Selecting Cox-2 inhibitor celecoxib induces epithelial-mesenchymal transition in human lung cancer cells via activating MEK-ERK signaling

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Introduction
Lung cancer is the leading cause of cancer-related mortality in both men and women worldwide, where non–small cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases. NSCLC is a highly metastatic cancer with limited treatment options. Most cases presented locally advanced (37%) or metastatic (38%) disease when diagnosed (1). There has been modest improvement in the survival rate of NSCLC patients over the past two decades, with 5-year relative survival of NSCLC patients, which urges the investigation to re-evaluate this strategy for NSCLC treatment. In this study, we observed that celecoxib treatment at clinically relevant concentrations induced epithelial-mesenchymal transition (EMT) in NSCLC cells regardless of Cox-2 status, which, however, was not recapitulated using another Cox-2 inhibitor, etodolac. Celecoxib-stimulated EMT in turn promoted cell invasion and rendered cells resistant to chemotherapy. Further mechanistic investigation by disrupting the integrity of signaling pathways using specific inhibitors or RNA interference revealed that celecoxib-induced EMT in NSCLC cells is indispensable of transforming growth factor-β1/Smad signaling. Instead, the activated MEK-ERK/SNAIL1 signaling largely accounted for celecoxib-induced EMT. Taken together, our study reveals the diverse impacts of Cox-2 inhibitors on EMT in NSCLC cells independent of Cox-2 inhibition, where celecoxib treatment leads to metastasis and chemoresistance via EMT induction. These findings reveal the increased risks of cancer metastasis and chemoresistance by applying Cox-2 inhibitors, celecoxib in particular, in clinical trials of NSCLC treatment and urge intensive preclinical assessment before proceeding to clinical application.

Materials and methods
Cell line and reagents
Human lung adenocarcinoma A549 cells, HCC827 cells, human breast adenocarcinoma MCF-7 cells and Madin-Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). A549 cells and MCF-7 cells were maintained in high glucose–Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY) at 37°C in a humidified 5% CO2 atmosphere. MDCK cells were maintained in Eagle’s minimal essential medium with 10% FBS. HCC827 cells were cultured in RPMI-1640 supplemented with 10% FBS. Cells were trypsinized and seeded in 12-well tissue culture plates. Twenty-four hours later, plating cells were treated with TGF-β1, celecoxib (Sigma, St. Louis, MO) or etodolac (Sigma) for different period in serum-free DMEM medium. Celecoxib and etodolac were dissolved in dimethyl sulfoxide at 10 mM as stock solution. The final concentration of dimethyl sulfoxide did not exceed 0.1% (v/v). Control cells in all the experiments throughout this study were treated with dimethyl sulfoxide as vehicle control. MEK/ERK and phosphatidylinositol-3 phosphate kinase (PI3K) and AKT inhibitors (AZD6244, Selleckchem; wortmannin, Sigma; MK-2206, Selleckchem) were used to study the concerned signal pathways. The rabbit antihuman antibodies, such as E-cadherin, phospho-Smad2, phospho-Smad3 (Ser423/425), Smad3, phosphor-AKT (S473), AKT, phosphor-ERK (T202/Y204), ERK, phosphor-MEK (S217/221), MEK and

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638
Celecoxib induces EMT in lung cancer

Celecoxib induces EMT regardless of TGF-β1 stimulation

We first examined the effect of celecoxib on EMT in A549 cells. TGF-β1, which plays a major role in EMT induction, was utilized to stimulate EMT occurrence at the concentration of 1 ng/ml (18). The alterations of E-cadherin and vimentin expression, the hallmarks of EMT, were detected following treatment with various concentrations of celecoxib in the presence of TGF-β1. Celecoxib treatment effectively decreased Cox-2 expression (Figure 1E and F) and meanwhile aggravated TGF-β1 stimulation, as determined by both western blot analysis (Figure 1E–H) and immunostaining (Supplementary Figure 1A, available at Carcinogenesis Online). These data suggest that the impact of celecoxib on EMT process is dispensable of TGF-β1 signaling. These findings were further confirmed in MDCK cells, which is the most widely used experimental model to study the molecular mechanisms of EMT (Supplementary Figure 1B, available at Carcinogenesis Online).

Celecoxib promotes cell invasion and renders cells resistant to chemotherapy

EMT has been known to render tumor cells increased invasive potentials and resistant to chemotherapy (11). We next assessed the invasive capabilities of cells after celecoxib treatment using matrigel invasion assay. Cells were treated with increasing concentrations of celecoxib for 18 h. A significant increase in the number of A549 cells invaded through basement membrane–like matrigel barriers was observed when exposed to 4 or 6 μM of celecoxib compared with the untreated cells (P ≤ 0.001; Figure 2A and B). At the same concentration, celecoxib treatment did not apparently affect the viability of the cells, as shown by unaffected cell number (P = 0.634, Figure 2C). These findings were also recapitulated in MDCK cells where celecoxib treatment promoted cell invasion in a dose-dependent manner (Figure 2D and E).

Enhanced tumor cell invasiveness are often associated with elevated matrix metalloproteinases (MMPs) activities (19). We therefore assessed the impact of celecoxib on the activity of MMPs. A549 cells were treated with various concentrations of celecoxib for 24 h and MMP activity in the supernatant was measured using gelatin zymography assay. The results showed an increased MMP-2 activity in A549 cells after treated with 4 or 6 μM celecoxib but the MMP-9 activity was not apparently affected (Supplementary Figure 2A and B, available at Carcinogenesis Online). These data suggested that MMP-2 may be involved in the enhanced cell invasion upon celecoxib treatment.

In addition to cell invasion, EMT is known to result in the resistance to chemotherapy. We then tested the sensitivity of A549 cells to chemotherapy in the presence of celecoxib. It was found that the cytotoxicity of cisplatin or paclitaxel in A549 cells was significantly reduced by the addition of celecoxib (Figure 2F and G), consistent with previous report (20). These findings collectively indicated that celecoxib treatment attenuated efficacy of chemotherapy via inducing EMT.

Celecoxib-triggered EMT process is dispensable of Cox-2 status

We further asked whether celecoxib stimulated EMT via modulating Cox-2 activity. To this end, MCF-7 cells, which barely express Cox-2 protein (Figure 3A (21)), were treated with celecoxib followed by the detection of EMT markers. As observed in A549 cells, celecoxib similarly decreased E-cadherin expression along with increasing expression of vimentin in a time- and dose-dependent manner (Figure 1A–D). Likewise, celecoxib was able to decrease the expression of E-cadherin and increase the expression of vimentin in absence of TGF-β1 stimulation, as determined by both western blot analysis (Figure 1E–H) and immunostaining (Supplementary Figure 1A, available at Carcinogenesis Online). These data suggest that the impact of celecoxib on EMT process is dispensable of TGF-β1 signaling. These findings were further confirmed in MDCK cells, which is the most widely used experimental model to study the molecular mechanisms of EMT (Supplementary Figure 1B, available at Carcinogenesis Online).
Indeed, etodolac inhibited EMT process, as evidenced by the upregulated E-cadherin and downregulated vimentin in A549 cells regardless of TGF-β1 stimulation (Figure 3H and I, and Supplementary Figure 3, available at Carcinogenesis Online), in agreement with previous reports in bladder and gastrointestinal cancer cells (22,23).
Celecoxib induces EMT in lung cancer

Together, it appeared that the cellular impacts of celecoxib in causing EMT induction in NSCLC cells were apart from Cox-2 activity and PGE2 level.

The activated MEK/ERK signaling accounts for celecoxib-stimulated EMT

We next proceeded to investigate the molecular basis behind celecoxib-stimulated EMT. A series of signaling pathways have been associated with EMT induction, including the MEK/ERK axis, TGF-β1/Smad signaling and PI3K/AKT pathway that have well-validated role in EMT modulation (11). The status of these pathways upon celecoxib treatment was examined in A549 cells by measuring the level of phosphorylated ERK, AKT, MEK, Smad2 and Smad3. Regardless of TGF-β1 treatment, phosphorylation of ERK, AKT and MEK were substantially elevated by celecoxib treatment in a dose- and time-dependent manner (Figure 4A–D). In contrast, the phosphorylation of Smad2/3 was barely affected, largely ruling out the implication of TGF-β1 signaling in celecoxib-stimulated EMT (Figure 4A and B). These observations were recapitulated in MCF-7 (Supplementary Figure 4A, available at Carcinogenesis Online) and HCC827 cells (Supplementary Figure 4B, available at Carcinogenesis Online). These results suggested that celecoxib-induced EMT is probably associated with MEK/ERK and PI3K/AKT signaling instead of TGF-β1/Smad pathways.

Fig. 2. Celecoxib promotes cell invasion and renders cells resistant to chemotherapy. Invasive assessment in both A549 cells and MDCK cells was detected using transwell assay. (A) Representative images of A549 cells that invaded through the filters (×100 magnification). (B) Quantification of invasive cells. The bars indicate mean ± SD. The significance of the difference was assessed using student’s t-test. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. (C) Effect of celecoxib on the proliferation of A549 cells. A549 cells were incubated with celecoxib in serum-free medium for 72 h and cell number was counted with trypan blue staining. (D) Representative images of MDCK cells that invaded through the filters (×100 magnification). (E) Quantification of invasive MDCK cells performed as described in (B). (F, G) IC50 of cisplatin and paclitaxel in A549 cells in the presence of celecoxib. The significance of the difference was assessed using student’s t-test. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
Upon upregulated upstream signaling, transcription factors including SNAIL1, SNAIL2/Slug, ZEB1, ZEB2/SIP1 and Twist proteins are known to bind to E-box elements in the promoter region of E-cadherin, leading to transcriptional repression and induction of the mesenchymal phenotype (11). Therefore, we also probed the alterations of E-box binding transcription factors and found the increased expression of SNAIL1 after celecoxib treatment in A549 cells (Figure 4E and F), suggesting that SNAIL1 may be involved in celecoxib-induced EMT process.

To further dissect the role of PI3K/AKT and MEK/ERK signaling pathways in celecoxib-induced EMT, a series of specific inhibitors and siRNAs were applied in the presence of celecoxib in A549 cells. The treatment of AKT inhibitor MK-2206 failed to restore celecoxib-stimulated downregulation of E-cadherin (Figure 5A), which was recapitulated by using AKT1 siRNA (Figure 5C). These results largely rule out the contribution of the activated AKT1 signaling, which was due to PI3K as verified by the PI3K inhibitor wortmannin in Figure 5B, to the enhanced EMT by celecoxib.

In contrast, MEK inhibitor AZD6244 was found to effectively block celecoxib-induced activation of ERK, SNAIL1 and rescue the alteration of EMT hallmarks (Figure 5D and E), suggesting that the MEK/ERK signaling mainly accounts for celecoxib-stimulated EMT. Consistently, disruption of MEK1 and MEK2 using specific siRNAs partially restored decreased E-cadherin and increased vimentin levels by celecoxib treatment. The combination of MEK1 and MEK2 siRNA further exaggerated the effect comparing to either single MEK1 or MEK2 siRNA (Figure 5F–H). Taken together, these results suggest a key role of MEK/ERK signaling in celecoxib-induced EMT. The intensified EMT by the disruption of PI3K/AKT signaling is probably due to the compensatorily activated MEK signaling (Figure 5A and B).

Blockage of MEK/ERK signaling impedes celecoxib-stimulated cell invasion

We finally were intrigued to determine whether blockage of MEK/ERK signaling was able to impede celecoxib treatment–stimulated cell invasion. Both A549 and MDCK cells were pretreated with MEK inhibitor AZD6244 for 2h prior to exposure to celecoxib. The invasive potentials were assessed using matrigel invasion assay. Consistent with previous findings, celecoxib treatment strikingly promoted the cell invasion in both A549 and MDCK cells. In contrast to that, pre-treatment with AZD6244 largely blocked celecoxib-enhanced invasive capability (Figure 6A–C). These data further validated the key role of MEK/ERK pathway in celecoxib-caused EMT and suggested the blockage of MEK/ERK signaling could be an approach to prevent celecoxib-promoted cancer malignancy.

Discussion

Metastasis is observed in nearly 40% of lung cancer patients at the time of diagnosis and accounts for most lung cancer mortality. EMT, a biological process that turns polarized epithelial cells into
Celecoxib induces EMT in lung cancer

The mesenchymal phenotype, via multiple biochemical changes, is critical in the development of invasiveness and metastatic potential of human cancers. The enhanced EMT process in cancer cells has been known to increase the risk of metastasis and is associated with poor prognosis of multiple types of cancer (11). In this study, we have discovered that Cox-2 inhibitor celecoxib at clinically relevant concentrations promotes NSCLC cells to undergo EMT and renders cell-enhanced invasive potentials and chemoresistance. We have noticed that a recent study has reported that another Cox inhibitor–induced cellular morphological change and migration via EMT in NSCLC cells (24).

**Fig. 4.** Celecoxib activates MEK/ERK, PI3K/AKT but not TGF-β1/Smads pathways in A549 cells. Cells were maintained in 12-well plates with serum-free medium for 24 h before treated with celecoxib and/or TGF-β1. The expression of p-Smad2, p-Smad3, p-MEK, p-ERK, p-AKT and SNAIL1 were detected by western blot analysis. (A) The effect of celecoxib on signal pathways after 1 h treatment followed by TGF-β1 induction for 15 min. (B) The effect of celecoxib on signal pathways after 1 h treatment. (C) Time-dependent activation of signal pathways by celecoxib treatment for different time periods. (D) Semiquantification of western blot bands shown in (B). Densitometric analysis of p-MEK, p-ERK and p-AKT bands were performed and normalized with that of β-actin. The bars indicate mean ± SD. The significance of the difference was assessed using student’s t-test. *P ≤ 0.05. (E) The effect of celecoxib on SNAIL1 expression in A549 cells. (F) Semiquantification of SNAIL1 expression shown in (E) performed as described in (D). ***P ≤ 0.001.
failed to achieve survival benefit by introducing celecoxib into the conventional regimen in the NSCLC treatment. These findings appear contradictory to the previously identified association between high Cox-2 expression and the poor prognosis of NSCLC patients (8). We further resolved the puzzle by showing that celecoxib-induced EMT is independent of its Cox-2 inhibitory activity. First, celecoxib induced EMT process in Cox-2 non-expressing MCF-7 cells. Second, another selective Cox-2 inhibitor etodolac did not resemble celecoxib in its effect on EMT. Celecoxib and etodolac both inhibited the PGE2 synthesis but the effect on EMT varies, further supporting that celecoxib-induced EMT in A549 cells is independent of PGE2 level and Cox-2 protein. Indeed, the variable effects of Cox-2 inhibitors on EMT have been reported in a broad range of literatures, suggesting a structure-relevant effect of the compounds (23,24). These evidences together imply the importance of comprehensive evaluation of Cox-2 inhibitors in preclinical studies prior to the clinical application.

Of note, in spite of the celecoxib-enhanced potentials in invasion and chemoresistance of NSCLC cells revealed in this study, the clinical trials thus far have not observed the poorer clinical outcomes, although not improved either by combining celecoxib with chemotherapies or targeted therapies in unselected stage IIIB/IV NSCLC, renal cell cancer and breast cancer patients (25–27). However, subset analysis revealed that NSCLC patients with high expression of intratumoral Cox-2 benefited from celecoxib treatment (28). Likewise, patients with low Cox-2 expression displayed shorter median time to progression (TTP) and overall survival compared with Cox-2 high expression cohort or entire study population in advanced renal cancer and breast cancer patients (26,27). We reasoned that benefit of celecoxib in patients with high Cox-2 expression group was due to the primary pharmacological effects of celecoxib via inhibiting Cox-2, whose overexpression is a validated prognostic biomarker for poor clinical outcome. This benefit, which may be partially compromised due to its EMT induction side effects, dominates the overall clinical outcome in Cox-2 overexpressing patients. Conclusively, in patients who barely express Cox-2 and therefore cannot benefit from Cox-2 inhibition, the side effects of celecoxib dominates the clinical outcome among this patient subset. Moreover, we also cannot rule out the impact of the fact that patients enrolled in these studies were mostly at late stage of advanced cancers, which may not be able to properly reflect the further intensified tumor progression caused by celecoxib treatment.

To explore the molecular basis behind celecoxib-induced EMT will provide insights into the mechanism of action of this drug and more importantly create chances to overcome its side effects. By profiling a panel of EMT-related pathways, we observed the simultaneously upregulated MEK/ERK, PI3K/AKT signaling in NSCLC cells upon celecoxib treatment, consistent with previous studies (29,30). It was also noticed that celecoxib at the concentrations of up to 50 µM exhibited opposite effect on ERK and AKT activity and led to associated impacts on cell proliferation (31,32). Given the fact that the maximum plasma concentration of celecoxib in human following a single oral
Celecoxib induces EMT in lung cancer

dose of 800 mg was 5.6 \mu M (33), the effect of celecoxib in a range of 2–8 \mu M, as applied in this study, is believed to be more physiologically relevant. We then followed this clue to elucidate the possible roles of these two pathways in celecoxib-induced EMT. Intervention of MEK/ERK signaling using both small-molecule inhibitors and specific siRNAs reversed celecoxib-stimulated EMT and upregulation of SNAIL1, suggesting celecoxib-induced EMT was mediated by the activated MEK/ERK/SNAIL1 pathway. In contrast, PI3K/AKT signaling inhibition led to an opposite effect, which, we speculated, was probably due to the compensatory activation of MEK/ERK signaling. Indeed, the compensatory regulation of these two pathways has been widely observed in a broad scenario (34,35). The increased AKT phosphorylation following MEK inhibition by AZD6244 has been observed in various human cancer cells, including A549 cells (36,37). Moreover, activated AKT by insulin-like growth factor I was found to suppress ERK activation via phosphorylation of inhibitory sites in Raf N-terminus (38), suggesting that MEK/ERK and PI3K/AKT pathways negatively regulate each other. Given the complicated cross-regulation of these two pathways, it is challenging to dissect the exact role of these two pathways in EMT regulation upon celecoxib treatment. A possible working model is that the relative potency of the two pathways stimulated by celecoxib would determine the eventual outcomes of the EMT phenotype. Considering the heterogeneity of cancer cells, the intervention of celecoxib may lead to opposite effects, which should be aware of when assessing the impact of celecoxib on EMT in a broader range of cancer cells.

In summary, our results suggest that Cox-2 inhibitor celecoxib treatment in NSCLC cells leads to the activation of MEK/ERK/SNAIL1 pathway and in turn stimulates EMT process. The diversity of Cox-2 inhibitors in chemical structures and the heterogeneity of cancer cells appear accounting for the variable effects of Cox-2 inhibitors on EMT process. Our results provided insights into the effects of Cox-2 inhibitors on EMT and its associated tumor cell invasion and chemoresistance, which trigger us to re-evaluate the current anticancer strategy of introducing Cox-2 inhibitors into the clinical regimen.

**Supplementary material**

Supplementary Figures 1–4 can be found at http://carcin.oxfordjournals.org/
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References

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