

## Original Article

# HGF/MET signaling promotes glioma growth via up-regulation of Cox-2 expression and PGE2 production

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**Abstract:** Cyclooxygenase2 (Cox-2) is well known for glioma growth through up-regulation of prostaglandin E2 (PGE2) levels. MET, a hepatocyte growth factor (HGF) receptor, is also frequently high expressed in glioma, which promotes glioma growth and invasion. Here, we demonstrate that HGF/MET signaling can promote PGE2 production in glioma cells via Cox-2 up-regulation. RNA inhibition of MET suggested that MET signaling is essential for Cox-2 up-regulation. Moreover, HGF could enhance Cox-2 expression and PGE2 release. Knockdown of Cox-2 inhibited growth-promoting effects of HGF, suggesting that HGF/MET functioned via Cox-2/PGE2 pathway. Therefore, our work reveals a critical relationship of Cox-2/PGE2 and HGF/MET signaling in promoting glioma cells proliferation. Further, targeting MET and Cox-2 may represent an attractive target therapy for glioma.

**Keywords:** GBMs, Cox-2, MET, PGE2

## Introduction

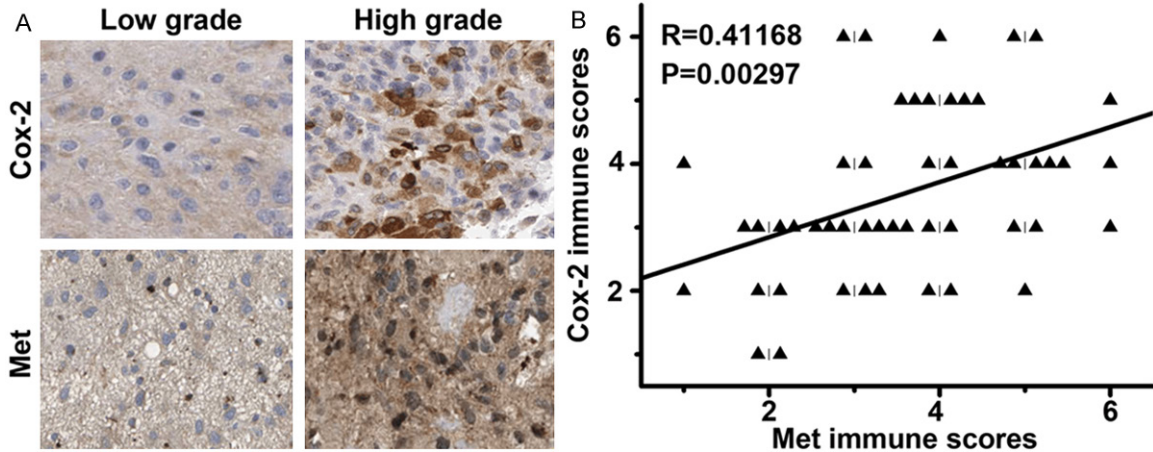
Glioblastoma is the most malignant human primary brain tumor with poor survival despite multimodality treatment [1-3]. Cox-2 is one of the famous cyclooxygenase enzymes, catalyzing the synthesis of prostanoids by converting arachidonic acid into prostaglandin E2 [4, 5]. Previous studies demonstrated that Cox-2 was negatively correlated with glioma prognosis [6-8]. Additionally, recent evidences indicates that Cox-2 is essential for glioma proliferation and invasion, which suggesting a critical role of Cox-2 in glioma initiation and development [9, 10].

Prostaglandin E2 (PGE2) is one of the most abundant Cox-2 metabolites in tumor tissues [4, 11]. So far, Cox-2/PGE2 pathway could influence most of the hallmarks of cancer, such as colon cancers [4, 12]. These signaling could directly activated PGE2-dependent downstream pathways, including Ras-mitogen-activa-

ted protein kinase (MAPK) and so on [13]. Such pathways are well known for mediators of carcinogenesis [14-17].

HGF signaling is one of critical tyrosine kinase in different cell types [18-20], and *in vivo* studies have demonstrated function of Cox-2 in MET-driven hepatocellular tumor progression and HGF-induced tumor angiogenesis [21]. Despite the importance of Cox-2 in glioma, the latent regulation mechanism of Cox-2 by HGF/MET in glioma cells was not clarified [22, 23]. Meanwhile, silencing MET expression in glioma could significantly impair tumor growth [24], leading targeting HGF/MET signaling therapeutically in most of cancers promising [19, 25]. Thus, the mechanisms by which HGF/MET signaling promote glioma survival deserves more attention.

Here, we reported that HGF/MET signaling promotes PGE2 release in glioma cells by up-regulating Cox-2 expression. What's more, inhibition



**Figure 1.** Expression of MET and Cox-2 in glioma samples. A. Expression of Cox-2 and MET in low grade and high grade glioma specimens were assessed by IHC assay. A representative figure in shown. B. Correlation between MET and Cox-2 expressions from human samples (n=50).

of Cox-2 attenuated HGF induced proliferation in glioma. To our knowledge, this is the first report highlighting a critical role for HGF/MET signaling in the regulation of PGE2 production in glioma cells.

#### Materials and methods

##### Tissue samples and clinical data

Fifty paraffin-embedded glioma specimens with clinical data were collected from the 2<sup>nd</sup> Affiliated Hospital of Harbin Medical University. This study was approved by the hospital institutional review board and written informed consent was obtained from all patients.

##### Cell culture and transfection

Human U87 and U251 glioblastoma cells were obtained from the China Academia Sinica Cell Repository (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Los Angeles, CA, USA) supplemented with 10% fetal bovine serum (Gibco), and incubated at 37°C in a 4% CO<sub>2</sub> atmosphere.

##### Oligonucleotides

Cox-2 siRNA, MET siRNA were chemically synthesized by GenePharma, Shanghai, China. Cells were transfected with Cox-2 siRNA, MET siRNA oligonucleotides (200 pmol each) using Lipofectamine 2000 (Invitrogen). Cells transfected with scrambled oligonucleotides (scrambled) were used as control.

##### Quantitative real-time polymerase chain reaction (qRT-PCR)

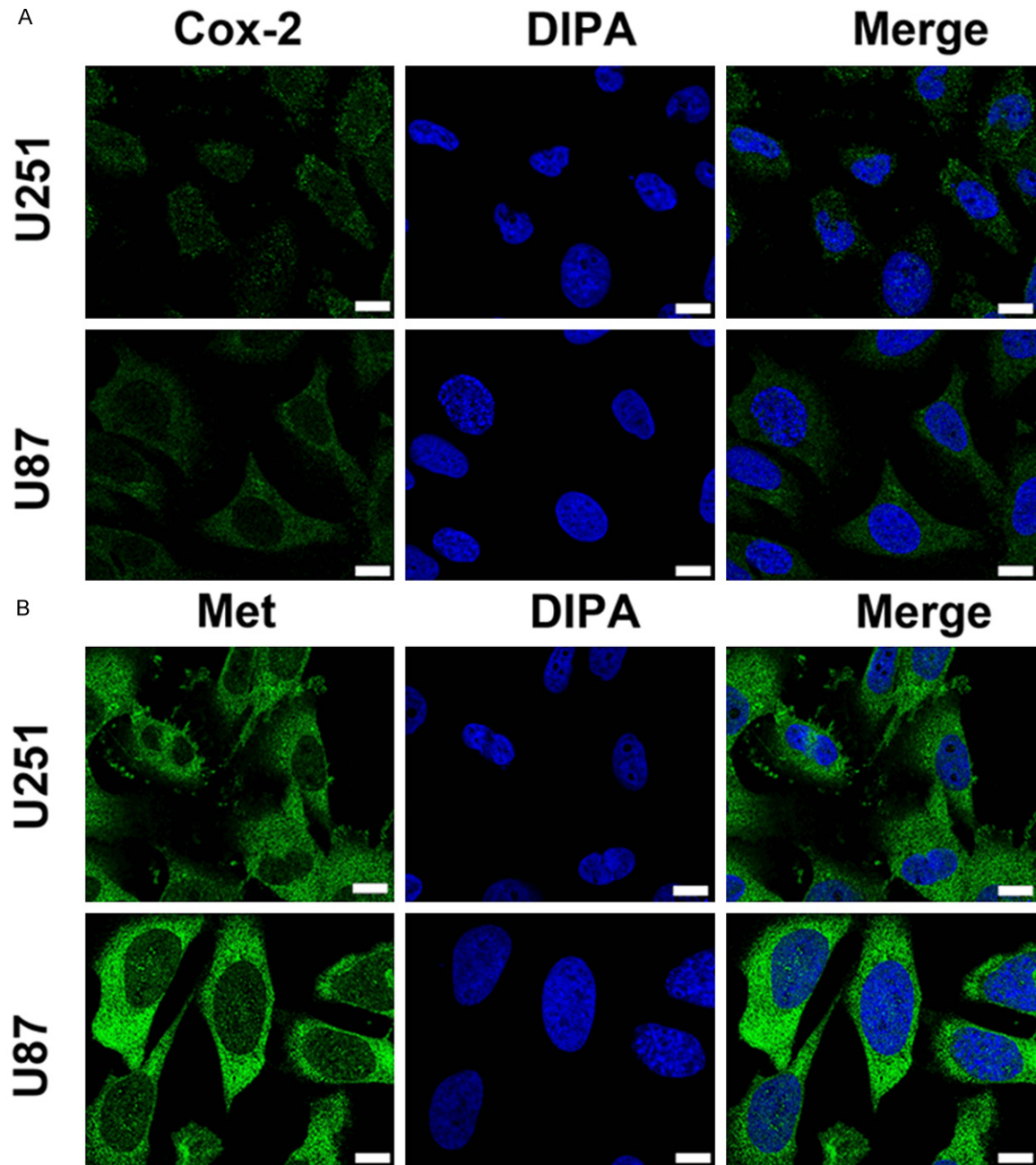
For qRT-PCR assays, total RNA was extracted using RNAiso Reagent (Takara, Japan). Reverse transcription was performed with PrimeScript RT reagent Kit (Takara) according to the manufacturer's instructions. The qRT-PCR conditions were performed as followed: DNA denaturation (94°C for 4 min), 40 cycles of amplification (94°C for 40 s, 42°C for 40 s, data collection (72°C for 40 s). qRT-PCR was performed on an ABI 7400 thermocycler (Applied Biosystems) using SYBR Premix Ex Taq™ (Perfect Real Time) Kits (TaKaRa) according to the manufacturer's instructions.

##### MTT proliferation assay

After transfected with scramble or siRNA, cell count and MTT assay were used as previously described [26]. The absorbance values of each well were measured with a microplate spectrophotometer (Molecular Devices; Sunnyvale, CA) at 450 nm. All proliferation experiments were performed in triplicate.

##### Western blot (WB) and immunohistochemistry (IHC)

WB and IHC assay were performed as previously described [8]. Briefly, WB and IHC assays were performed using antibodies against MET and Cox-2 (1:1000 dilution for WB and 1:100 dilution for IHC, Santa Cruz, USA), and GAPDH (1:1000 dilution for WB, Santa Cruz, USA). IHC



**Figure 2.** Expression of MET and Cox-2 in glioma cell lines. A. Expression of Cox-2 in U251 and U87 was assessed by IF assay. B. Expression of MET in U251 and U87 was assessed by IF assay.

scores were performed using a semiquantitative grading system as previous study [26].

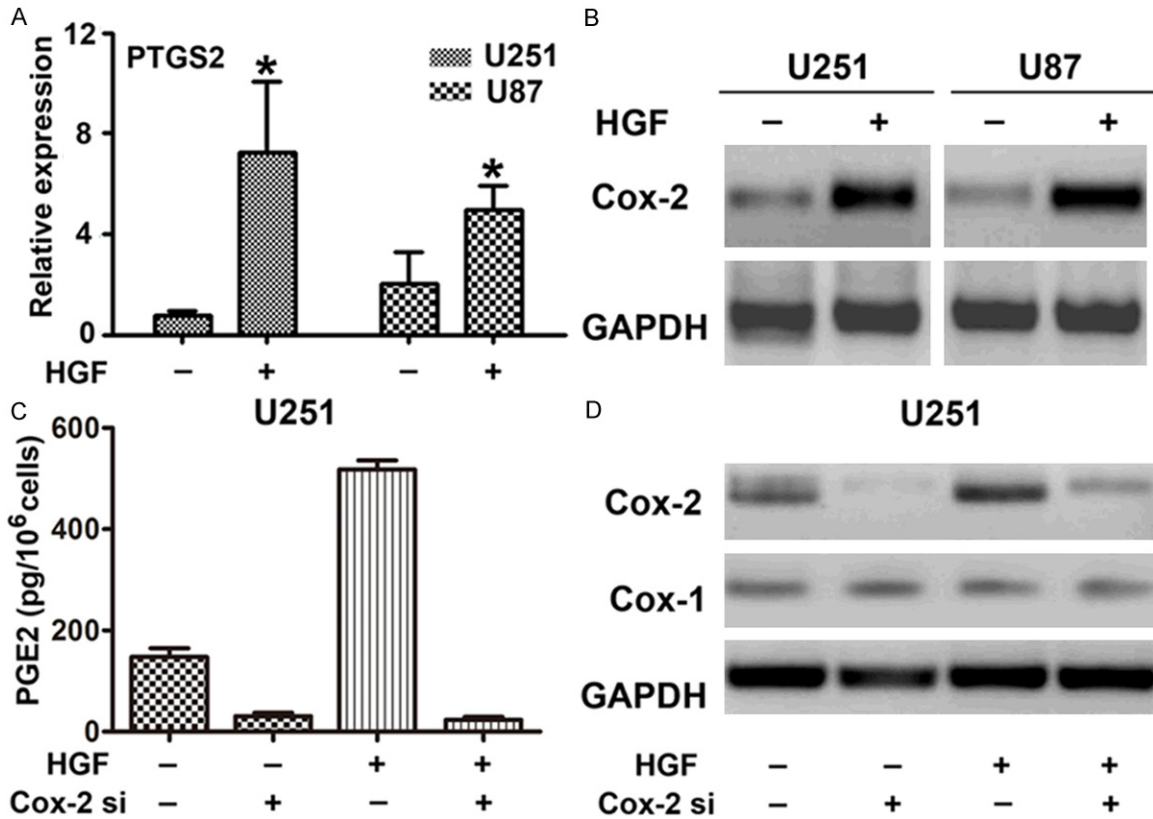
#### *Nude mouse tumor xenograft model*

Nude mouse tumor xenograft model was made as previous study [26]. 4-week-old female nude mice were subcutaneously injected with U87 glioma cells. The tumor volume was measured twice a week, using the formula: volume = length × width<sup>2</sup>/2. Then, mice were divided into

two groups randomly after tumor volume reached 40 mm<sup>3</sup>. Each group was treated with 10 μL Lipofectamine containing MET siRNA or scrambled once every 3 days for 14 days by local injection at multiple sites.

#### *Statistical analysis*

The significance between 2 groups was performed with Student t test and for multiple groups was estimated with ANOVA. *P* < 0.05



**Figure 3.** HGF up-regulates Cox-2 expression and promotes PGE2 accumulation in glioma cells. A. U87 and U251 were cultured in DMEM supplemented with 10% FBS and then stimulated using vehicle or HGF for 48 h at doses of 30 ng/ml. qRT-PCR was carried out for COX-2 mRNA. B. Cells were stimulated with vehicle or HGF for 48 h at doses of 30 ng/ml. Western Blot assay was carried out for COX-2. C. Cells were stimulated with 30 ng/ml HGF for 48 h with or without Cox-2 siRNA. PGE2 levels in the culture medium were subsequently analyzed using a commercial enzyme-linked immunosorbent assay and standardized to cell number. D. Cells were treated as in C and western blot analysis carried out to examine expression of COX-1 and COX-2 following HGF treatment.

was considered statistically significant. Statistics were performed using the SPSS 19.0 software (SPSS, Chicago, IL).

**Results**

*Expression of MET and Cox-2 in glioma samples and cell lines*

Since both MET and Cox-2 are involved in gliomagenesis [6, 8, 22], we firstly explored the expression of MET and Cox-2 protein in different grade glioma samples (n=50). In agreement with previous studies, MET and Cox-2 were indeed high expressed in glioma samples, and generally higher expressions were evident in high grade than low grade samples (Figure 1). Overall, Cox-2 and MET were positively correlated in all grade glioma e (Figure 1, R=0.41168, P=0.00297). Furthermore, both MET and Cox-2

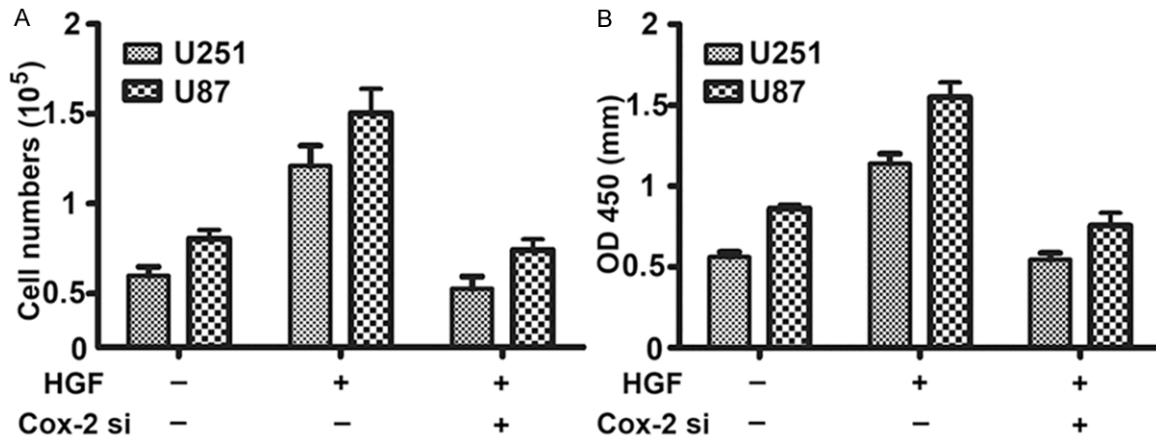
were also detected in U251 and U87 *in vitro* (Figure 2). Thus, both MET and Cox-2 were high expressed in glioma.

*HGF up-regulates Cox-2 expression and induces PGE2 release in glioma cells*

Owing to the proliferation and invasion promoting effects of Cox-2 and MET during gliomagenesis, we aimed to investigate regulatory mechanism of Cox-2 and hypothesized that Cox-2 could be induced by HGF in glioma cells. To testify this hypothesis, we detected Cox-2 expression of U251 and U87 cells after HGF stimulation. Cox-2 mRNA levels were significantly increased (Figure 3A) and Cox-2 protein expression were highly induced (Figure 3B) after HGF treatment for 48 h.

Plenty evidences points towards an essential role for the Cox-2 metabolite PGE2 in the pro-





**Figure 4.** Inhibition of Cox-2 attenuates HGF-stimulated glioma cell growth. A. U251 and U87 cells were pre-treatment with vehicle or Cox-2 siRNA for 4 h before treatment with 30 ng/ml HGF for 48 h. Cell number was assessed by cell counting. B. U251 and U87 cells were pre-treatment with vehicle or Cox-2 siRNA for 4 h before treatment with 30 ng/ml HGF for 48 h. Cell proliferation was assessed by MTT assay.

motion of gliomagenesis [6, 9]. We further investigated whether PGE2 levels increased after HGF treatment. Results demonstrated that HGF stimulation significantly increased PGE2 production and this increase could be inhibited after transfecting with Cox-2 siRNA (Figure 3C). However, no change of Cox-1 was observed after HGF stimulation (Figure 3D), which suggesting that Cox-2 expression is important for HGF-driven PGE2 accumulation.

#### *Inhibition of Cox-2 attenuates HGF-stimulated glioma cell growth*

As we demonstrated that HGF/MET signaling promotes Cox-2 up-regulation and PGE2 production, we next investigated the role of Cox-2 during HGF-promoted proliferation using cell counting and MTT assay. After 24 h, HGF induced U251 and U87 cells growth, and this effect was significantly inhibited by Cox-2 knock-down (Figure 4A, 4B), suggesting that HGF proliferation-promoting effects is dependent on the production of PGE2 induced by Cox-2 over-expression.

#### *MET inhibition reduces Cox-2 expression in glioma cells*

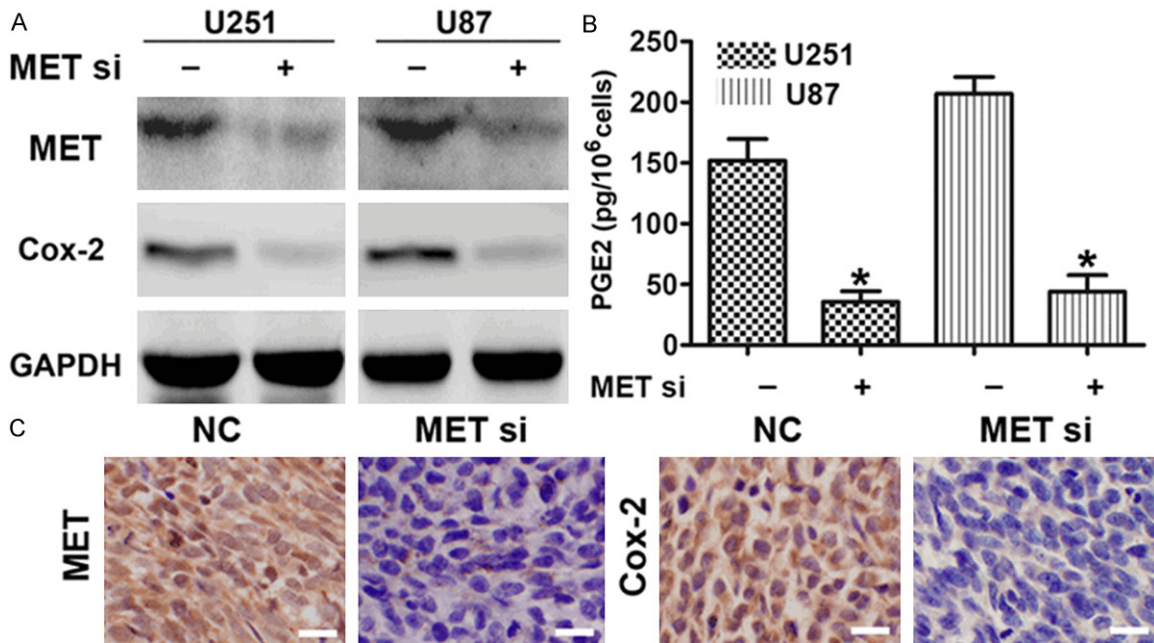
Given that U87 and U251 cells express high MET levels, we investigated whether reduction of MET inhibit Cox-2 expression and PGE2 accumulation. MET siRNA treatment lead to downregulation of Cox-2 and PGE2 at 48h *in vitro* (Figure 5A, 5B). Further, *in vivo* MET siRNA treatment also decreased expression of Cox-2 and PGE2 (Figure 5C), which reveals an impor-

tant relationship between HGF/MET signaling and PGE2 in glioma.

#### **Discussion**

Our previous studies demonstrated the important roles for Cox-2 and MET in glioma, their relationship was largely unknown. In this study, we first reveal that HGF/MET signaling is an important regulator for Cox-2/PGE2 pathway in glioma. Further, inhibition of Cox-2 restrained HGF-induced glioma cell growth, suggesting an important role for Cox-2 as a mediator of HGF/MET signaling pathway. We reported that the MET downregulation suppressed Cox-2 expression and PGE2 biogenesis and propose that targeting MET may represent an attractive target therapy for glioma.

Plenty of studies suggest that MET-targeted therapy may benefit patients with various types of cancer [25]. ARQ 197, a recently developed small molecule MET inhibitor, is currently in Phase I clinical trials, which is well tolerated, and inhibited intratumoral c-MET signaling (c-MET Inhibitor ARQ 197) [27]. We have a hypothesis that Cox-2/PGE2 mediate glioma growth promoting effects of HGF/MET and low-expression of MET reduces PGE2 production leading to anti-tumor effects. Evidence supports our hypothesis, because HGF stimulation induced Cox-2 expression in MET-positive thyroid papillary carcinoma cells and given that Cox-2 inhibition prevents HGF-driven invasiveness [28]. Besides, our study was consistent with previous reports in other various tumor



**Figure 5.** MET inhibition reduces Cox-2 expression in glioma cells. A. U251 and U87 were treated with the MET siRNA in standard growth medium for 48 h before lysis and western blotting for MET, Cox-2 and GAPDH. B. PGE2 levels in the culture medium were analyzed using a commercial enzyme-linked immunosorbent assay and standardized to cell number. C. Expression of Cox-2 and PGE2 *in vivo* analyzed by IHC after treatment with MET siRNA.

types, such as gastric epithelial cells [18], squamous cell carcinoma cells [29] and lung carcinoma cells [30] and identifies Cox-2 as a downstream of HGF/MET signaling. However, this study first reported that HGF/MET induced Cox-2 expression increases PGE2 levels in glioma. Moreover, inhibition of MET reduced Cox-2 expression as well as Cox-2 mediated PGE2 production. Therefore, targeting MET may be a useful therapeutic strategy to reduce PGE2 by reducing Cox-2 expression.

In that HGF stimulation could modulate PGE2 synthesis and degradation, we propose that PGE2 may be an important mediator for HGF/MET mediated cell survival. Studies revealed PGE2 can directly activate MET downstream independent of HGF in colon cancer cells [13, 31]. Meanwhile, our works demonstrate that Cox-2 inhibition restrain HGF-promoted cell proliferation. Generally, these results emphasized the benefits of disrupting this regulation therapeutically and knockdown of Cox-2 may have particular merits for glioma clinical therapy with high tumor MET or HGF expression.

In conclusion, our study demonstrates a critical role for HGF/MET signaling in the promotion of

PGE2 biogenesis in glioma cells, by inducing Cox-2 expression. Given the important role of PGE2 during glioma development, therapeutically inhibition of MET and Cox-2 may represent a useful approach for glioma patients.

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**Disclosure of conflict of interest**

None.

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## MET/PGE2 signaling in glioma cells

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