear with RNAi treated group. The mechanism that how could COX-2 regulates S ZEB2 and nail expression needs further study.

In previously study, it was found that PGE_2 modulated transcriptional repressors of E-cadherin and thereby regulates COX-2-dependent E-cadherin expression in NSCLC in autocrine or paracrine fashion (Dohadwala et al., 2006). However, many COX-2 functions do not dependent on PGE_2 production, in order make clear whether COX-2 regulated EMT is dependent of PGE_2 cascade completely or not, RNA interference was used to silence the COX-2 gene in this experiment. After being treated with RNAi, MDA-MB-231 cells appearance to more epithelial, from fibroblastoid spindle-shaped to compact, and cobblestone-like epithelial structures cells. Results of immunohistochemistry, real-time PCR and western blotting indicate that the expression of COX-2 protein and mRNA were decreased obviously, upregulation of E-cadherin and downregulation of vimentin were more obviously than that of celecoxib treated group. Celecoxib can inhibit the function of COX-2, reduce the generation of PGE_2, while RNAi targeting for COX-2 reduce the generation of PGE_2 through decreasing the expression of COX-2. Although the PGE_2 level of culture medium has no obvious difference between the celecoxib and RNAi treated group, the modulation of them on EMT is different obviously. Those results suggest that the COX-2 inhibition mediated reverse of EMT was only partly dependent on PGE_2 cascade.

In conclusion, the role mechanisms of COX-2 in the development and progression of breast cancer are probably through promoting the proliferation and invasion of breast cancer cells, howbeit, the signal pathway that COX-2 involved in the process need to be clarified. our study shows that COX-2 dependent pathways contribute considerably to EMT development of breast cancer cells only partially dependent on PGE_2; downregulation of COX-2 resulted in Snail decreasing could be partially responsible for reversal of EMT too. The finding may be helpful to elucidate the mechanism of selective COX-2 inhibitors in the prevention and reverse of EMT of breast cancer.

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