β2-AR Activation promotes cleavage and nuclear translocation of Her2 and metastatic potential of cancer cells

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Research

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Abstract

Background The prolonged hypersecretion of catecholamine induced by chronic stress may correlate with various steps of malignant progression of cancer and β2-AR overexpressed in certain cancer cells may translate the signals from neuroendocrine system to malignant signals by interacting with oncoproteins such as Her2. Crosstalk of the cell signaling pathways mediated by β2-AR and Her2 may promote a stronger or more sustained biological response. However, the molecular mechanisms underlying cross-communication between β2-AR and Her2 mediated signaling pathways are not fully understood.

Methods In this study, the effects of adrenergic signaling on Her2 cleavage were evaluated by various assays, such as western blot, immunofluorescence and immunohistochemistry. In order to reveal the mechanism about Her2 cleavage triggered by β2-AR activation, the molecular and pharmacological means were employed. By using in vitro and in vivo assay, the influences of the crosstalk between β2-AR and Her2 on the bio-behaviors of tumor cells were demonstrated.

Results Our data demonstrate that catecholamine stimulation activates the expression and proteolytic activity of ADAM10 by modulating the expression of miR-199a-5p and SIRT1 and also confirm that catecholamine induction triggers the activities of γ-secretase, leading to shedding of Her2 ECD by ADAM10 and subsequent intramembranous cleavage of Her2 ICD by presenilin-dependent γ-secretase, nuclear translocation of Her2 ICD and enhanced transcription of tumor metastasis-associated gene COX-2 . Chronic stimulation of catecholamine strongly promotes the invasive activities of cancer cells in vitro and spontaneous tumor lung metastasis in mice. Furthermore, the nuclear localization of Her2 was significantly correlated with overexpression of β2-AR in human breast cancer tissues.

Conclusion This study illustrates that adrenergic signaling activation triggers Her2 cleavage, resulting in enhanced invasive and metastasis activities of cancer cells. Our data also reveal that an unknown mechanism by which the regulated intramembrane proteolysis (RIP) initiated by β2-AR activation controls a novel Her2-mediated signaling transduction under physiological and pathological conditions.

Background

Activation of β2-AR-mediated signaling can amplify multiple receptor-signaling pathways (1–3). Crosstalk of the cell signaling pathways mediated by β2-AR and receptor tyrosine kinases (RTK) may promote a stronger or more sustained biological response in a variety of target tissues (4–7). Our previous study discloses a positive feedback loop comprised by β2-AR and Her2: the chronic stimulation of catecholamine induces β2-AR-mediated up-regulation of Her2 expression, whereas Her2 overexpression promotes autocrine release of catecholamine, leading to elevation of β2-AR level (8). Several lines of evidence implicate that the prolonged hypersecretion of catecholamine induced by chronic stress may correlate with higher occurrence of malignancies in various organs (9–13) and β2-AR overexpressed in certain cancer cells may translate the signals from neuroendocrine system to malignant signals by interacting with oncoproteins such as Her2 (8, 14, 15). However, the molecular mechanisms

underlying cross-communication between β2-AR and Her2 mediated signaling pathways are largely unexplored.

Receptor localization plays a crucial role in gathering of paracrine signals from adjacent cells (16, 17). Unlike epidermal growth factor receptor (EGFR) that constantly shuttles and recycles through the cell, Her2 as a member of EGFR family is a highly internalization-resistant receptor and primarily resides on the plasma membrane of epithelial cells, though its nuclear localization has been documented (18–20). Nuclear translocation of Her2 was proposed to be related to endocytic internalization in a full-length form and mediated by a conventional nuclear importing system associated with the nuclear pore complex (21). However, there was contradictory evidence regarding nuclear localization of Her2 (16, 17). It was also reported that soluble Her2 C-terminal fragment (CTF) synthesized by alternative initiation of translation was located in the nucleus (22). In accordance with this study, we observed that Her2 intracellular domain (ICD) but not full-length Her2 was exclusively distributed in the nucleus (23). Correspondingly, a nuclear localization signal was identified within the sequences of Her2 ICD. It has been known that Her2 extracellular domain (ECD) can be separated proteolytically from full-length Her2 and detected in cultural media of human breast cancer cell line SKBR3 as well as in sera of patients with breast cancer (24). Occurrence of Her2 ECD is a marker for production of the N-terminally truncated and membrane-associated Her2 fragment with a molecular weight of 95 kDa (p95Her2), which possesses constitutive ligand independent activity and enhanced transforming efficiency (25, 26). Elevation of soluble Her2 ECD level in the sera of patients has been correlated with recurrence, nodal metastasis, worse prognosis and poor response to hormone therapy, chemotherapy and targeted therapy in clinical studies (24, 27). However, the molecular mechanisms whereby Her2 is cleaved are poorly understood.

Materials And Methods

Cell culture and treatment

MCF-7, BT474, MDA-453 and SKOV3 cells are obtained from the American Type Culture Collection. The MCF-7/Her2 cells stably overexpressing Her2 were established in our laboratory as described previously (28). For the treatment with β-AR agonists and antagonist, the cells were incubated overnight in a serum-free medium and then treated with 10 μM epinephrine, 10 μM norepinephrine, 5 or 10 μM isoproterenol (ISO), 0.5 or 2 μM ICI-118,551, 1 or 5 μM DAPT or 1 μM ATEN for indicated time point. For the treatment with γ-secretase antagonist, the cells were treated with 0.5 or 2 μM L685458.

Western blot

The whole-cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were probed with the primary antibodies against ADAM10 (Abcam), the C-terminus and N-terminus of Her2 (Cell Signaling), phosphorylated Her2 (Thermo) and phosphorylated Akt, GFP, Presenilin 1, Presenilin 2, Nicastrin, PEN2, COX-2, Na/K-ATPase, Histone H3, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-tubulin (Cell Signaling) and SIRT1 (Santa Cruz).

Bands were visualized by the Enhanced Chemiluminescence System (Amersham Pharmacia Biotech). All experiments were performed in duplicate.

Real-time RT-PCR

Expression of miR-199a-5p in SKOV3 and MDA-453 cells was detected by miRNA expression assay kit (GenePharma). For quantization of COX-2 expression, real-time RT-PCR was performed with the primers 5’-TTCAAATGAGATTGTGGGAAAATTGCT-3’ (sense) and 5’-AGATCATCTCTGCCTGAGTATCTT-3’ (antisense). The results were analyzed using the comparative threshold cycle method with β-actin as an internal control with the primers 5’-GTGGGGCGCCCCAGGCACCA-3’ (sense) and 5’-CTTCCTTAATGTCACGCACGATTTC-3’ (antisense). The experiments were performed three times independently.

In vitro γ-secretase assay

In vitro γ-secretase activities were measured as described previously (29). The cells were resuspended in lysis buffer and lysed by passing through a 30-guage needle attached to a 1 ml syringe. The membrane pellets were incubated at 37 °C for 2 h in 50 µl of assay reaction buffer (pH 6.5) containing 12 µM specific fluorogenic substrate (Calbiochem). The fluorescence was measured using SpectraMax M5 spectrometer (Molecular Devices). The experiments were performed in duplicate.

Immunofluorescence and confocal microscopy

The MCF-7/Her2-GFP cells were treated with 100% methanol at -20 °C for 10 min and then nuclei stained with 1 µg/ml DAPI (Sigma). For immunofluorescent labeling, MCF-7 cells were fixed in 100% methanol at -20 °C for 10 min. After washing and blocking, the primary antibody against the C-terminus of Her2 was applied to the cells for 45 min at 37 °C. Binding was detected with Alexa fluor 549 (Red)-labeled secondary antibody (Invitrogen). Nuclei were stained with DAPI. For detection by using the antibody against the N-terminus of Her2, binding was detected with Alexa fluor 488 (Green)-labeled secondary antibody (Invitrogen). Nuclei were stained with PI. Fluorescence images were collected under a laser scanning confocal microscope (Radiance 2100, BioRad) with Laser-Sharp 2000 software (Bio-Rad). This experiment was repeated at least twice.

Preparation of nuclear extracts and oligonucleotide pulldown assays

The 5’-biotinylated double-stranded oligonucleotides (5’-ATAAACTTCAAATTTCAGTA-3’) corresponding to the positions − 1770 to -1750 of the COX-2 promoter were synthesized by Invitrogen Biotechnology. The same sequences that are not biotinylated were used as the competitors. The biotinylated oligonucleotides containing the mutated Her2 binding sequences (5’-ATAAACTGACCCGGGAAGTA-3’), in which conserved nucleotides were replaced, and the sequences lacking the Her2 binding motif (5’-ATAAACTTCAAATTTCAGTA-3’) were also synthesized. SKOV3 cells were treated with 5 µM of ISO for 0 or

9 h under serum-free conditions after overnight starvation. The nuclear extracts were prepared by using a Nuclear-Cytosol Extraction Kit (Applygen Technologies). 200 μg of the nuclear extracts was incubated at 4 °C for 4 h with each pair of the oligonucleotides previously coupled to Dynabeads M-280 (Invitrogen). The protein/DNA complexes were separated with a Dynal magnet. Binding of Her2 was detected by Western blot with the antibodies against N-terminus or C-terminus of Her2. The experiments were performed in duplicate.

Invasion assay

Cell invasion assay kit (Cell Biolabs) was employed to determine the invasive activities of SKOV3 cells. In brief, SKOV3 cells chronically stimulated by 5 μM ISO or unstimulated cells were resuspended in serum-free medium. 2.5×10^3 cells were transferred to the hydrated Matrigel chambers. After 48 h, noninvasive cells were removed from the upper surface and the invasive cells on the lower surface of the membrane were fixed, stained with Cell Stain provided by the manufacturer and observed under a microscope (Nikon). The experiment was performed in duplicate.

In vivo tumor model

Five to six-week-old athymic female BALB/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. A total of 0.1 ml of the cell suspension (10^8 cells/ml) was injected s.c. in the right upper flank of the mice. After tumor cell injection for four days, the mice were treated daily i.p. with PBS or ISO (10 mg/kg). Each group contained eight mice. Sixty days following tumor implantation, mice were sacrificed. Primary tumors were dissected, weighted and fixed in formalin. The lungs of the mice were autopsied, fixed and photographed.

Immunohistochemistry

Paraffin-embedded tissue sections were dewaxed and gradually hydrated. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide. After antigen retrieval, the sections were incubated with rabbit monoclonal antibodies against the N-terminus and C-terminus of Her2 (Cell signaling) and β2-AR (Abcam). Following washing with PBS, the sections were incubated with horseradish peroxidase-conjugated goat-anti-rabbit antibody. The color was developed by incubation with 3, 3’-diaminobenzidine solution. Staining was assessed microscopically by two independent pathologists. Photomicrographs were taken with an Olympus microscope. Omission of the primary antibody and substitution by non-specific rabbit IgG at the same concentration were used as negative controls.

Clinical samples

All clinical tissue samples were obtained from the Affiliated Hospital of Xuzhou Medical University with the informed consent of patients and with approval for experiments from the Hospital.

Statistical analysis

Data were expressed as mean ± SD. For comparisons among the groups in the experiments, an ANOVA test was performed. For evaluation of consistency between β2-AR expression and Her2 nuclear localization in the tumor tissues, Kappa coefficients were calculated. $P < 0.05$ was considered statistically significant.

Results

Catecholamine stimulation induces cleavage and phosphorylation of Her2

When we treated human breast cancer cell line BT474 with β-AR agonist isoproterenol (ISO), an extra ~ 80 kDa molecular mass was accumulated in a time-dependent manner as detected by Western blot with the antibody against the C-terminus of Her2 (Fig. 1a). Similar result was also obtained in human ovarian cancer cell line SKOV3 (Fig. 1b), suggesting that the 80 kDa fragment may represent Her2 CTF and appearance of the 80 kDa Her2 fragment (p80Her2) was associated with ISO stimulation. To determine whether the generation of p80Her2 is mediated by activation of β-AR, we used β-AR activators including ISO (5 μM) and naturally occurring catecholamines, epinephrine (10 μM) and norepinephrine (10 μM), to treat human breast cancer cell line MCF-7. As shown in Fig. 1c, both full-length Her2 and p80Her2 were remarkably increased. Pretreatment with the specific inhibitor of β2-AR ICI 118,551 (1 μM) strikingly impaired the effect of ISO on the formation of p80Her2, whereas the specific inhibitor of β1-AR ATEN (1 μM) had only marginal effect (Fig. 1d), indicating that activation of β2-AR is a prerequisite for the generation of p80Her2.

P95Her2 was assumed to contain the transmembrane and cytoplasmic domains (24). To clarify whether p80Her2 derives from proteolysis or alternative initiation of translation, we constructed MCF-7 cells expressing Her2-GFP fusion protein (MCF-7/Her2-GFP). Western blot analysis with the antibody against GFP showed that a new product, whose size perfectly fits the molecular weight of p80Her2-GFP fusion protein (~ 100 kDa), appeared after exposure of the cells to ISO (Fig. 1e), testifying that the p80Her2 fragment is a product cleaved from full-length Her2. We examined the soluble Her2 ECD in the cultural supernatant of SKOV3 cells by using the antibody against the N-terminus of Her2. Concomitant with markedly increased p80Her2 in the whole cell lysates, an about 100 kDa protein was clearly detected in the cultural supernatant of the cells (Fig. 1f). Notably, p80Her2 was apparently smaller than the membrane-associated p95Her2. We hypothesized that p95Her2 may undergo the secondary cleavage. Moreover, the phosphorylation of both full-length Her2 and p80Her2 was prominently enhanced in a time-dependent manner, indicating a rapid posttranslational modification of Her2 following ISO stimulation (Fig. 1g).

Catecholamine modulates the cleavage of Her2 ECD by promoting ADAM10 expression through downregulation of

miR-199a-5p and upregulation of SIRT1

The ectodomain cleavage of the transmembrane proteins is generally mediated by membrane-associated metalloproteases under the regulation of multiple signaling pathways such as the activation of PKC (30, 31). Earlier studies showed that Her2 ECD shedding could be suppressed by the broad-spectrum metalloprotease inhibitors TAPI, batimastat and the tissue inhibitor of metalloproteases-1 (24). A previous study by utilizing the siRNAs selectively inhibiting ADAM10 expression suggested that ADAM10 may be one of the proteases responsible for Her2 cleavage (32). However, shedding of Her2 is inefficient in contrast to the majority of shedding events. In addition, how the sheddase is controlled under physiological conditions is unclear. To test whether ADAM10 is engaged in catecholamine-induced Her2 ECD cleavage, we examined the expression of ADAM10 in MCF-7 cells stably transfected with Her2 expression plasmid (MCF-7/Her2). We found that ISO stimulation induced a significant upregulation of ADAM10 expression, which was obviously coherent with the accumulation of p80Her2 (Fig. 2a). A recent study indicates that the NAD-dependent deacetylase SIRT1 regulates the transcription of the gene encoding ADAM10 by direct interaction with ADAM10 promoter (33). The data in Fig. 2b showed that epinephrine stimulation dramatically promoted the expression of SIRT1 in a time-dependent manner in MDA453 and SKOV3 cells. SIRT1 was recently identified as a direct target of miR-199a-5p (34). Interestingly, the expression of miR-199a-5p was strikingly repressed in SKOV3 and MDA453 cells treated with epinephrine as determined by real-time RT-PCR analysis (Fig. 2c). The experimental study has demonstrated that β2-AR can activate an antiapoptotic signal through Gi-dependent coupling to phosphatidylinositol 3'-kinase/Akt pathway and the activated Akt is sufficient for inducing downregulation of miR-199a-5p in cardiac myocytes (35). We noticed that the phosphorylation of Akt was significantly enhanced by epinephrine stimulation, accompanied with reduction of miR-199a-5p in MDA453 and SKOV3 cells (Fig. 2b and 2c). Furthermore, the epinephrine-induced shedding of Her2 by ADAM10 was completely inhibited by ADAM10 specific siRNA (Fig. 2d), verifying that catecholamine modulates the cleavage of Her2 ECD by promoting ADAM10 expression through downregulation of miR-199a-5p and thus upregulation of SIRT1. It has been known that the stimulation of β2-AR with the agonists leads to the shedding of heparin-binding EGF-like growth factor by ADAM17, which is also the major sheddase for Her3 and Her4 (36, 37), and subsequent activation of EGFR in an autocrine/paracrine manner (4, 5). The findings in this study unambiguously demonstrate that ADAM10 activities induced by catecholamine stimulation mediate cleavage and activation of Her2 tyrosine kinase, indicating that transactivation of Her2 is mediated by β2-AR through a different mechanism.

γ-secretase activity induced by catecholamine-stimulation is responsible for the generation of p80Her2 ICD

Generation of CTFs from a transmembrane receptor involves cleavage within the transmembrane domain by γ-secretase-catalyzed proteolytic processing, whereas the activity of γ-secretase is proposed to be regulated by the ADAM-mediated ECD cleavage of transmembrane receptor (38). Soluble Her4 ICD is produced by the sequential activities of ADAM-17 and γ-secretase after binding of its ligand heregulin or

activation of protein kinase C (PKC) by 12-O-tetradecanoylphorbol-13-acetate (39). However, proteolytic cleavage of Her2 by γ-secretase has not been reported. As mentioned previously, p80Her2, the product of Her2 cleavage triggered by catecholamine stimulation is somehow smaller than p95Her2. It was recently found that activation of β2-AR provoked γ-secretase activity in HEK293 cells (40). We speculated that the membrane-associated Her2 ICD may undergo the secondary cleavage after shedding of Her2 ECD, possibly by γ-secretase. The expression of γ-secretase components, presenilin 1 (PS1), PS2, nicastrin and PEN-2 was present in MCF-7, MCF-7/Her2 and SKOV3 cells (Fig. 3a). When we treated MCF-7 and SKOV3 cells with ISO, γ-secretase activities, determined by fluorogenic substrate assay, were prominently elevated in both cell lines (Fig. 3b and 3c). In comparison with the known γ-secretase substrates CD44, Notch and E-cadherin for homology of the amino acid sequences of the transmembrane domain Her2 dose not share high homology with these sequences. However, these transmembrane domains including that of Her2 frequently harbor several valine residues, some of which have been identified as the potential γ-secretase cleavage sites (Fig. 3d). To investigate the role of γ-secretase in Her2 intramembranous processing, we utilized the selective γ-secretase inhibitor L685,458 and a dipeptidic γ-secretase specific inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT). Pretreatment with L685,458 or DAPT significantly blocked the production of p80Her2 in a concentration-dependent manner (Fig. 3e). The polytopic membrane proteins PS1 and PS2 are suggested to be the catalytic components of an active γ-secretase complex. We employed the specific siRNAs targeting PS1 and PS2 to transfect SKOV3 cells. Knock-down of either PS1 or PS2 alone or both extraordinarily prevented the formation of p80Her2 (Fig. 3f and Fig. S1), demonstrating that γ-secretase activity induced by catecholamine-stimulation is responsible for the generation of p80Her2 ICD.

Catecholamine stimulation mediates nuclear translocation of Her2 ICD efficiently

Nuclear translocation of Her2 as a full-length molecule was investigated in certain cell lines (18). In contrast to other Her family members, the nuclear entry of Her2 was much less efficient. The regulatory mechanisms of nuclear translocation of full-length Her2 receptor under physiological conditions remain elusive. To illustrate the subcellular distribution of Her2, we traced the intracellular trafficking of GFP-tagged Her2 after treating MCF-7/Her2-GFP cells with ISO. In unstimulated cells ectopic overexpressed Her2-GFP was defined at the cytoplasmic membrane (Fig. 4a). No evidence for Her2-GFP in the nucleus was found. However, in the presence of ISO, nuclear Her2 was readily visualized (Fig. 4b). In addition, Her2-GFP proteins were also observed in the intracellular membrane systems. By immunofluorescence using the monoclonal antibodies against both N- and C-terminus of Her2 we observed that Her2 molecules were predominantly located at the cytoplasmic membrane and no substantial nuclear Her2 could be detected with either antibody in unstimulated SKBR3 breast cancer cells (Fig. 4b and 4c). The data are consistent with several previous observations that Her2 did not localize in the nucleus of several breast cancer cell lines spontaneously (16). However, after treatment with ISO for 60–90 min, Her2 was massively migrated into the nuclei (Fig. 4b). Notably, nuclear Her2 could be easily detected by the antibody against the C-terminus of Her2 (Fig. 4b) but not by the antibody against the N-terminus of Her2

(Fig. 4c). Consistent with these data, p80Her2 was detected in the nuclei of ISO-simulated SKOV3 cells by cellular fractionation and Western blot (Fig. 4d) but not in the unstimulated cells, suggesting that it was Her2 ICD liberated by γ-secretase cleavage that entered the nuclei. Moreover, the features for uniform distribution of nuclear Her2 was distinguishingly different from those in the previous observation that Her2 in the nuclear appeared as discrete punctate spots under conventional culture conditions. Altogether, these results indicate that catecholamine stimulation mediates nuclear translocation of Her2 ICD efficiently.

Her2 ICD physically binds to the promoter of COX2 gene and drives transactivation of COX2 gene

Sequential cleavage of transmembrane receptors can rapidly transform membrane-associated proteins into soluble effectors, which enter the nucleus and regulate the transcription of their target genes (31). To determine the functional significance of p80Her2 in the nucleus, we isolated the nuclear proteins from SKOV3 cells and performed oligonucleotide pull-down assay. A previous study identified the Her2-associated sequence (HAS), which was located at 1750 nucleotides upstream from the transcriptional initiation site in COX-2, a known target gene of Her2 (18). We utilized the sequence as an oligonucleotide probe. By oligonucleotide pull-down assay we could reproducibly detect the association of p80Her2, but not p185Her2, with the oligonucleotide probes containing the HAS sequence in ISO-simulated cells. Identical Western blot with the mutated HAS sequence or nonspecific oligonucleotides exhibited no specific signal (Fig. 5a). In addition, the binding of p80Her2 with the HAS sequence was strongly impaired or utterly abolished by the specific competitors in a dose-dependent manner. Furthermore, the expression of COX-2 at both mRNA and protein levels was remarkably upregulated by ISO stimulation (Fig. 5b and 5c). These data provide further evidence to confirm that under catecholamine stimulation Her2 ICD migrates into the nucleus, physically binds to the promoter of COX2 gene and drives transactivation of COX2 gene.

Catecholamine stimulation strongly promotes the invasive activities of cancer cells in vitro and spontaneous tumor lung metastasis in mice

In an effort to determine the effects of catecholamine stimulation on the biological behaviors of tumor cells and relevance of Her2 nuclear localization in tumor development and metastasis, we investigated whether catecholamine stimulation confers proliferation and invasion potential to Her2-overexpressing tumor cells in a human ovarian cancer xenograft model. The treatment with ISO daily did not accelerate tumor growth in mice, compared with the control group (Fig. S2). However, ISO stimulation significantly promoted the invasive capacity of SKOV3 cells in in vitro invasion assay using Matrigel invasion chamber and caused spontaneous lung metastasis in nude mice (Fig. 6a, 6b and Fig. S3). Metastatic

colonization was observed by gross examination and microscopic inspection of tissue sections (Fig. 6b and Fig. S3). Surprisingly, the nuclear staining of Her2 was mainly observed in the metastatic tumor tissues by immunohistochemical labeling with the antibody against the C-terminus of Her2 (Fig. 6c), whereas nuclear Her2 was rarely seen in the primary tumors (Fig. S4). Moreover, using the antibody against the N-terminus of Her2 nuclear Her2 could not be detected, but only membrane-anchored Her2 was signaled (Fig. 6c).

To gain further insights into correlation of Her2 nuclear localization with β2-AR activation, we examined the expression of both Her2 and β2-AR in 55 Her2 positive human breast cancer tissues. In the tissues expressing relatively low level of β2-AR, Her2 molecules were predominantly distributed at the cytoplasmic membrane (Fig. 6d, case 1). Nevertheless, in the tissues expressing high level of β2-AR, nuclear Her2 was strongly positive (Fig. 6d, case 2). In 62.6% of the tissues with strong immunoreactivity for anti-β2-AR antibody (20/32) nuclear Her2 was positive. Only 13% of tissues expressing low level of β2-AR (3/23) displayed positive staining of nuclear Her2 (Table S1). The difference between two groups was highly significant ($p < 0.0002$). However, the kappa coefficient for β2-AR expression and Her2 nuclear localization was moderate (0.46), suggesting that other molecular mechanisms may be involved in the nuclear translocation of Her2. Simultaneous staining of β2-AR and nuclear Her2 was also observed in human ovarian cancer tissues (Fig. S5). The data demonstrate that nuclear localization of Her2 is intimately associated with the overexpression of β2-AR, activation of β2-AR-mediated signaling pathway and breast cancer metastasis.

Discussion

The present study demonstrates that catecholamine-induced β2-AR activation triggers shedding of Her2 ECD by ADAM10 and subsequent intramembranous cleavage of Her2 ICD by presenilin-dependent γ-secretase, resulting in nuclear translocation of p80Her2 and enhanced transcription of target genes (Fig. 7). The regulated intramembrane proteolysis (RIP) has been highlighted as a novel mode of proteolysis-dependent signal transduction. It is known that RIP is well-controlled process and induced by specific agonists and intracellular signaling pathways (41, 42). The ADAMs as key sheddases for transmembrane receptors are now emerging as signaling scissors in diverse signal transduction pathways. The expression of ADAMs is not constitutive but highly inducible and sensitive to the stimuli from microenvironment. Many of ADAM substrates involved in signaling events are dysregulated in cancers or during tumor progression (31, 43). Our data confirm that catecholamine stimulation activates the expression and proteolytic activity of ADAM10 by modulating the expression of miR-199a-5p and SIRT1 and that catecholamine induction triggers the activities of γ-secretase, demonstrating that catecholamine-induced β2-AR activation play decisive roles in cleavage and nuclear translocation of Her2 under physiological and pathological conditions.

Elevated p95Her2 level was considered to be an independent prognostic factor in breast cancer and p95Her2 found to associate with Her3 exclusively. Therapeutic antibody Herceptin did not block p95Her2 activation and its downstream signaling (24). Our study shows that catecholamine stimulation strongly

promoted the invasive activities of cancer cells in vitro and spontaneous tumor lung metastasis in mice. We noticed that the nuclear localization of Her2 was conspicuous in metastatic lung tissues. Furthermore, overexpression of β2-AR significantly correlated with Her2 nuclear localization in human breast caner tissues, implicating clinical significance of the crosstalk between β2-AR and Her2. Interestingly, a recent phase II clinical trial showed that β-blockade with propranolol reduces biomarkers of metastasis in breast cancer (44). It is conceivable that ADAM10 and γ-secretase shedding of Her2, p95Her2/Her3 heterodimer formation, subsequent activation of the PI3K-AKT pathway, nuclear translocation of Her2 ICD and ultimate transactivation of tumor metastasis-associated genes may account for the resistance to targeted therapy and metastatic progression in breast cancer. Nuclear Her2 and β2-AR level may be important markers for the progression and metastasis of Her2 overexpressing cancers.

The potential role of psychosocial stress in malignancy has been postulated long ago. A number of studies have provided compelling evidence for the significance of the neuroendocrine system in development, progression and metastasis of a variety of cancers. The recent findings from laboratory and clinical investigations pointed out the role of β2-AR in the development of breast, ovarian and gastric cancers. Neurotransmitters as an important determinant can directly affect the biological behaviors of cancer cells through β2-AR-mediated signaling pathway (10). Our previous study and other studies indicate that chronic stimulation by catecholamine triggers stronger mitogenic effect and metastatic capability in cancer cells, implicating that neuroendocrine and sympathetic nervous systems execute important functions in tumor progression (8, 10, 45–48). The present study elucidates the association between catecholamine-induced β2-AR activation, cleavage of Her2 and invasive/metastatic capacity of tumor cells, reveals a novel molecular mechanism for induction of ADAM10 and γ-secretase, generation and nuclear translocation of p80Her2 and reiterates a potential role of stress-related catecholamine release and persistent activation of β2-AR in breast and ovarian cancer invasion and metastasis. As Her2 is a flagship molecular target of modern oncology (19, 49), understanding the signaling characteristics of crosstalk between β2-AR and oncoproteins, such as Her2, may shed a new light on complex signaling network of human malignancy and improve the current paradigm of personalized cancer therapy.

Conclusions

In the present study, we demonstrate that catecholamine-induced β2-AR activation triggers shedding of Her2 ECD by ADAM10 and subsequent intramembranous cleavage of Her2 ICD by presenilin-dependent γ-secretase, resulting in nuclear translocation of Her2 ICD and enhanced transcription of tumor metastasis-associated gene COX-2. Catecholamine stimulation strongly promotes the invasive activities of cancer cells in vitro and spontaneous tumor lung metastasis in mice. Furthermore, the nuclear localization of Her2 was significantly correlated with overexpression of β2-AR in human breast caner tissues, indicating that catecholamine-induced β2-AR activation play decisive roles in tumor progression and metastasis.

Abbreviations

β2-AR: β2-adrenergic receptor; ADAM10: A Disintegrin And Metalloprotease 10; ECD: Extracellular domain; ICD: Intracellular domain; RIP: Regulated intramembrane proteolysis; RTK: Receptor tyrosine kinases; EGFR: Epidermal growth factor receptor; CTF: C-terminal fragment; p95Her2: Her2 fragment with a molecular weight of 95 kDa; ISO: Isoproterenol; PCR: Polymerase chain reaction; RT-PCR: Reverse transcription-PCR; PBS: Phosphate buffered saline; IgG: Immunoglobulin G; GFP: Green fluorescent protein; DAPT: N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester

Declarations

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Authors' contributions

DL, JZ and MS provided the idea and designed the study. DL, XX, SL, XZ, AT, LZ and WM performed the experiments. DL, XX, SL, performed data analysis. DL and MS drafted the manuscript. All authors reviewed the manuscript and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

This study was approved by the ethical committees of Xuzhou Medical University. All clinical tissue samples were obtained from the Affiliated Hospital of Xuzhou Medical University with the informed consent of patients and with approval for experiments from the Hospital.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Figures

Figure 1

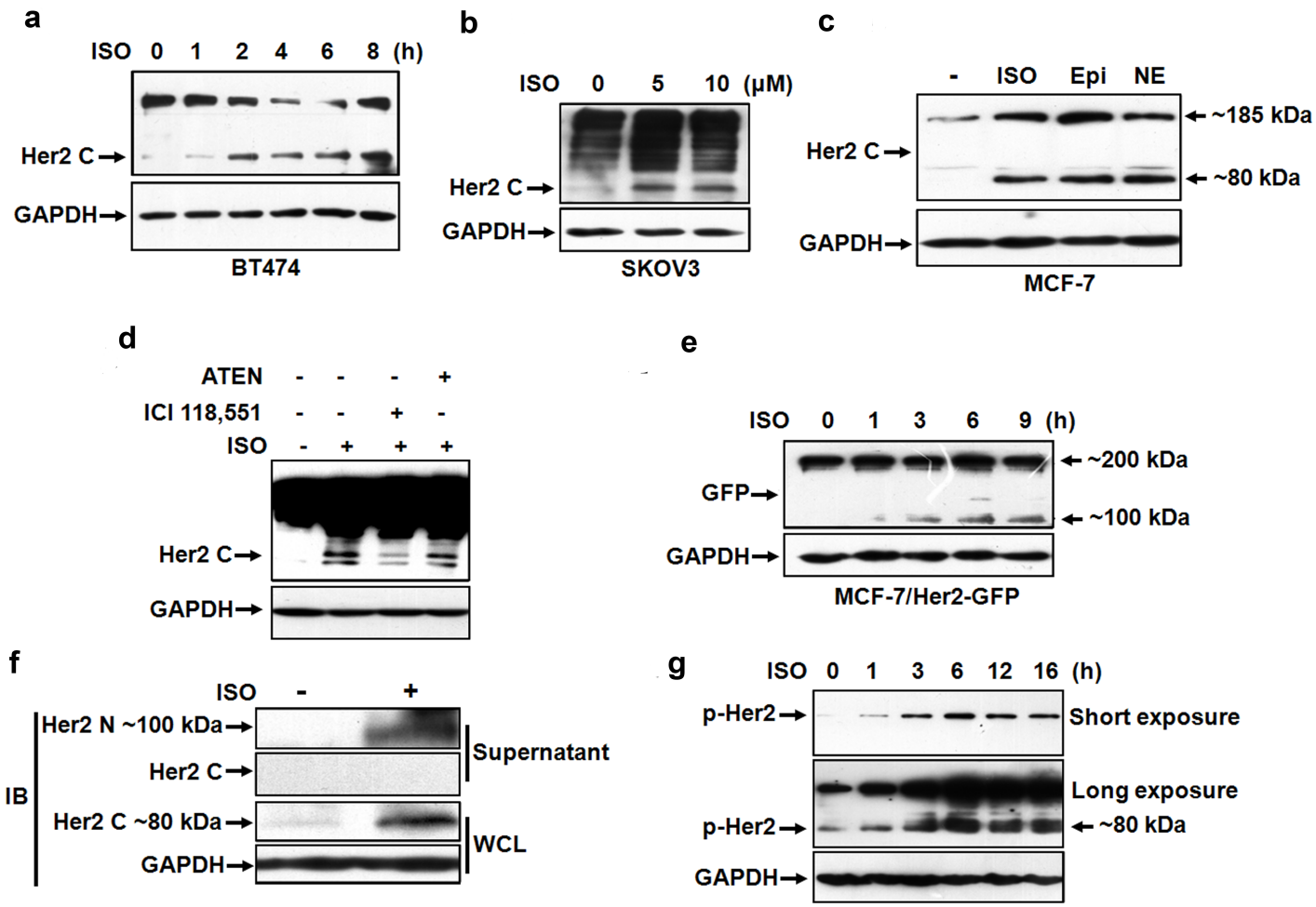

Figure 1

Catecholamine stimulation induces cleavage and phosphorylation of Her2. a BT474 cells were starved overnight and then treated with 5 μM ISO for 0, 1, 2, 4, 6 or 8 h. The expression of Her2 was analyzed by Western blot with the antibodies against the C-terminus of Her2. Equal loading was verified by detection of GAPDH. b SKOV3 cells were treated with 0, 5 or 10 μM ISO for 6 h. c MCF-7 cells were treated with 5 μM ISO, 10 μM epinephrine or 10 μM norepinephrine. d MCF-7 cells were pretreated with 1 μM ICI-118,551 or 1 μM ATEN for 1 h and then incubated with 5 μM ISO. e MCF-7/Her2-GFP cells were treated with 5 μM ISO for 0, 1, 3, 6 or 9 h. Western blot was performed by using the antibody against GFP. f The supernatant and whole cell lysates of SKOV3 cells treated with 5 μM ISO were collected and the fragments of Her2 detected by the antibodies against the N-terminus and C-terminus of Her2. g SKOV3 cells were treated with ISO for 0, 1, 2, 6, 12 or 16 h. Phosphorylation of Her2 was detected by Western blot.

Figure 2

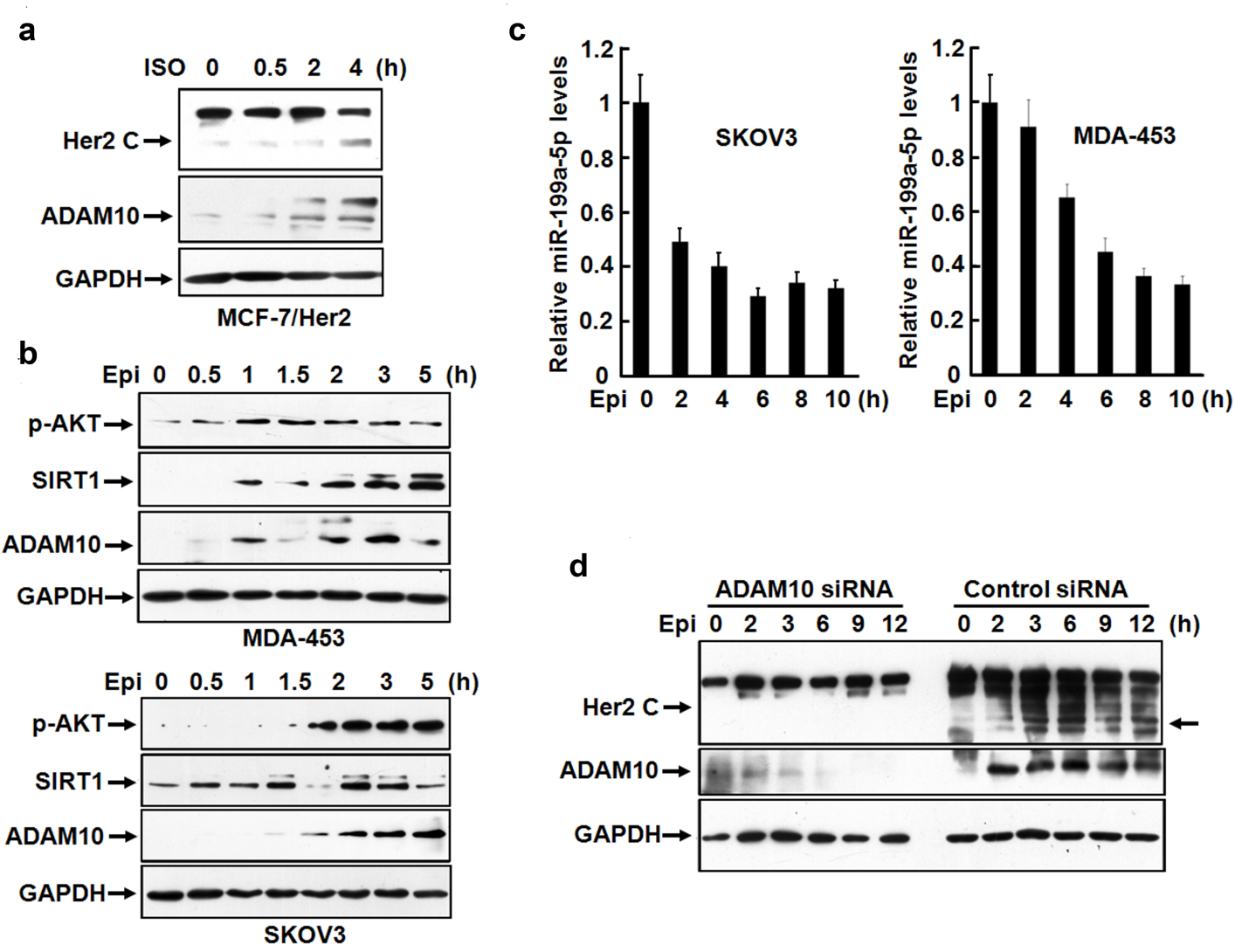

Figure 2

Catecholamine induces the cleavage of Her2 ECD by promoting ADAM10 expression. a MCF-7/Her2 cells were treated with 5 μM ISO for 0, 0.5, 2 or 4 h. The expression of Her2 and ADAM10 was analyzed by

Western blot with the antibodies against the C-terminus of Her2 and ADAM10. b MDA-453 and SKOV3 cells were treated with 10 μM of epinephrine for 0, 0.5, 1, 1.5, 2, 3, 5 or 7 h. Phosphorylated Akt, SIRT1 and ADAM10 were analyzed by Western blot. c SKOV3 and MDA453 cells were treated with 10 μM of epinephrine for 0, 2, 4, 6, 8 or 10 h. The expression of miR-199a-5p was analyzed by real-time PCR. d SKOV3 cells were transfected with the specific siRNA targeting ADAM10 or control siRNA. After transfection for 48 h, the cells were treated with 10 μM of epinephrine for 0, 2, 3, 6, 9 or 12 h and the expression of Her2 and ADAM10 was detected by Western blot with the antibodies against the C-terminus of Her2 and ADAM10.

Figure 3

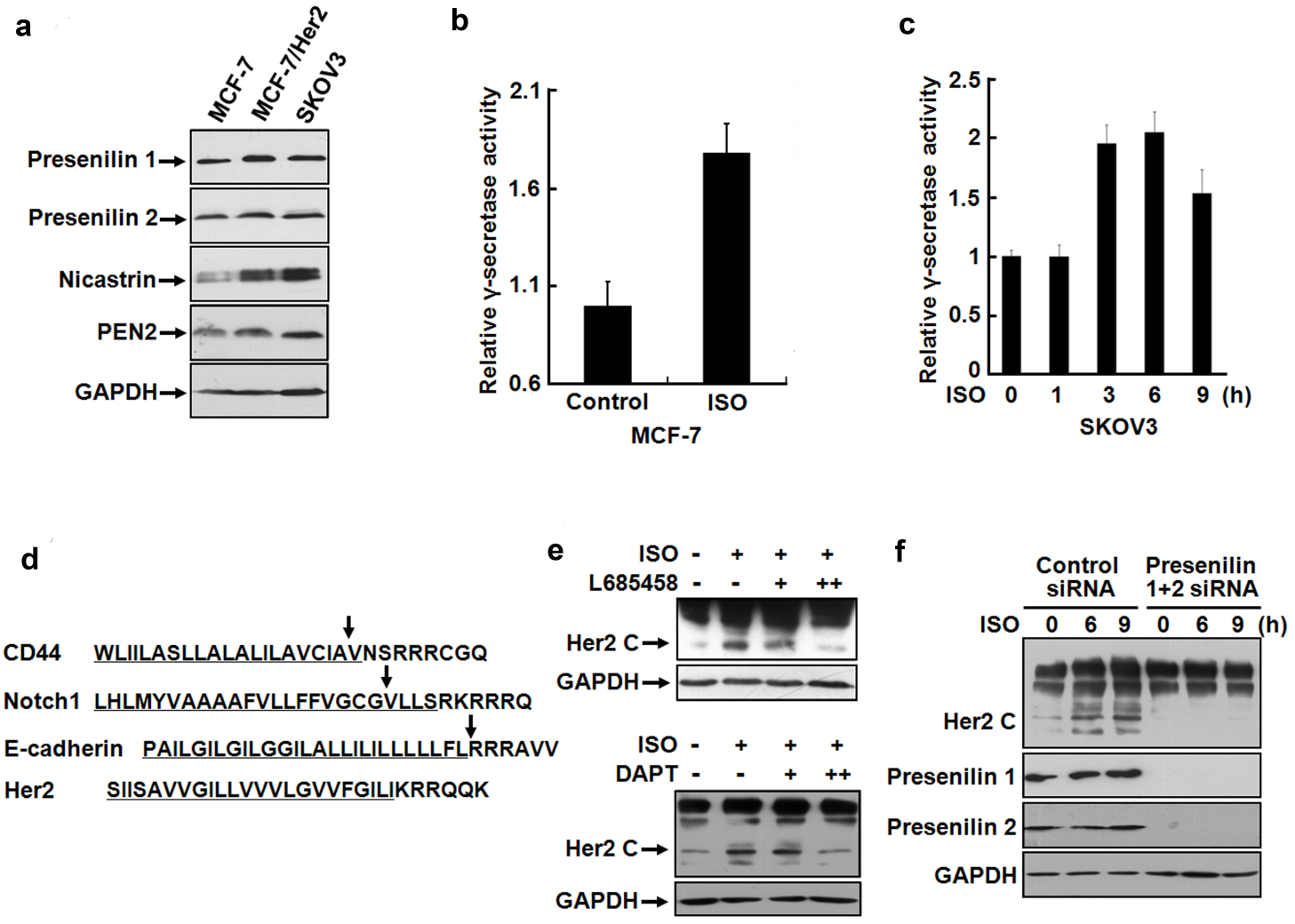

Figure 3

γ-secretase activity induced by catecholamine-stimulation is responsible for the generation of p80Her2 ICD. a The expression of presenilin 1, presenilin 2, nicastrin and PEN-2 in MCF-7, MCF-7/Her2 and SKOV3 cells was analyzed by Western blot. b The cell lysates from MCF-7 cells treated with 5 μM ISO were prepared and γ-secretase activities measured. c SKOV3 cells were treated with 5 μM of ISO for 0, 1, 3, 6 or 9 h. The activities of γ-secretase were measured. d Comparison the known γ-secretase substrates CD44, Notch and E-cadherin with Her2 for homology of the amino acid sequences of the transmembrane

domain. e SKOV3 cells were pretreated with 0.5 and 2 μM L685458 or 1 and 5 μM DAPT and then incubated with 5 μM ISO. The expression of Her2 was analyzed by Western blot. f SKOV3 cells were transfected with the siRNA targeting presenilin 1 and presenilin 2. After transfection for 48 h, the cells were treated with 5 μM ISO for 0, 6 or 9 h and the expression of Her2, presenilin 1 and presenilin 2 was detected by Western blot.

Figure 4

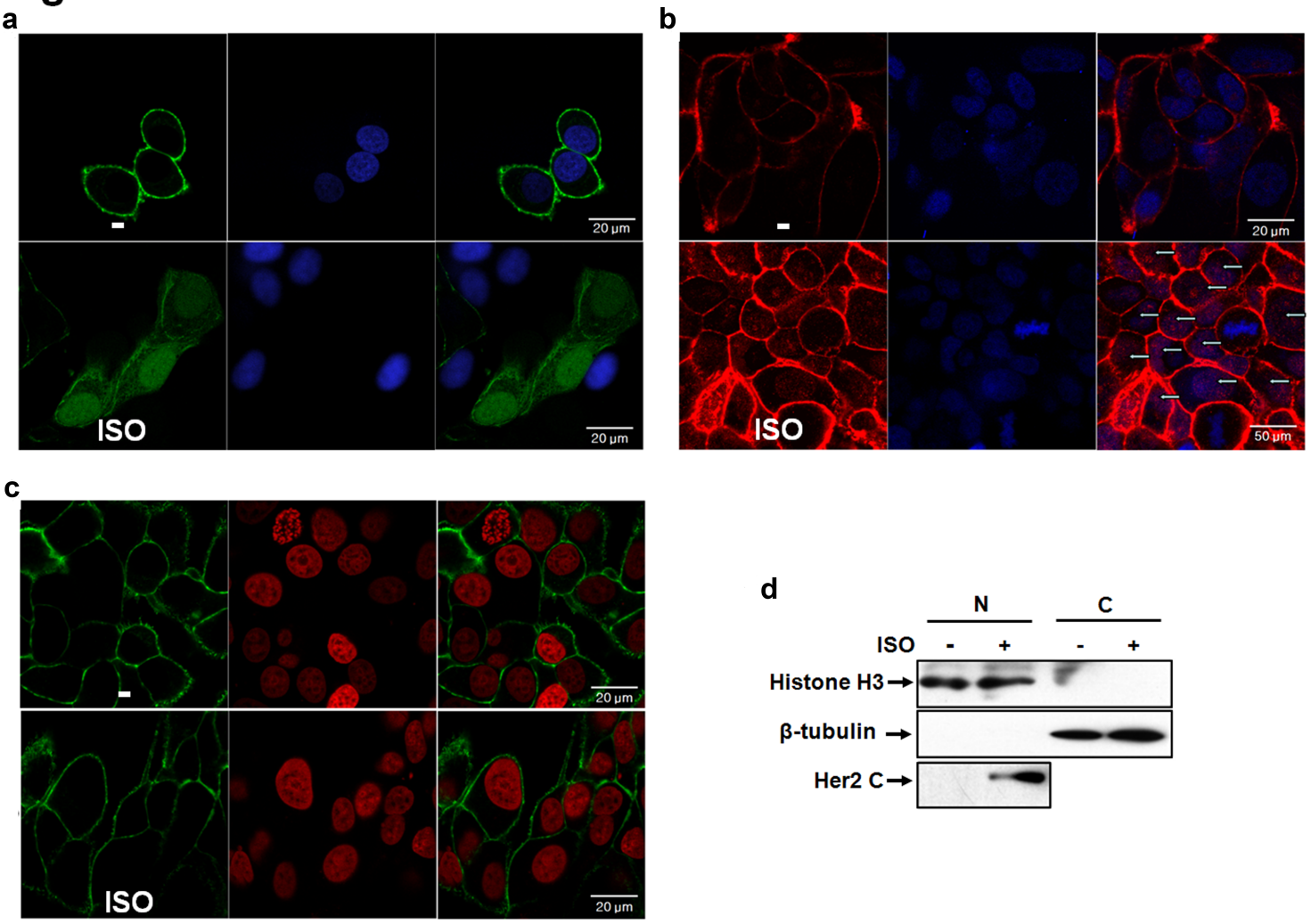

Figure 4

Catecholamine stimulation mediates nuclear translocation of Her2 ICD. a MCF-7/Her2-GFP cells were treated with 5 μM of ISO. The subcellular localization of Her2-GFP fusion protein was observed under a confocal microscope. The nuclei were stained with 1 μg/ml DAPI. Bar = 20 μm b MCF-7 cells were labeled with the antibody against the C-terminus of Her2. Binding was detected with Alexa fluor 549 (Red)-labeled secondary antibody. Nuclei were stained with DAPI. Bar = 20 μm (upper panel) or 50 μm (lower panel) c The MCF-7 cells were incubated with the antibody against N-terminus of Her2 and binding was detected with Alexa fluor 488 (Green)-labeled secondary antibody. Nuclei were stained with PI. Bar = 20 μm d SKOV3 cells were treated with 5 μM of ISO for 0 or 9 h after overnight starvation. The nuclear extracts were prepared using a Nuclear-Cytosol Extraction Kit. The expression of Her2 was analyzed by the

antibody against the C-terminus of Her2. Detection of histone H3 and β-tubulin was used as the indicators of nuclear and cytoplasmic proteins.

Figure 5

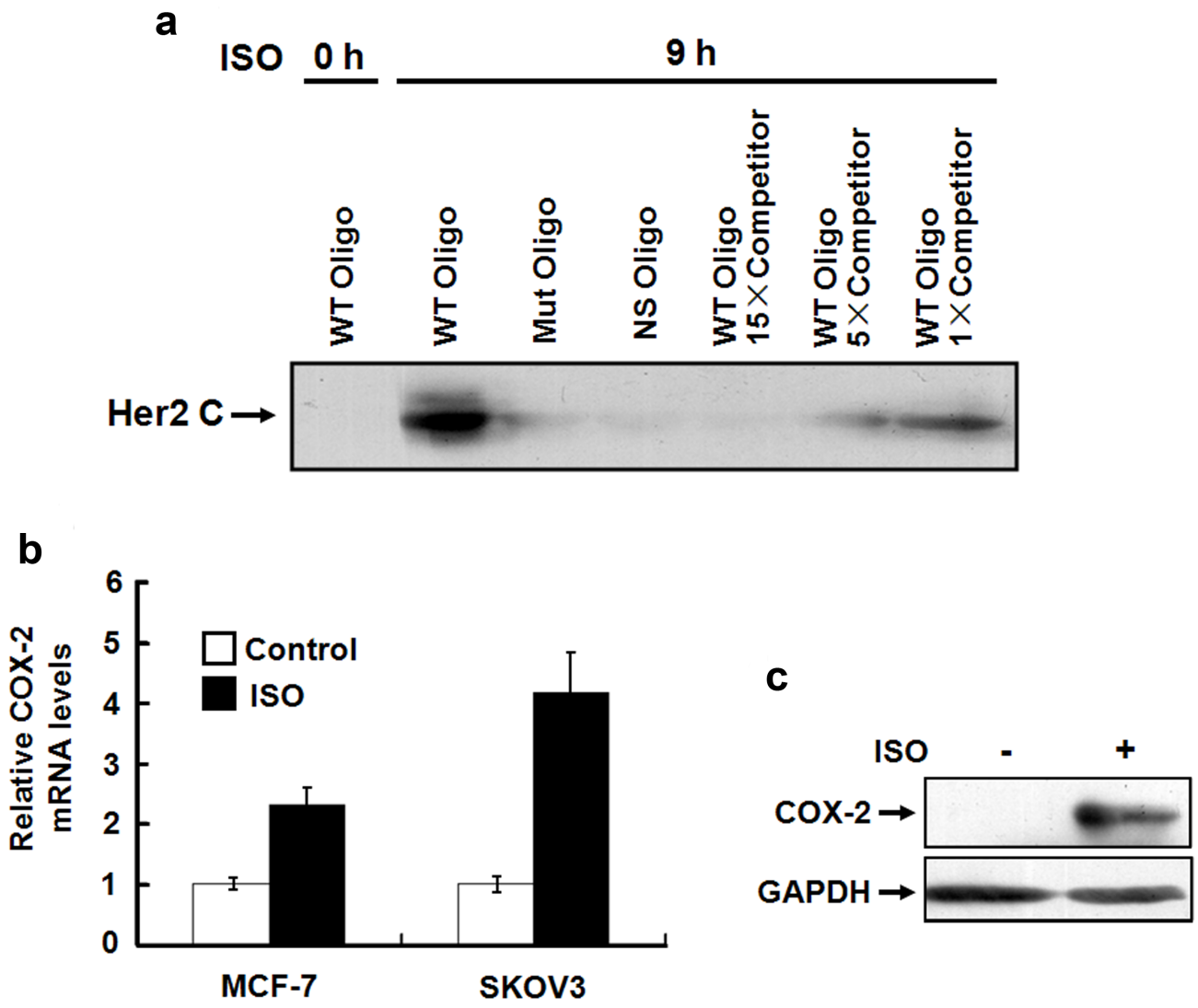

Figure 5

Her2 ICD physically binds to the promoter of COX2 gene and drives transactivation of COX2 gene. a 200 μg of the nuclear extracts was incubated with the 5'-biotinylated double-stranded oligonucleotides (5'-ATAAACTTCAAATTTCAGTA-3') corresponding to the positions -1770 to -1750 of the COX-2 promoter previously coupled to Dynabeads M-280. The protein/DNA complexes were separated with a Dynal magnet, denatured in SDS sample buffer and subjected to SDS-PAGE. Binding of Her2 was detected by Western blot with the antibodies against N-terminus or C-terminus of Her2. The same double-stranded sequences that are not biotinylated were used as the competitors. The biotinylated oligonucleotides containing the mutated Her2 binding sequences (5'-ATAAACTGACCCGGGAAGTA-3') and the sequences

lacking the Her2 binding motif (5'-ATAAACTTCAAATTTCAGTA-3') were used as the controls. b and c MCF-7 and SKOV3 cells were treated with 5 μM of ISO. The expression of COX-2 at the mRNA and protein levels was detected by real-time RT-PCR and Western blot.

Figure 6

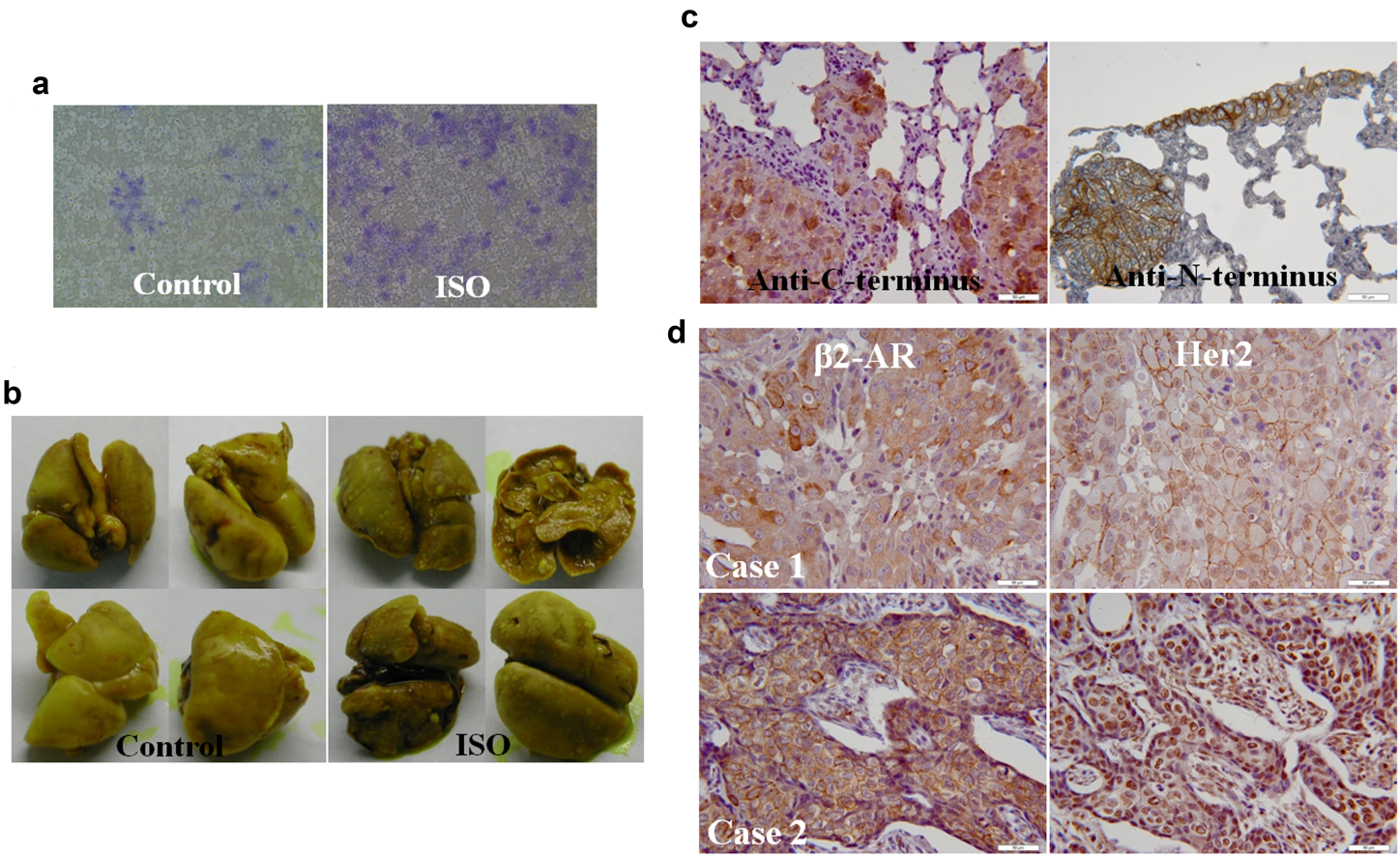

Figure 6

Catecholamine stimulation promotes the invasive and metastatic potentials of tumor cells. a SKOV3 cells were chronically stimulated by 5 μM ISO and invasive activities of the cells assessed by a Cell invasion assay kit. The invasive cells were fixed and stained. b A total of 0.1 ml of the cell suspension (108 cells/ml) was injected s.c. in the right upper flank of five to six-week-old athymic female BALB/c nude mice. After tumor cell injection for four days, the mice were treated daily i.p. with PBS or ISO (10 mg/kg). Eight mice were used in each group. Sixty days following tumor implantation, mice were sacrificed. The lungs of the mice were autopsied, fixed and photographed. c Lung metastatic tumors were dissected. Paraffin-embedded tissue sections were stained with rabbit monoclonal antibodies against N-terminus and C-terminus of Her2 and β2-AR. d The expression of β2-AR and Her2 in breast cancer tissues was analyzed by immunohistochemistry with the antibodies against β2-AR and C-terminus of Her2. Bar = 50 μm

Figure 7

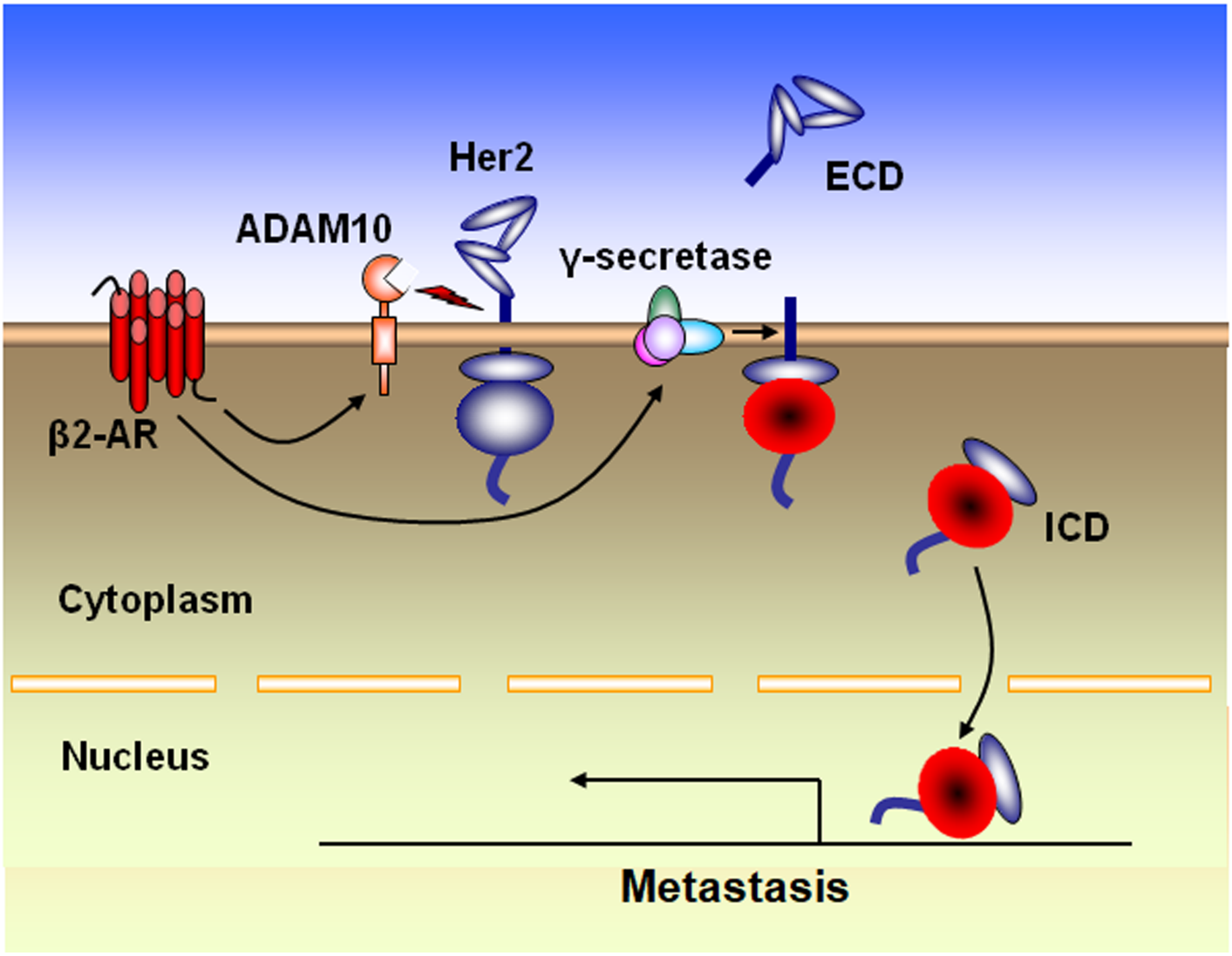

Figure 7

The molecular mechanism of cleavage and nuclear translocation of Her2. Catecholamine-induced β2-AR activation triggers shedding of Her2 ECD by ADAM10 and subsequent intramembranous cleavage of Her2 ICD by presenilin-dependent γ-secretase, resulting in nuclear translocation of p80Her2 and enhanced transcription of target genes.

Supplementary Files

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