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# Celecoxib potentially inhibits metastasis of lung cancer promoted by surgery in mice, via suppression of the PGE2-modulated β-catenin pathway

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## HIGHLIGHTS

- We studied anti-metastasis effects in models with endogenous and exogenous PGE2.
- We firstly investigated unilateral pneumonectomy-induced A549 cells metastasis.
- Celecoxib inhibited PGE2-induced metastasis by suppressing GSK3β-β-catenin pathway.
- We provide a new rationale for the antimetastatic activity of celecoxib.

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## ABSTRACT

Surgery is the major treatment method for non-small cell lung cancer. It has been reported that plasma PGE2 level is increased following surgery and stress which promotes lung cancer metastasis. In the present study, two animal models were used to confirm the effects of exogenous and endogenous prostaglandin E2 (PGE2) on metastasis of lung cancer cells. We found that both PGE2 level and A549 metastasis were enhanced in mice with unilateral pulmonary resection following tail vein injection of lung cancer A549 cells. Both endogenous PGE2 level and pulmonary metastatic nodules were significantly reduced by celecoxib. A549 metastases were increased in mice after exogenous PGE2 injection. In the animal models, celecoxib inhibited lung cancer cell metastasis induced by exogenous PGE2. Therefore, we focused on the effects of celecoxib on the downstream pathway of PGE2 in vitro and found that celecoxib inhibited PGE2-induced A549 migration and invasion, which were evaluated by wound healing and Transwell experiments. The expression of protein and mRNA of MMP9 and E-cadherin following treatment with PGE2 were suppressed and increased by celecoxib, respectively; however, MMP2 showed no change. A549 cell invasion and up-regulation of the expression of MMP9 and down-regulation of E-cadherin induced by PGE2 were inhibited by FH535, an inhibitor of β-catenin. Deletion of β-catenin by siRNA abrogated celecoxib-induced inhibition of MMP9 up-regulation and E-cadherin down-regulation by treatment of PGE2. Furthermore, we found that the level of β-catenin together with GSK-3β phosphorylation was inhibited by celecoxib. In conclusion, celecoxib inhibits metastasis of A549 cells in the circulation enhanced by PGE2 after surgery by not only inhibiting endogenous PGE2 expression, but also by suppression downstream of PGE2 via the GSK-3β-β-catenin pathway.



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## 1. Introduction

Surgery remains an essential therapeutic approach for most solid malignancies, including non-small cell lung cancer (NSCLC). However, surgery is also a risk factor for the promotion of pre-existing micro-metastases and the initiation of new metastases through several mechanisms, including the release of prostaglandins and stress hormones (Friedrich et al., 1999; Howe, 2007; Muzii et al., 1996; Singh-Ranger et al., 2008). Prostaglandins are paracrine and endocrine lipids that mediate pain and inflammation. Prostaglandin E2 (PGE2) is abundantly secreted by endothelial cells in damaged tissue following mechanical trauma. PGE2 is recognized by four different subtypes of G protein-coupled receptors (EP1-4), and activation of these receptors causes an elevation in PKA and c-AMP that mediates immunocyte suppression (Su et al., 2008; Sugimoto and Narumiya, 2007). It has been reported that PGE2 activates PI3K and the protein kinase, Akt, by free G protein βγ subunits and the direct association of a G protein subunit with Axin1 through its heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptor EP2. This results in the inactivation and release of GSK3β from the destruction complex and the consequent intracellular accumulation of β-catenin (Castellone et al., 2005). β-catenin accumulation eventually results in its nuclear translocation. In the nucleus, β-catenin binds to members of the TCF/LEF family of transcription factors, thus modulating expression of a broad range of target genes which are associated with cell survival, proliferation and metastasis (Fodde and Brabletz, 2007).

Elevated levels of tumor cyclooxygenase-2 (COX-2) and its metabolite PGE2 contribute to a decrease in E-cadherin, augmentation of cancer motility and invasiveness, resistance to apoptosis, and suppression of antitumor immunity (Castelao et al., 2003; Chell et al., 2006; Wang and Dubois, 2010). COX-2 has been identified as one of four key 'metastasis progression' genes, which collectively synergize to mediate both tumor development and metastasis to other organs (Gupta et al., 2007). Therefore, COX-2 inhibitors have been reported to inhibit metastasis via suppression of PGE2 production. However, the influence of COX-2 inhibitors on the PGE2-induced downstream signal pathway in NSCLC cells remains unknown.

Celecoxib is a NSAID that specifically inhibits COX-2. It has significant anti-inflammatory and analgesic properties but lesser toxicity than other NSAIDs such as aspirin and ibuprofen, which inhibit both COX-1 and COX-2 (Harris et al., 2000). In this study, we investigated the effect of celecoxib on plasma PGE2 level and metastasis of A549 cells intravenously inoculated in mice receiving unilateral pneumonectomy.

## 2. Materials and methods

### 2.1. Cell culture and reagents

The human lung cancer cell line A549 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A549 cells were cultured in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen). Cultures were maintained in an atmosphere containing 5% $CO_2$ (Forma Scientific). Prior to the experiments, cell viability greater than 95% was confirmed by trypan blue staining. Antibodies were obtained as follows: anti-MMP2, anti-MMP9, anti-E-cadherin, anti-β-catenin, anti-GSK-3β and anti-β-actin were purchased from Santa Cruz Biotechnology (CA, USA); IRDyeTM 800-conjugated secondary antibodies were obtained from Rockland, Inc. (Philadelphia, USA). Chemicals were obtained as follows: DMSO, FH535, Celecoxib for cell experiments were purchased from Sigma Chemical (St. Louis, MO, USA); and Celecoxib for animal experiments from Pfizer Pharmaceuticals Ltd. (CA, USA); and the PGE2 ELISA kit from R&D System (MN, USA). BALB/c nude mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China).

### 2.2. In vivo animal model and lung metastasis (Backhus et al., 2006)

Female BALB/c nude mice weighing 18–22 g were randomly assigned to the following five treatment groups (6 mice per group): control; mice were injected with PGE2 (1 mg/kg); mice were injected with PGE2 (1 mg/kg) and celecoxib (100 mg/kg); mice received pneumonectomy; mice received pneumonectomy and celecoxib (100 mg/kg). A549 cells were prepared as a suspension of $10^6$ cells in 150 μL saline and injected using a 29-gauge needle into the lateral tail vein under sterile conditions. Celecoxib administration (100 mg/kg) began one day before tumor cell injection until the mice were sacrificed.

After the mice were sacrificed, the lungs were rinsed with 5% picric acid solution. Lung metastasis was determined by counting the total number of metastatic nodules in each lung. The animal study was carried out according to the State Food and Drug Administration of China regulations on animal care.

### 2.3. Pneumonectomy (Gibney et al., 2011)

Surgery was carried out on the same day as A549 cell injection. After general anesthesia and intubation, the animal was maintained on a Flexivent rodent ventilator (SCIREQ, Montreal, QC, Canada) at 200 bpm, 10 mL/kg, and PEEP of 2 cm $H_2O$ with a pressure limited constant flow profile. Pneumonectomy was performed through the fifth intercostal space via a left thoracotomy. With minimal manipulation of the lung, the hilum was ligated with a 5–0 surgical silk tie (Ethicon, Somerville, NJ, USA) and excised. A recruitment maneuver involving a 3 s ramp to 30 cm $H_2O$ and a 3 s plateau was performed while closing the thoracotomy with a 3-0 silk stitch (Ethicon). When spontaneous muscle activity returned, the animal was extubated and transferred to a warming cage.

### 2.4. Wound healing assay (Kim et al., 2010)

A549 cells were seeded in six-well plates and incubated overnight in starvation medium. Cell monolayers were wounded using a sterile 200-μL pipette tip, washed with starvation medium to remove detached cells, and incubated with the indicated agents for 24 h. Phase contrast pictures were taken with an inverted microscope using a magnification of 200.

### 2.5. Cell proliferation assay (Yin et al., 2011)

Cells were plated on 96-well plates at 4000 cells in 100 μL per well and detected in quadruplicate at 24 h. 10 μL Cell Counting Kit-8 (Dojindo) was added to each well and viable cells were measured by absorbance at 450 nm after incubation at 37 °C for 3 h. Absorbance values were normalized to the medium control.

### 2.6. Transwell assay

Pre-chilled serum-free RPMI-1640 was mixed with matrigel (1:5; BD Biosciences) for the tumor invasion assay. Prior to this assay, cells were pretreated with PGE2 or celecoxib for 24 h. The upper hanging millicell chambers (Millipore, Billerica, MA, USA) were filled with 100 μL of the mixture inserted into the 24-well plates, and the matrigel was allowed to solidify at 37 °C for 4 h. After solidification, 50,000 cells were trypsinized, washed, resuspended in RPMI-1640 containing 0.01% FBS (Gibco), and placed evenly in the upper chambers. The lower chambers which contained 5% FBS