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TITLE PAGE

Lapatinib-mediated COX-2 expression via EGFR/HuR interaction enhances the aggressiveness of triple-negative breast cancer cells

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RUNNING TITLE PAGE

Running title: lapatinib-induced metastasis in TNBCs

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Abbreviations:

EGFR: Epidermal Growth Factor Receptor

HER2: Human Epidermal Growth Factor Receptor 2

ER: Estrogen Receptor

PgR: Progesterone Receptor

TNBCs: Triple-negative Breast Cancers

UTR: Un-translated Region

miR-7: MicroRNA-7

COX-2, PTGS2: Cyclooxygenase-2

ARE: AU-rich element

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ABSTRACT

Lapatinib, a dual EGFR/HER2 kinase inhibitor, showed clinical benefits in advanced HER2-positive breast cancer patients. Because some triple-negative breast cancers (TNBCs) frequently overexpress EGFR, the anti-tumor activity of lapatinib in such diseases was also tested. However, the results showed a worse event-free survival rate. It remains unknown whether and how lapatinib elicits the aggressiveness of such cancer cells. In this study, our results demonstrated that lapatinib facilitated axillary and lung metastases of triple-negative MDA-MB-231 breast cancer cells without affecting their viability, leading to worse survival in orthotopic xenograft mice. The lapatinib-increased motility was attributed by the elevation of EGFR through the downregulation of microRNA-7 and by the subsequent overexpression of cyclooxygenase-2 (COX-2). Strikingly, independent of its kinase activity, the elevated EGFR at least partly stabilized COX-2 expression by enhancing the binding of HuR to *COX-2* mRNA. Our results suggest that lapatinib may increase the migration and invasion of MDA-MB-231 cells by upregulating EGFR and COX-2 through the downregulation of microRNA-7, providing a potential explanation for the worse clinical outcome of TNBC patients who receive lapatinib-based treatment. These findings also shed new light on the molecular mechanism of *COX-2* mRNA stabilization by EGFR in a kinase-independent manner.

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Introduction

Lapatinib (Tykerb®, GW-572016), an oral dual tyrosine kinase inhibitor of the EGFR and HER2 receptors, has been approved for the treatment of advanced HER2-positive breast cancer patients who fail to respond to chemotherapy or treatment with trastuzumab (anti-HER2 monoclonal antibody)(Rusnak et al., 2001a; Rusnak et al., 2001b). Lapatinib inhibits tyrosine phosphorylation of HER2, in turn attenuating the downstream signaling pathways that promote tumor progression(Spector et al., 2005; Xia et al., 2002). The anti-tumor activity of lapatinib in several cancer types, particularly in breast cancer, has been shown in preclinical and phase I/II trials(Nelson and Dolder, 2006). Patients with HER2-positive metastatic or inflammatory breast cancer showed response rates of 25% and 50%, respectively, to lapatinib(Gomez et al., 2008; Johnston et al., 2008). The results from phase III trials with lapatinib in HER2-positive diseases further showed an encouraging outcome with a significant improvement over time in disease progression due to the addition of lapatinib to capecitabine (Cameron et al., 2008; Geyer et al., 2006) or paclitaxel (Di Leo et al., 2008).

Although the majority of the clinical benefits associated with treatment with lapatinib were achieved in patients with HER2-positive breast cancers, the use of lapatinib in HER2-negative patients continues to be of interest(Amir et al., 2010; Boussen et al., 2010; Finn et al., 2009a; Frassoldati et al., 2008; Johnston et al., 2009). In up to 80% of HER2-, estrogen receptor (ER)-, and progesterone receptor (PgR)-negative (triple-negative) breast cancers (TNBCs), the EGFR protein is expressed and is considered a potential therapeutic target (Siziopikou et al., 2006; Siziopikou and Cobleigh, 2007). Thus, lapatinib is also currently being tested as a monotherapy or in combination with other systemic therapies for triple-negative or other HER2-negative breast cancers (Dhillon; Sharma). Unfortunately, lapatinib has shown a lack of dramatic efficacy in overall HER2-negative diseases in most of these studies. It is noteworthy that the addition of lapatinib to paclitaxel was actually found to worsen the clinical outcomes, with shorter median event-free survival in triple-negative and HER2/PgR-negative patients (Finn et al., 2009b). This possible negative influence of

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lapatinib-based treatment on HER2-negative diseases might become a major concern for these vigorous clinical trials. However, it remains unknown whether and how lapatinib elicits its negative effects on these cancer cells.

Here, we show that treatment with lapatinib greatly facilitated metastasis of triple-negative MDA-MB-231 breast cancer cells to the axillary lymph nodes and lung without affecting their proliferation, and it elicited a worse survival rate in an orthotopic xenograft mouse model. The enhanced migration and invasion involved the increase in EGFR expression through the downregulation of microRNA-7 and the subsequent elevation of COX-2 expression. Intriguingly, the elevated expression of EGFR, independent of its kinase activity, was able to stabilize COX-2 expression by enhancing the binding of HuR to *COX-2* mRNA without affecting its promoter activity. Our finding that lapatinib enhances the metastasis of TNBCs through the induction of EGFR and COX-2 expression may provide a plausible explanation for the worse clinical outcome observed in some HER2-negative patients who receive lapatinib-based treatment.

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Materials and Methods

Cell lines

Human breast cancer cell lines (SkBr3, BT474, MDA-MB-231, MDA-MB-157, and HS-578T) and their derivatives were cultured in DMEM/F-12 with 10% fetal bovine serum. Lapatinib-resistant cancer cell clones were established by treatment with gradually increasing concentrations of these drugs for over one month. The sensitivities of these established cancer cells to lapatinib were tested using the MTT assay or by cell number counting. These lapatinib-resistant clones were cultured in the presence of 1 μM lapatinib.

Antibodies and reagents

Anti-EGFR antibodies for immunoprecipitation (IP) and immunoblotting (IB) (SC-03) were purchased from Thermo Scientific and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-COX-2, anti-HER2, anti-HER3, and anti-HER4 antibodies were from Santa Cruz Biotechnology. Celecoxib, and NS-398 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Lapatinib was purchased from LC Laboratory (Woburn, MA). The validated siRNA oligonucleotide targeting EGFR and the non-targeting control siRNA were purchased from Dharmacon (Lafayette, CO) and Santa Cruz Biotechnology (Santa Cruz, CA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay

In vitro cell viability was measured using an MTT colorimetric assay. Briefly, cells (5×10^3 cells per well) were seeded in 96-well plates for 24 h and subsequently subjected to treatments as indicated. After the treatments, the relative cell amounts were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method on a daily basis. MTT (Sigma) at 1 mg/ml was added to each well. After a 3-hr incubation,

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the medium was removed, and the MTT was dissolved in 100 μl of DMSO. The absorbance was measured at 570 nm, and the relative proliferation index for each day was determined using the absorbance at day 0 as the standard.

Wound healing and transwell migration/invasion assays

For the *in vitro* wound healing assay, confluent monolayers of cells were scratched, and the migration distance of the cells into the scratched area was measured in 10 randomly chosen fields. The Student's *t*-test was used to determine the statistical significance. For the transwell migration assay, 1×10^5 cells were plated in the top chamber onto a non-coated membrane (24-well insert; pore size, 8 μm; Corning Costar). For the invasion assay, 1×10^5 cells were plated in the top chamber onto a Matrigel-coated membrane. Each well was coated freshly with Matrigel (60 μg; BD Bioscience) before the invasion assay. In both assays, the cells were plated in medium without serum or growth factors. After incubation for 24 hours, the cells that did not migrate or invade through the pores were removed with a cotton swab. Cells on the lower surface of the membrane were fixed with methanol and stained with crystal violet. The cells that migrated or invaded through the membrane were counted under a light microscope (40×, five random fields per well) prior to the application of DMSO and measurement of the absorbance.

Animal studies

All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of China Medical University and Hospital (No. 100-61-N). Female severe combined immunodeficient (SCID) mice at 4 to 6 weeks of age were used in the orthotopic tumor-xenograft model. For the primary tumor growth assay, viable cells [3×10^6; re-suspended in a 1:1 mixture of PBS and growth factor–reduced

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Matrigel (BD Biosciences) in a total volume of 50 μL] were injected orthotopically into the mammary gland. The primary tumor growth rates were analyzed by measuring the tumor length (L) and width (W) with calipers and by calculating the volume with the formula $LW^2/2$. Axillary and lung metastases were observed after euthanasia.

RNA isolation, reverse transcription (RT), polymerase chain reaction (PCR), real-time PCR quantification

Total RNA was extracted from the cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For microRNA, the reverse transcription (RT) reactions contained 2 μg of RNA, 50 nmol/L of the stem-loop RT primer, 0.25 mmol/L of each deoxynucleotide triphosphate, 50 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen), 1× RT buffer, 10 mmol/L DTT, and 4 units of RNase inhibitor. The stem-loop RT primer for hsa-miR-7 was designed according to mature miRNA sequence (Sanger Center miRNA Registry, http://microrna.sanger.ac.uk/sequences/). The sequences of the RT primers are as follows: hsa-miR-7 RT primer, 5'-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACAACA-3'; U48 RT primer, 5'-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTCAGCG-3'. Real-time PCR was performed in reactions containing 0.5 μmol/L of each forward and reverse primer, 0.1 μmol/L of the Universal Probe Library Probe #21 (Roche), the 1× LightCycler TaqMan Master, and 2 μL of cDNA using a Roche LightCycler 480 Real-Time PCR system. U48 small nuclear RNA was used as an internal control. The sequences of the forward primers were as follows: miR-7, 5'-GCGGCGTGGAAGACTAGTGAT-3'; U48, 5'-CGGCGGTAACTCTGAGTGTGT-3'. The reverse primer for all of the above sets of genes was 5'-GTGCAGGGTCCGAGGT-3'. For *EGFR, COX-2,* and *actin* mRNA, 1 μg of total RNA was subjected to RT with an oligo-dT primer using a reverse transcriptase kit (Invitrogen). Equal amounts of cDNA (2 μL) were subjected to PCR and amplified with 30 cycles using the following primers: *EGFR*, forward 5'-GTTGATATCATGCGACCCTCCGGGACG-3' and

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reverse 5'-GGTTCTAGATCATGCTCCAATAAATTC-3'; *COX-2*, forward 5-'GCTTAAACAGGAGCATCCTGA-3' and reverse 5'-GGGTAATTCCATGTTCCAGC-3'; *Actin*, forward 5'-CTGGAACGGTGAAGGTGACA-3' and reverse 5'-AAGGGACTTCCTGTAACAATGCA-3'. The PCR products were subjected to 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. Real-time PCR reactions containing 0.3 μl of cDNA, 0.3 μl of the forward and reverse primers, 5 μl of 2X SYBR Green (Roche), and 1.4 μl of distilled water were performed with a Roche LightCycler 480 Real-Time PCR system.

MicroRNA overexpression

The microRNA mimic oligonucleotides for hsa-miR-7 were purchased from Dharmacon and transfected into cells with DharmaFECT 1 (Dharmacon, Lafayette, CO) based on the manufacturer's instructions. Forty-eight hours after transfection, the cells were used for the migration assay, the invasion assay, the luciferase reporter assay, and the immunoblotting assay.

Reporter gene assay

The luciferase reporter gene containing full-length 3' untranslated region (UTR) of the human *EGFR* gene was a gift from Dr. Keith Giles (Western Australian Institute for Medical Research). The *COX-2* promoter luciferase reporters were a gift from Prof. Wen-Chang Chang (Taipei Medical University, Taipei, Taiwan). The *EGFR* promoter was amplified with primers (forward 5'-CGATGGTACCTTCTCCTGCGGGAGCTACAG-3' and reverse 5'-CGATCTCGAGGGGCACTCTTGGATAGGCAA-3') from genomic DNA extracted from MDA-MB-231 cells. The segment (from -828 to +1199) of the *EGFR* promoter was cloned into the pGL4.22[*luc2CP*/Puro] vector (Promega) at the *Kpn*I and *Xho*I sites. Cells that were 60-70% confluent in 24-well plates were transfected with the indicated plasmids using

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Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The firefly luciferase reporter gene construct and pTK-Renilla luciferase construct (for normalization) were co-transfected at 1 μg each per well. After transfection for 48 h, the cell lysates were prepared and the luciferase activity was measured according to manufacturer's instructions (Dual-Luciferase Reporter Assay System, Promega).

RNA Immunoprecipitation Assay

The total lysates were prepared with lysis buffer [3 mM MgCl2, 40 mM KCl, 5% glycerol, 0.5% Nonidet P-40, 2 mM DTT, 10 mM HEPES, pH7.5, containing protease, phosphatase inhibitors and RNase OUT (0.5 unit/μl)] and the lysate concentration was adjusted to 2.5 μg/μl with lysis buffer. One milligram of each lysate was mixed with 400 μl NT2 buffer (150 mM NaCl, 1 mM MgCl2, 0.05% Nonidet P-40, 50 mM Tris, pH7.4, 40 mM EDTA) and subjected to immunoprecipitation with 1 μg HuR or IgG control monoclonal mouse antibody (Santa Cruz Biotechnology) at room temperature for 2 h. The protein G beads were pelleted, washed with NT2 buffer, containing 0.1% sodium deoxycholate, and suspended in 0.5 ml TRIzol reagent (Invitrogen). After extraction of total RNA, RT and PCR were conducted with oligo-dT and specific primers for COX-2, respectively.

Statistical analysis

All results are presented as the mean ±SD. A two-tailed Student's *t*-test was used to calculate the statistical significance between the groups. The significance of the difference in the survival rate was analyzed by a log-rank test based on the Kaplan-Meier test. The tumor volume was analyzed by a two-sided *t*-test.

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Results

Treatment with lapatinib enhanced the metastasis ability of triple-negative breast cancer (TNBC) cells both *in vitro* and *in vivo*.

As found in most triple-negative breast tumor tissues, EGFR expression was detected in the triple-negative MDA-MB-231, MDA-MB-157, and HS-578T beast cancer cell lines as well as in HER2-positive SkBr3 and BT474 cell lines (Fig. 1A). To explore the long-term effect of lapatinib-based treatment on TNBCs, these three TNBC cell lines were treated with gradually increasing concentrations of lapatinib for over one month to establish lapatinib-resistant clones (named 231/Lap, 157/Lap, and HS-578T/Lap, respectively). The growth rates of these 231/Lap (Fig. 1B, upper panel), 157/Lap (Fig. 1B, middle panel), and HS-578T/Lap (Fig. 1B, lower panel) cells remained comparable to their parental cells. However, the 231/Lap cells were found to possess a higher migration ability compared with MDA-MB-231 cells in both the wound healing migration assay (Fig. 1C) and the transwell cell migration assay (Fig. 1D). The increases in migration, examined by either the wound healing migration assay or the transwell cell migration assay, were also observed in 157/Lap (Fig. 1E) and HS-578T/Lap (Fig. 1F) cells compared with their parental cells, respectively. This increased migration ability was not affected when lapatinib was removed from the medium of the 231/Lap cells (Supplemental Fig. S1). Moreover, the ability of the 231/Lap (Fig. 1G) and HS-578T/Lap (Fig. 1H) cells to invade into the matrix gel was also stronger than that in the parental cells.

To further validate the pro-aggressive effects of lapatinib on TNBCs *in vivo*, MDA-MB-231 or 231/Lap#6 cells were orthotopically injected into the mammary fat pads of NOD-SCID mice. Over a 5-week period after implantation, the growth rates of the resulting primary tumors from both cell lines were similar in the absence of lapatinib, and treatment with lapatinib also did not affect the tumor size in mice bearing MDA-MB-231 cells (Fig. 1I). Nonetheless, the survival rate of the 231/Lap#6 group was significantly lower than that of mice injected with MDA-MB-231 cells ($p=0.021$, log-rank test); consistently, lapatinib

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treatment also obviously decreased the survival rate of the mice that received MDA-MB-231 cells (p=0.017, log-rank test) (Fig. 1J). No macrometastasis to the axillary lymph node, lung or other organs was found in any of the 18 mice with MDA-MB-231 tumors at the end of the 5-week experiment. Strikingly, 6 of 18 mice with 231/Lap#6 tumors and 2 of 9 lapatinib-treated mice with MDA-MB-231 tumors showed axillary lymph node metastasis. Moreover, 3 of 8 mice with axillary metastasis further showed lung metastasis (Fig. 1K). Taken together, these findings suggested that treatment with lapatinib renders TNBC cells that possess a higher propensity to metastasize to the axillary lymph node and lung.

The increased EGFR expression via downregulation of microRNA-7 mediated lapatinib-enhanced cell migration.

Next, we investigated the underlying mechanisms of lapatinib-facilitated cell migration and invasion. Because lapatinib functions as an EGFR/HER2 dual tyrosine kinase inhibitor, we investigated whether the enhanced migration of the 231/Lap cells involved changes in these receptor tyrosine kinases. As shown in Figure 2A, the 231/Lap clones retained their HER2-negative status, but they showed a remarkable increase in EGFR expression. Furthermore, there was no significant change in HER4 expression, but an obvious decrease in HER3 expression was observed in the 231/Lap cells. Although the 231/Lap clones express a higher EGFR protein level, EGF-induced activation of EGFR, as measured by EGFR tyrosine phosphorylation, was completely blocked in these cells in the presence of lapatinib (Fig. 2B). The lapatinib-increased EGFR expression was also observed in the 157/Lap and the HS-578T/Lap cells (Fig. 2C), suggesting that lapatinib may increase EGFR expression in TNBCs. To examine whether the increased EGFR expression, independent of its tyrosine kinase activity, contributes to the enhancement of cell migration in response to lapatinib treatment, lapatinib-resistant cells were transfected with EGFR siRNA to silence EGFR expression and then subjected to a wound healing migration assay. Indeed, when EGFR expression was knocked down by two different siRNA duplexes, the cell migration

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of 231/Lap cells was dramatically reduced in wound healing assays (Figs. 2D and 2E). Similar results were also observed in 231/Lap cells in the transwell migration (Fig. 2F) and invasion (Fig. 2G) assays, revealing the crucial roles of kinase-inactive EGFR in enhancing the migration and invasion of TNBCs in response to long-term treatment with lapatinib.

We next investigated the mechanism by which EGFR expression was upregulated by lapatinib treatment. The level of EGFR mRNA was increased in 231/Lap cells (Figs. 3A and 3B). However, the *EGFR* promoter activity was comparable between MDA-MB-231 and 231/Lap#6 cells (Fig. 3C), suggesting that the increased EGFR expression may be due to the stabilization of *EGFR* mRNA rather than augmentation of *EGFR* transcription. Consistent with this hypothesis, the activity of the *EGFR* 3' untranslated region (UTR) was much higher in 231/Lap#6 cells as measured by the luciferase-reporter assay (Fig. 3D). MicroRNA-7 (miR-7) has been reported to target the 3' UTR of *EGFR* mRNA and cause its degradation (Li and Carthew, 2005; Webster et al., 2009). Interestingly, the level of miR-7 was lower in 231/Lap (Fig. 3E), 157/Lap, and HS-578T/Lap (Fig. 3F) cells compared with the parental cell lines. Restoration of the levels of miR-7 dramatically attenuated the *EGFR* 3'UTR activity (Fig. 3G) and EGFR protein expression in the 231/Lap#6 cells (Fig. 3H). Ectopic expression of miR-7 also reduced the migration (Fig. 3I) and invasion (Fig. 3J) of 231/Lap#6 cells. Collectively, these results indicated that chronic treatment with lapatinib may increase the EGFR level by downregulating miR-7 expression, which in turn promotes the aggressiveness of TNBCs.

The upregulated COX-2 expression by EGFR accounted for the lapatinib-enhanced metastasis of TNBCs.

We further explored how augmented EGFR expression increased the migration and invasion of 231/Lap cells. The epithelial-mesenchymal transition (EMT), a key developmental program, is often activated during cancer metastasis(Iwatsuki et al., 2010).

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However, the characteristic features of the EMT were not observed in 231/Lap cells compared with those in their parental cells (Supplemental Fig. S2). It has been reported that cyclooxygenase-2 (COX-2, also named PTGS2) is an enzyme frequently associated with EGFR overexpression(Araki et al., 2004; Zhu et al., 2010) and cancer metastasis(Wang et al., 2007). Thus, involvement of COX-2 expression in lapatinib-mediated metastasis was thus examined. We found that the COX-2 protein expression was significantly increased in all three lapatinib-resistant cell lines, including 231/Lap, 157/Lap, and HS-578T/Lap cells (Fig. 4A). The *COX-2* mRNA level in 231/Lap cells was also elevated (Fig. 4B) in parallel to the increase in the EGFR level (Fig. 2A). Treatment with two COX-2 selective inhibitors, celecoxib (30 μM) or NS-398 (30 μM), for 24 h can significantly reduce the migration ability of 231/Lap#6 cells (Fig. 4C) without affecting their viability (Supplemental Fig. S3). Under these conditions, without affecting the growth rate of 231/Lap#6 tumors (Fig. 4D), treatment with celecoxib (10 μg/g/day) also reduced the metastasis rate to the axillary lymph nodes (Fig. 4E) and improved the survival rate of mice with 231/Lap#6 tumors (Fig. 4F), suggesting that the induction of COX-2 by lapatinib may worsen triple-negative diseases by increasing the aggressiveness of cancer cells. Indeed, by examining the protein expression of tumors from the experiments of Figures 1I-K, both primary and axillary metastatic xenograft tumors from 231/Lap#6 cells expressed higher levels of EGFR and COX-2 protein in comparison with the xenograft tumors from MDA-MB-231 cells (Supplemental Fig. S4). Within the group of MDA-MB-231 cells, treatment with lapatinib can also slightly enhance the EGFR and COX-2 expression in the axillary metastatic tumors (Supplemental Fig. S4), indicating that both EGFR and COX-2 play important roles in the lapatinib-facilitated aggressiveness of TNBCs.

To address whether the increased EGFR mediates the induction of COX-2 expression in these lapatinib-resistant clones, the inhibitory effect of EGFR siRNA on COX-2 expression was examined. We found that the decrease in EGFR expression by both EGFR siRNA and miR-7 could dramatically attenuate the COX-2 protein expression in 231/Lap#6 cells (Fig. 5A). To further support this finding, the effect of ectopic EGFR expression on the COX-2

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level was examined in 231/Lap#2 cells, which express a moderate degree of EGFR and COX-2. As shown in Figure 5B, overexpression of myc-EGFR slightly increased COX-2 expression in a dose-dependent manner. Surprisingly, this effect was not attenuated but further enhanced when the EGFR activity was blocked by lapatinib treatment, suggesting that EGFR might at least in part regulate COX-2 expression independent of its kinase activity. Next, we explored how the EGFR regulates COX-2 expression in a kinase-independent manner in 231/Lap cells. Targeting *EGFR* mRNA by two different siRNA duplexes diminished the *COX-2* mRNA level in 231/Lap#6 cells (Fig. 5C), indicating that EGFR is involved in the regulation of the *COX-2* mRNA level. Similarly to the protein and mRNA levels, the *COX-2* promoter activity in 231/Lap cells was also higher than that in MDA-MB-231 cells (Fig. 5D). However, attenuation of EGFR expression did not affect the induction of *COX-2* promoter activity (Fig. 5E). Altogether, these results implied that EGFR mediated lapatinib-facilitated aggressiveness of TNBCs by inducing COX-2 expression post-transcriptionally.

EGFR enhanced *COX-2* mRNA stabilization through interaction with HuR

To further study whether the elevated EGFR is involved in the post-transcriptional regulation of COX-2, 231/Lap#6 cells were transfected with EGFR siRNA followed by termination of *de novo* mRNA biosynthesis with actinomycin D. Indeed, silencing EGFR expression by siRNA time-dependently facilitated the reduction of *COX-2* mRNA (Fig. 6A) and protein levels (Fig. 6B) in the presence of actinomycin D, indicating that EGFR may contribute to maintaining a constantly high level of COX-2 expression by regulating *COX-2* mRNA in 231/Lap cells. HuR, an RNA-binding protein, has been widely reported to bind to and stabilize *COX-2* mRNA expression (Cok et al., 2003). Our finding raises the possibility that EGFR may stabilize *COX-2* mRNA through interaction with HuR. Our data showed that the interaction between EGFR and HuR was enhanced in 231/Lap#6 cells as measured by immunoprecipitation assays with an anti-EGFR antibody followed by immunoblot assays

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with an anti-HuR antibody or vice versa (Fig. 6C). Silencing EGFR by siRNA reduced HuR binding to *COX-2* mRNA in 231/Lap#6 cells in RNA-immunoprecipitation assays (Fig. 6D), suggesting that EGFR may cooperate with HuR to stabilize *COX-2* mRNA expression. Taken together, our results indicated that EGFR expression was elevated by treatment with lapatinib due to the downregulation of miR-7. The augmented EGFR in turn facilitated cell migration and invasion by stabilizing *COX-2* mRNA expression through an interaction with HuR (Fig. 6E).

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Discussion

In addition to the promising efficacy in HER2-positive breast cancer, the use of lapatinib in HER2-negative diseases, especially in triple-negative cancers due to frequent overexpression of EGFR, is of interest and currently being tested (Dhillon; Sharma). However, the use of lapatinib has been found to elicit diverse effects in different sub-groups(Finn et al., 2009b; Johnston et al., 2009; Leary et al., 2010; Mayer and Arteaga, 2010). Our findings suggest that long-term treatment with lapatinib may render triple-negative cells more aggressive with lymph node and lung metastasis through downregulation of miR-7 and sequential overexpression of EGFR and COX-2. These results provide a possible molecular mechanism that may explain how lapatinib-based treatment worsens the clinical outcome in breast cancer patients with triple-negative tumors(Finn et al., 2009b). Several lines of evidence have further indicated that the phenotypes and mechanisms shown in this study are not due to a clonal effect during the selection of the lapatinib-resistant clones. First, short-term treatment with lapatinib for 48h can also enhance cell migration (Supplemental Fig. S5A) and induce EGFR and COX-2 expression (Supplemental Fig. S5B) in MDA-MB-231 cells. The slight induction of EGFR and COX-2 expression in response to lapatinib treatment for 1-3 days was also found in other TNBC cell lines, such as MDA-MB-157 (Supplemental Fig. S5C) and HBL-100 (Supplemental Fig. S5D) cells. Similarly, lapatinib-resistant HER2-positive SkBr3 and BT474 cells also express higher EGFR protein levels in comparison with their parental cells (Supplemental Fig. S5E). Furthermore, lapatinib treatment can increase tumor metastasis to the axillary lymph nodes and lungs in xenograft mouse models bearing parental MDA-MB-231 tumors (Fig. 1K). These results support the relevance of a relationship between the EGFR/COX-2 induction and the resistant phenotype.

Aberrant EGFR expression is ubiquitously found in most human solid tumors, including non-small-cell lung, head and neck, colorectal, breast, renal, breast, glioma, prostate, and pancreatic cancers, and it has been recognized as a poor prognostic marker for therapeutic efficacy and patient survival(Normanno et al., 2006). Gene amplification and

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mutations are the major causes of EGFR overexpression and signaling activation(Normanno et al., 2006). Transcriptional upregulation by hypoxia-inducible factor 2-α(Franovic et al., 2007), p53(Deb et al., 1994; Ludes-Meyers et al., 1996), Notch-1(Purow et al., 2008), or β-catenin(Guturi et al., 2012) also accounts for EGFR overexpression in human cancers. Recently, it was proposed that evasion of negative regulation by miRNA contributes to the enhancement of EGFR expression. Two miRNAs, including miR-7(Li and Carthew, 2005; Reddy et al., 2008; Webster et al., 2009) and miR-128b(Weiss et al., 2008), have been reported to directly target and regulate *EGFR* mRNA expression and are involved in photoreceptor differentiation in the Drosophila eye(Li and Carthew, 2005) and cancer development(Webster et al., 2009; Weiss et al., 2008). Our data also suggest that lapatinib increases EGFR expression in MDA-MB-231 cells through downregulation of miR-7 but not miR-128b (data now shown) rather than through the induction of *EGFR* promoter activity. The involvement of miR-7 downregulation in EGFR overexpression was also observed in other TNBC cell lines, including MDA-MB-157 and HS-578T cells, in response to treatment with lapatinib, suggesting a common effect induced by lapatinib in TNBCs. However, further investigation is needed to understand how lapatinib downregulates miR-7. In addition to being targeted by miR-7, EGFR has also been reported to induce miR-7 transcription relying on its tyrosine kinase activity(Chou et al., 2010), suggesting that miR-7 regulates EGFR expression through negative feedback. Therefore, inhibition of EGFR kinase activity by lapatinib may account for the downregulation of miR-7 and subsequent elevation of the EGFR protein.

Deregulation of COX-2 expression is associated with aggressive pathological parameters and poor prognosis in several human malignancies, including colorectal, ovarian, head and neck, and breast cancers(Wang et al., 2007). The enhancement of migration and invasion of lapatinib-resistant cells was at least partly due to COX-2 overexpression because blockage of COX-2 activity by selective COX-2 inhibitors can significantly reduce the cell mobility (Fig. 4C) and improve the survival rate of mice with 231/Lap#6 tumors (Fig. 4F). In addition to its potential role in tumor promotion and progression, overexpression of COX-2 has been

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suggested to promote resistance to EGFR TKIs in both EGFR-dependent and -independent manners(Kim et al., 2009; Krysan et al., 2005), and addition of specific COX-2 inhibitors can synergize the effect of EGFR TKIs in some preclinical studies(Buchanan et al., 2007; Choe et al., 2005; Zhang et al., 2005). The induction of COX-2 transcription by EGFR is well documented and mostly relies on EGFR tyrosine kinase activity(Rogers et al., 2005). However, silencing EGFR expression dramatically reduced the COX-2 protein levels in 231/Lap clones (Fig. 5A) without affecting its promoter activity (Fig. 5E). Instead of inducing promoter activity, our data revealed that the lapatinib-induced EGFR can increase COX-2 expression by enhancing HuR-dependent *COX-2* mRNA stabilization. Our unpublished data also indicate that the *COX-2* promoter activation in lapatinib-resistant clones is attributed to the NF-κB signaling pathway, rather than the kinase-inactive EGFR.

The stabilization of COX-2 by HuR has been well studied(Cok et al., 2003; Dixon et al., 2001). HuR regulates COX-2 mRNA stability by targeting to the AU-rich elements (AREs) within its 3'UTR(Cok et al., 2003; Dixon et al., 2001). The expression of HuR is associated with increased COX-2 expression in colorectal, ovarian, and breast cancers with a high tumor stage(Denkert et al., 2006; Denkert et al., 2004a; Denkert et al., 2004b). Phosphorylations of HuR by PKC, p38, Chk1, or Cdk1 have been reported to enhance its binding to target mRNAs or to facilitate its translocation from the nucleus to the cytoplasm, where it regulates the stability and translation of target mRNAs(Doller et al., 2007; Doller et al., 2008; Doller et al., 2011; Kim et al., 2010; Subbaramaiah et al., 2003). Protein interaction between HuR and β-catenin was recently found to cooperatively regulate COX-2 mRNA stability in colon cancer cells(Kim et al., 2012). Our data also suggest that EGFR may interact with HuR partly in a kinase-independent manner to maintain the COX-2 mRNA stability. Although further studies on the RNA-mediated interaction between EGFR and HuR are needed, these kinase-independent functions of EGFR, including stabilization of COX-2 expression and SGLT-1-mediated maintenance of adequate intracellular glucose levels(Weihua et al., 2008), may bring to light new molecular perspectives on drug

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resistance to EGFR TKIs. However, further studies are needed to better understand the underlying mechanisms.

Attempts to extend the clinical benefit of lapatinib from HER2-postive cancers to HER2-negative diseases are actively ongoing, but the clinical outcomes have varied substantially among different subgroups. Our findings indicate that instead of affecting cell viability, lapatinib promotes the metastasis of TNBCs through the upregulation of EGFR and COX-2, and our results do not favor the use of lapatinib in triple-negative diseases. Our results also elucidate a potential kinase-independent function of EGFR in stabilizing *COX-2* mRNA expression through interaction with HuR, indicating a possible link to EGFR TKI drug resistance.

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Author Contribution

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Conducted experiments: Huang, W.C., Wei, Y.L., Yu, M.C., Hsu, S.C., Tsai, S.L. Chen, W.S.

Contributed new reagents or analytic tools: Hsia, T.C., Tu, C.Y., Yu, Y.L., Yeh, MH., Huang, C.Y.

Performed data analysis: Huang, W.C., Hsia, T.C., Tu, C.Y., Chen, Y.J. Huang, T.C. Yen, C.J.

Wrote or contributed to the writing of the manuscript: Huang, W.C., Chen, Y.J., Hung, M.C.

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Footnotes

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Figure Legends

Fig. 1. The effects of long-term treatment with lapatinib on migration and invasion of TNBCs *in vitro* and *in vivo*.

A, The EGFR and HER2 protein expression in SkBr3, BT474, MDA-MB-231, MDA-MB-157, and HS-578T breast cancer cell lines was examined by Western blot analysis with anti-EGFR, anti-HER2 and anti-tubulin antibodies. B, The growth of MDA-MB-231 (231), MDA-MB-157 (157), and HS-578T cells and their lapatinib-resistant clones (231/Lap, 157/Lap and HS-578T/Lap) were measured using the MTT assay. C-F, The migration abilities of MDA-MB-231 (C) and MDA-MB-157 (E) cells and their lapatinib-resistant clones (231/Lap and 157/Lap) at the indicated time points were determined by wound-healing assays. The migration abilities of lapatinib-resistant MDA-MB-231 (D) and HS-578T (F) cells as well as their parental cells over 24 h were also determined by transwell migration assays. G and H, MDA-MB-231 (G), HS-578T (H), and their lapatinib-resistant clones were cultured in transwells coated with Matrigel, and the invaded cells were measured after 24 h. I-K, NOD-SCID mice were orthotopically implanted with MDA-MB-231 or 231/Lap#6 cells. The mice implanted with MDA-MB-231 cells were treated with vehicle or lapatinib (100 mg/g/day) after implantation until the end of the experiments. The tumor sizes in each group were measured every four days (I). Survival was analyzed by a log-rank test based on the Kaplan-Meier test (J). Treatment with lapatinib promotes the metastasis of MDA-MB-231 cells to the axillary lymph nodes and lungs in orthotopic xenograft mice. The total number and metastasis number of each group from two independent experiments are shown in (K). The quantitative data are represented as the mean ± SD of three independent experiments. *, $p< 0.05$; **, $p<0.01$; ***, $p<0.001$.

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Fig. 2. The Involvement of increased EGFR expression in cell migration and invasion of lapatinib-resistant TNBCs.

A-C, MDA-MB-231 cells and 231/Lap clones were treated with 100 ng/ml EGF for 15 min (B). Whole cell lysates prepared from MDA-MB-231 (A and B), MDA-MB-157 (C), and HS578T (C) cells and their lapatinib-resistant clones were subjected to immunoblotting analyses using the indicated antibodies. D and E, The migration of 231/Lap#6 cells transfected with EGFR siRNA for two days were examined by wound-healing assays for the indicated time. F and G, MDA-MB-231 cells and 231/Lap clones transiently transfected with or without EGFR siRNA were subjected to transwell migration (F) and invasion (G) assays. The quantitative data are presented as the mean ± SD of three independent experiments. *, $p< 0.05$; **, $p<0.01$; ***, $p<0.001$.

Fig. 3. The involvement of hsa-miR-7 downregulation in the EGFR expression of lapatinib-resistant TNBCs.

A-B, The *EGFR* mRNA levels of MDA-MB-231 and 231/Lap clones were analyzed by RT-PCR (A) and RT-qPCR (B). C-D, MDA-MB-231 and 231/Lap#6 cells were transfected with *EGFR* promoter (C) or 3'UTR (D) -luciferase reporters for 2 days and then analyzed using luciferase reporter assays. E-F, Total RNAs from MDA-MB-231 (E), MDA-MB-157 (F), and HS-578T (F) cells and their lapatinib-resistant clones were subjected to RT-qPCR analyses for miR-7 and U48 levels. G-H, 231/Lap#6 cells were transfected with increasing amounts of miR-7 for 2 days and then subjected to 3'UTR-luciferase reporter assays (G) or immunoblotting analyses (H). I-J, 231/Lap#6 cells were transfected with 100 nM miR-7 for 48 h and then subjected to transwell migration (I) and invasion (J) assays. The quantitative data are represented as the mean ± SD of three independent experiments. *, $p< 0.05$; **, $p<0.01$; ***, $p<0.001$.

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Fig. 4. The effects of elevation of COX-2 expression on the cell migration of lapatinib-resistant TNBCs.

A-B, The protein (A) and mRNA (B) levels of COX-2 in TNBCs and their lapatinib-resistant clones were measured by immunoblotting and RT-qPCR assays, respectively. C, 231/Lap#6 cells treated with 30 μM celecoxib and 30 μM NS-398 for the indicated time points were subjected to wound-healing assay. D-F, NOD-SCID mice were orthotopically implanted with 231/Lap#6 cells and treated with celecoxib (10 mg/g/day) after implantation until the end of the experiments. The tumor sizes of each group were measured every four days (D). The total number and metastasis number of each group from two independent experiments are shown (E). Survival was analyzed by a log-rank test based on the Kaplan-Meier test (F).

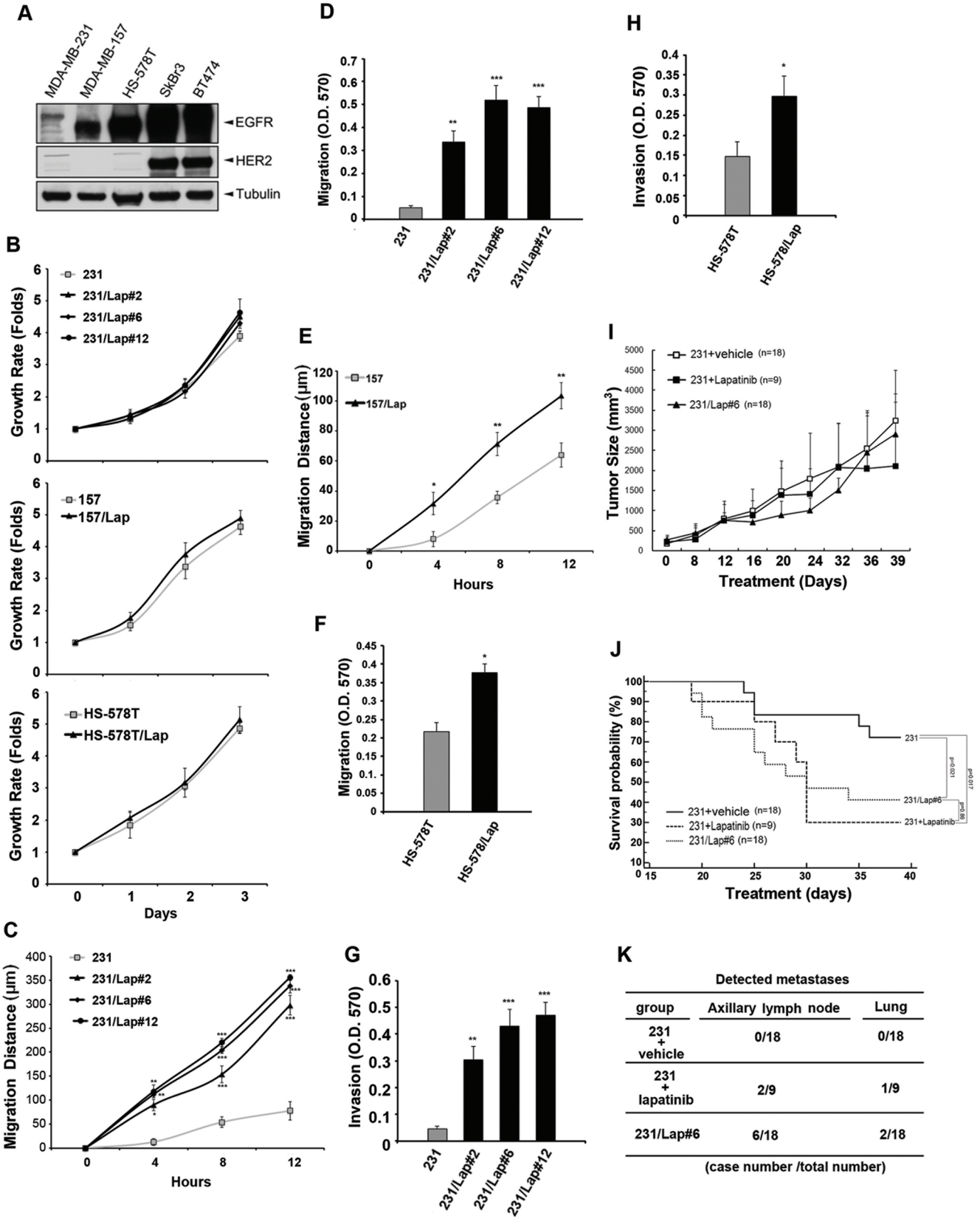
Fig. 5. The role of EGFR in the mRNA stability and promoter activity of *COX-2* in lapatinib-resistant TNBC cells.

A and C, 231/Lap#6 cells were transfected with 100 nM EGFR siRNA or miR-7 for 2 days, and then subjected to immunoblotting (A) and RT-qPCR (C). B, 231/Lap#2 cells transfected with Myc-EGFR and treated with or without 1 μM lapatinib for 48 h were subjected to immunoblotting analyses. D, MDA-MB-231 or 231/Lap clones were transfected with a *COX-2* promoter (−918/+49 bp)-luciferase reporter for 2 days and then subjected to luciferase activity assays. E, MDA-MB-231 or 231/Lap#6 cells were co-transfected with *COX-2* promoter-luciferase reporter and EGFR siRNA or control siRNA for 2 days and then subjected to luciferase activity assays and immunoblotting analyses.

Fig. 6. The involvement of EGFR in the binding of HuR to *COX-2* mRNA in lapatinib-resistant TNBCs.

A-B, 231/Lap#6 cells transfected with or without EGFR siRNA for 2 days followed by treatment with actinomycin D for the indicated time were subjected to RT-qPCR analyses (A) and immunoblotting (B). C, Total lysates prepared from MDA-MB-231 and 231/Lap#6 cells were subjected to IP/WB analyses with anti-HuR and anti-EGFR antibodies, respectively, or *vice versa*. D, 231/Lap#6 cells transfected with or without EGFR siRNA for 48 h were subjected to RNA-IP analyses with anti-HuR or IgG antibodies, followed by RT-PCR analyses. E, Illustration of the proposed model explaining how lapatinib facilitates the metastasis of triple-negative cells.

Fig.1



K

Detected metastases		
group	Axillary lymph node	Lung
231 + vehicle	0/18	0/18
231 + lapatinib	2/9	1/9
231/Lap#6	6/18	2/18

(case number /total number)

Fig.2

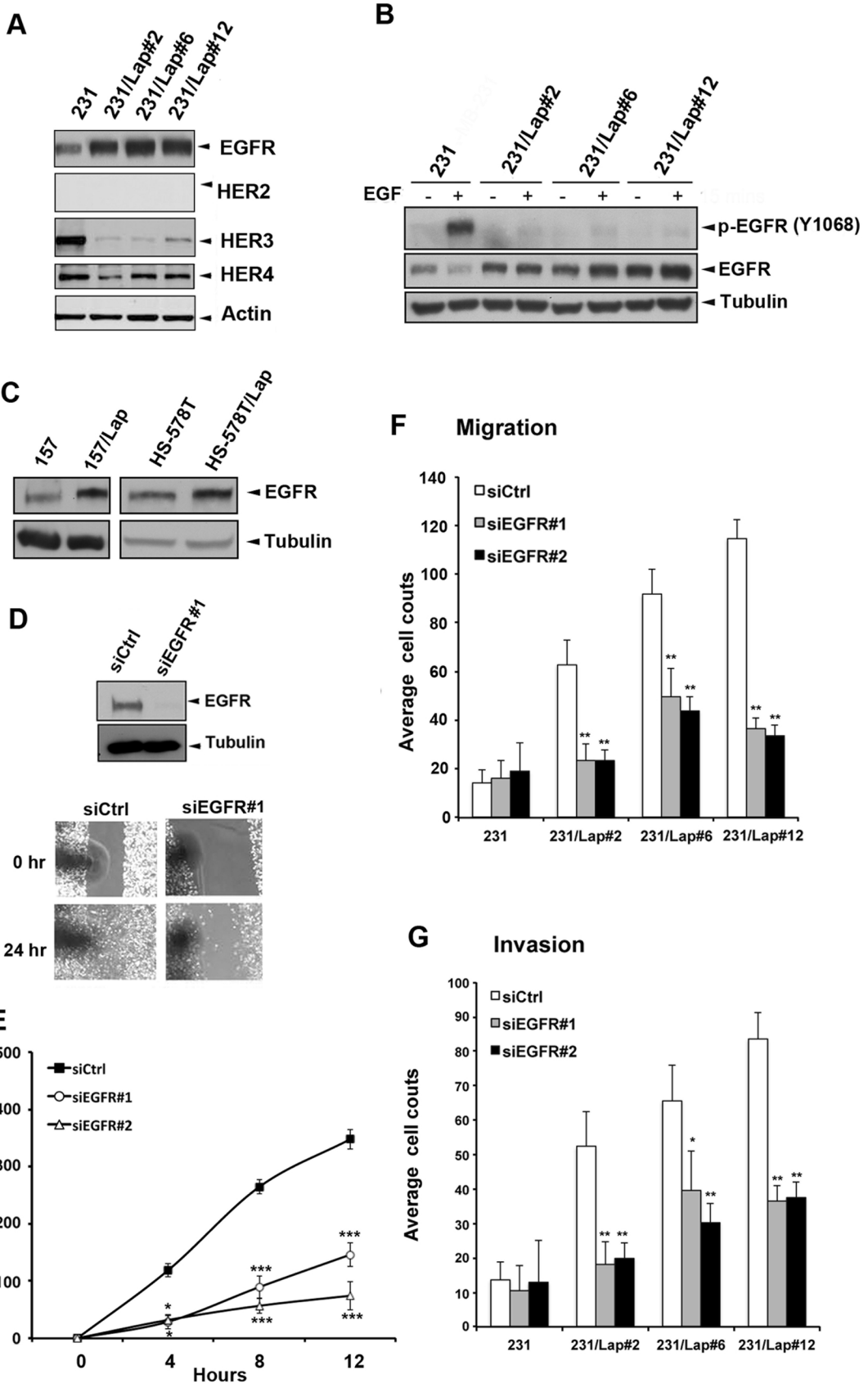

A
231
231/Lap#2
231/Lap#6
231/Lap#12
EGFR
HER2
HER3
HER4
Actin
B
231
231/Lap#2
231/Lap#6
231/Lap#12
EGF
- + - + - + - +
15 mins
p-EGFR (Y1068)
EGFR
Tubulin
C
157
157/Lap
HS-578T
HS-578T/Lap
EGFR
Tubulin
D
siCtrl
siEGFR#1
EGFR
Tubulin
siCtrl
siEGFR#1
0 hr
24 hr
E
Migration Distance (μm)
siCtrl
siEGFR#1
siEGFR#2
Hours
F
Migration
Average cell couts
siCtrl
siEGFR#1
siEGFR#2
231
231/Lap#2
231/Lap#6
231/Lap#12
G
Invasion
Average cell couts
siCtrl
siEGFR#1
siEGFR#2
231
231/Lap#2
231/Lap#6
231/Lap#12

Fig.3

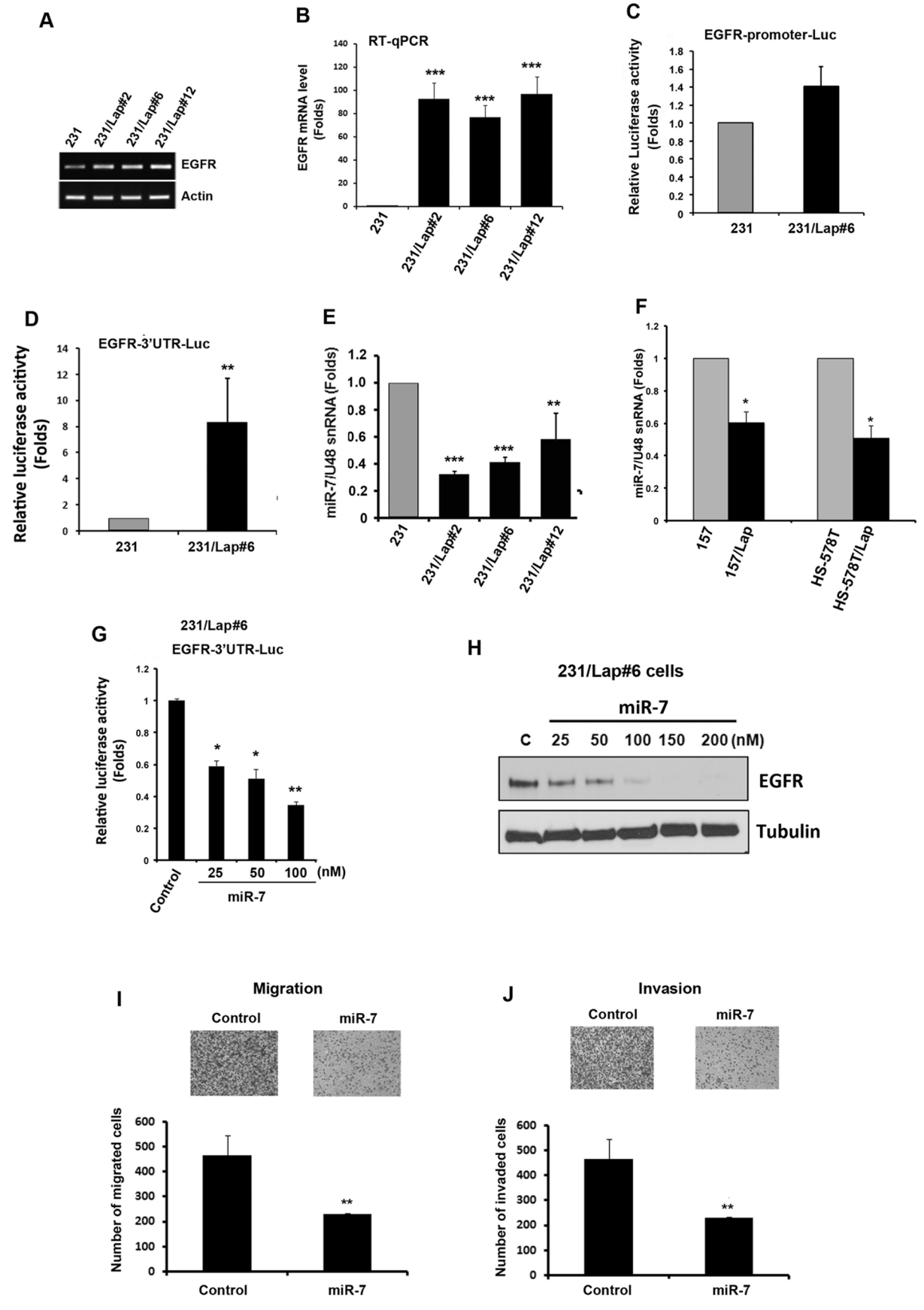
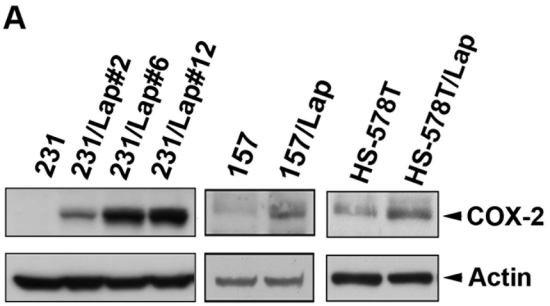
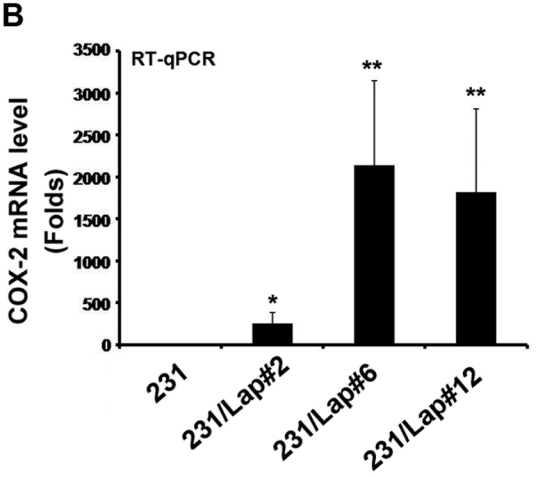
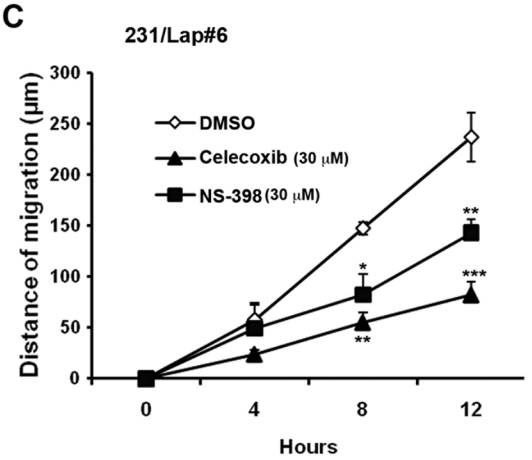
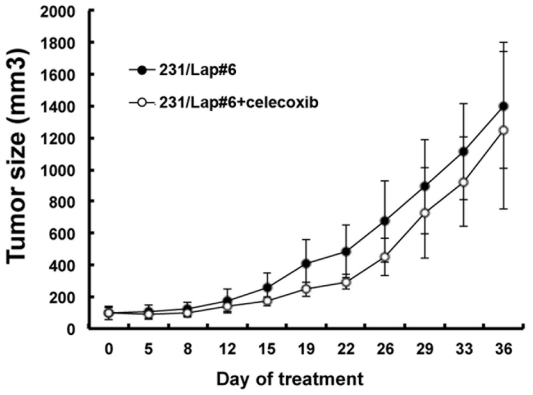

A
231
231/Lap#2
231/Lap#6
231/Lap#12
EGFR
Actin
B
RT-qPCR
EGFR mRNA level (Folds)
231
231/Lap#2
231/Lap#6
231/Lap#12
C
EGFR-promoter-Luc
Relative Luciferase activity (Folds)
231
231/Lap#6
D
EGFR-3'UTR-Luc
Relative luciferase acitivty (Folds)
231
231/Lap#6
E
miR-7/U48 snRNA (Folds)
231
231/Lap#2
231/Lap#6
231/Lap#12
F
miR-7/U48 snRNA (Folds)
157
157/Lap
HS-578T
HS-578T/Lap
G
231/Lap#6
EGFR-3'UTR-Luc
Relative luciferase acitivty (Folds)
Control
25
50
100
(nM)
miR-7
H
231/Lap#6 cells
miR-7
C
25
50
100
150
200
(nM)
EGFR
Tubulin
I
Migration
Control
miR-7
Number of migrated cells
Control
miR-7
J
Invasion
Control
miR-7
Number of invaded cells
Control
miR-7

Fig.4









E

	Lymph node metastsis	Metastasis rate
Vehicle	4/9	44.5%
Celecoxib	1/10	10%

(case /total)

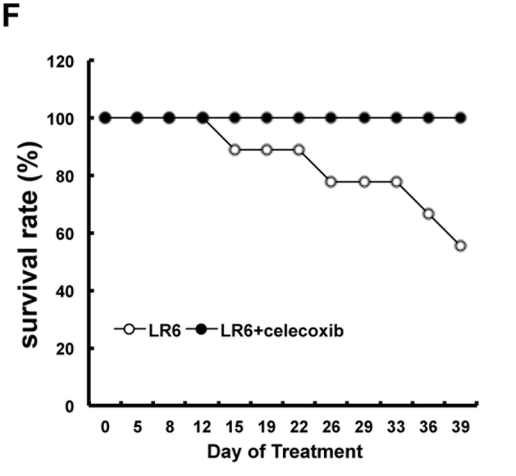


Fig.5

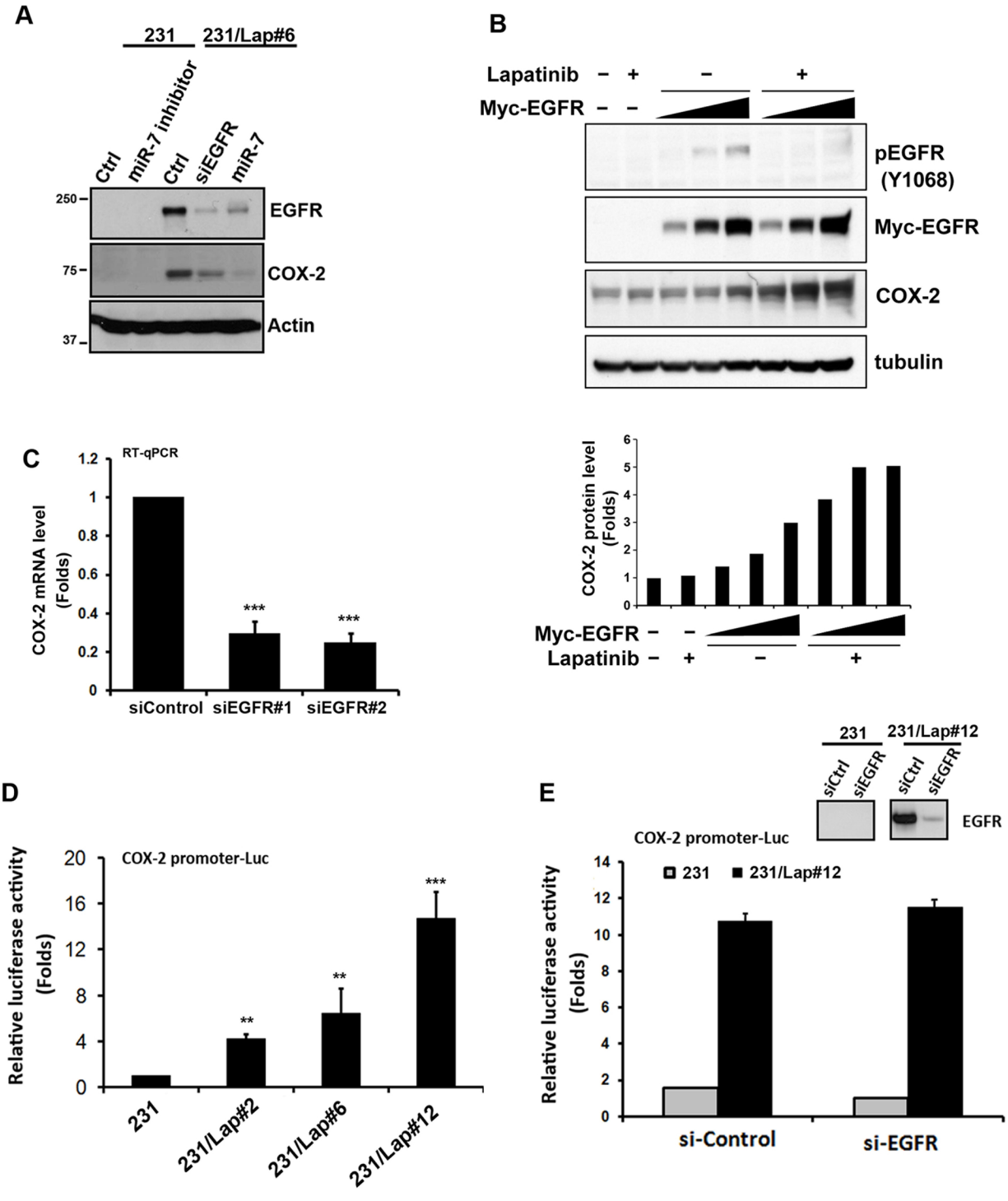
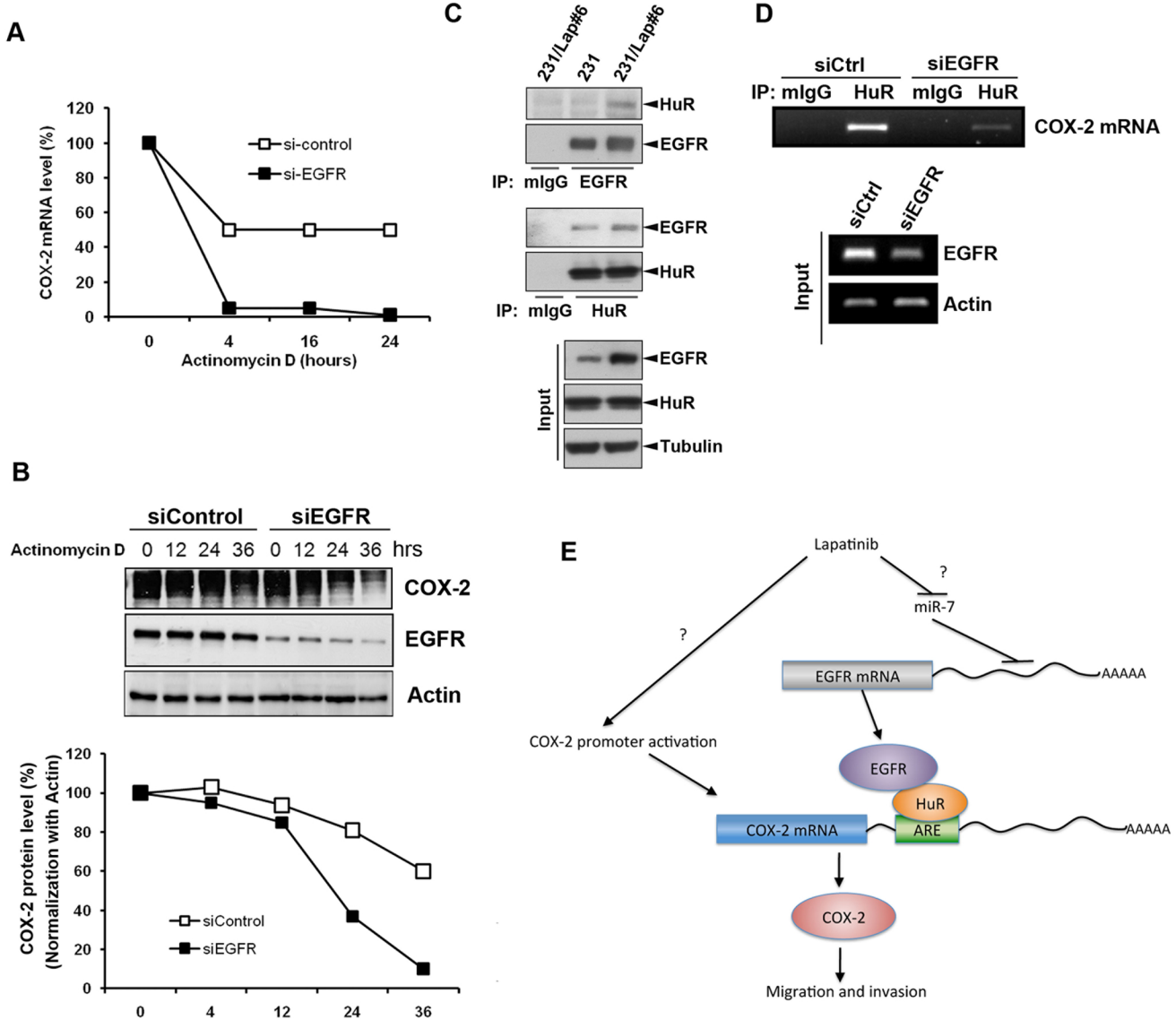

A
231
231/Lap#6
Ctrl
miR-7 inhibitor
Ctrl
siEGFR
miR-7
250
EGFR
75
COX-2
Actin
37
B
Lapatinib
Myc-EGFR
pEGFR (Y1068)
Myc-EGFR
COX-2
tubulin
C
RT-qPCR
COX-2 mRNA level (Folds)
siControl
siEGFR#1
siEGFR#2
COX-2 protein level (Folds)
Myc-EGFR
Lapatinib
D
COX-2 promoter-Luc
Relative luciferase activity (Folds)
231
231/Lap#2
231/Lap#6
231/Lap#12
E
231
231/Lap#12
siCtrl
siEGFR
EGFR
COX-2 promoter-Luc
Relative luciferase activity (Folds)
si-Control
si-EGFR

Fig.6


A
COX-2 mRNA level (%)
si-control
si-EGFR
Actinomycin D (hours)
B
siControl
siEGFR
Actinomycin D 0 12 24 36 0 12 24 36 hrs
COX-2
EGFR
Actin
COX-2 protein level (%) (Normalization with Actin)
siControl
siEGFR
Actinomycin D (hours)
C
231/Lap#6
231
231/Lap#6
HuR
EGFR
IP: mIgG EGFR
IP: mIgG HuR
Input
Tubulin
D
siCtrl
siEGFR
IP: mIgG HuR mIgG HuR
COX-2 mRNA
Input
EGFR
Actin
E
Lapatinib
?
miR-7
EGFR mRNA
AAAAA
COX-2 promoter activation
EGFR
HuR
COX-2 mRNA
ARE
COX-2
Migration and invasion