



Cancer Research

Substance P autocrine signaling contributes to persistent HER2 activation that drives malignant progression and drug resistance in breast cancer

Susana Garcia-Recio, Gemma Fuster, Patricia Fernandez-Nogueira, et al.

Cancer Res Published OnlineFirst September 12, 2013.

Updated version	Access the most recent version of this article at: doi: 10.1158/0008-5472.CAN-12-4573
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2013/09/12/0008-5472.CAN-12-4573.DC1.html
Author Manuscript	Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org .

Substance P autocrine signaling contributes to persistent HER2 activation that drives malignant progression and drug resistance in breast cancer

Susana Garcia-Recio¹⁻³, Gemma Fuster¹⁻², Patricia Fernandez-Nogueira¹⁻³, Eva M. Pastor-Arroyo¹⁻², So Yeon Park⁴, Cristina Mayordomo¹⁻², Elisabet Ametller¹⁻², Mario Mancino¹⁻², Xavier Gonzalez-Farre¹⁻², Hege Russnes⁵, Pablo Engel⁶, Domiziana Costamagna⁷, Pedro L. Fernandez⁸, Pedro Gascón¹⁻³; Vanessa Almendro¹⁻².

¹Department of Medical Oncology and ⁸Pathology, Hospital Clínic, ²Institut d'Investigacions Biomediques August Pi i Sunyer, ³Department of Medicine, University of Barcelona, Spain; ⁴Department of Pathology, Seoul National University Bundang Hospital, Seoul National University College of Medicine, Seongnam, Korea; ⁵Department of Genetics, Oslo University Hospital Radiumhospitalet, Norway; ⁶Department of Cell Biology, Immunology, and Neurosciences, Medical School, University of Barcelona, Spain; ⁷Department of Medicine and Experimental Oncology, Torino University, Italy.

Running title: Transmodulation of HER2 by SP in breast cancer

Keywords: Tachykinins, HER2, chemoresistance, breast cancer, transmodulation

Conflict of interest disclosure: The authors disclose no potential conflict of interest.

Correspondence to: Vanessa Almendro, PhD (almendro@clinic.ub.es) and Pedro Gascon, MD, PhD (gascon@clinic.ub.es); Edifici Cellex, C/ Casanova 143, Laboratori 1A 08036 Barcelona (Spain) / Phone number: +34 932275400 ext 4013/ Fax number: +34 934546520

ABSTRACT

ERBB receptor transmodulation by heterologous G-protein coupled receptors (GPCRs) generates functional diversity in signal transduction. Tachykinins are neuropeptides and proinflammatory cytokines that promote cell survival and cancer progression by activating several GPCRs. In this work, we found that the pain-associated tachykinin Substance P (SP) contributes to persistent transmodulation of the ERBB receptors EGFR and HER2 in breast cancer (BC), acting to enhance malignancy and therapeutic resistance. SP and its high-affinity receptor NK-1R were highly expressed in HER2+ primary breast tumors (relative to the luminal and triple negative subtypes), and were overall correlated with poor prognosis factors. In BC cell lines and primary cultures derived from BC samples, we found that SP could activate HER2. Conversely, RNAi-mediated attenuation of NK-1R, or its chemical inhibition, or suppression of overall GPCR-mediated signaling, all strongly decreased steady-state expression EGFR and HER2, establishing that their basal activity relied upon transdirectional activation by GPCR. Thus, SP exposure affected cellular responses to anti-ERBB therapies. Our work reveals an important oncogenic co-operation between NK-1R and HER2, thereby adding a novel link between inflammation and cancer progression that may be targetable by SP antagonists that have been clinically explored.

INTRODUCCION

Inflammation is a hallmark of cancer (1). Chronic inflammation is closely related to tumor development and progression; it supplies the tumor with a milieu of proinflammatory cytokines that can modulate the inflammation-related tumor cell signaling (2, 3). Some inflammatory conditions increase the risk of the onset of cancer by enhancing the proliferation and oncogenic potential of adjacent epithelia. In cancers that are already established, persistent inflammation enhances tumor growth and progression by activating several mechanisms that supports tumors, such as enhanced

angiogenesis and stromal cell recruitment. Inflammatory mediators can also cause genetic instability (4, 5) and favor the fixation of epigenetic alterations (6).

Several proinflammatory cytokines, such as chemokines, induce signal transduction through different families of G-protein coupled receptors (GPCRs). Activation of secondary messengers, such as PKC and adenylate cyclase, results in the diversification G-protein signal transduction (7, 8). In some cancer cell types, the mitogenic stimulus activated by G-proteins is partly mediated by transmodulation of the EGFR (epidermal growth factor receptor) (9, 10). The members of the ERBB family, including EGFR, HER2, HER3 and HER4, are receptors with tyrosine kinase (RTK) activity, and signaling networks triggered by them play a pivotal role in tumor initiation and progression (11, 12). In particular, HER2 is overexpressed in approximately 25% of breast cancer (BC) patients and is associated with worse prognosis and lower survival rates (13, 14). The precise pathways interconnected with RTK to fine tune cellular responses are still unknown, but emerging evidence indicates that transmodulation by heterologous GPCRs may play an important role in them (7, 8). It is particularly noteworthy that the crosstalk between GPCRs and RTKs allows for RTK activation even in the absence of ligands (7). Therefore, identifying of new modulators of RTKs activity could be highly useful in the search for new therapeutic targets.

In this work, we have investigated the transmodulation of the ERBB system by the neuropeptide/proinflammatory mediator Substance P (SP); a pleiotropic molecule synthesized by the *TAC1* gene. SP is related to processes of neurogenic inflammation, wound healing, hematopoiesis, microvasculature permeability, leukocyte trafficking, cell survival, and metastatic dissemination (15-19). The three classical members of the mammalian tachykinin family are SP and neurokinin A (NKA), both encoded by the *TAC1* gene, and neurokinin B (NKB) encoded by the *TAC3* gene. There is a third mammalian tachykinin gene (*TAC4*) that codifies for hemokinins and endokinins (15, 20, 21). These tachykinins exercise their biological functions through their preferential binding to the tachykinin receptors NK-1R (*TACR1*), NK-2R (*TACR2*) and NK-3R (*TACR3*), respectively, although each tachykinin can bind to the three tachykinin receptors with different affinity (22). NK-1R, the

high-affinity SP receptor, is a GPCR that is overexpressed in BC (19). In this work, we identify a new aberrant signaling network in BC based on the oncogenic co-operation between the tachykinergic system and HER2/EGFR signaling. The data presented here supports the idea that the SP produced within a proinflammatory setting can enhance tumor malignancy by activating HER2 and EGFR. In addition, we demonstrated that the network could be exploited for the development of new therapeutic strategies based on the dual inhibition of activation and transactivation mechanisms.

MATERIAL AND METHODS

Cell lines. All human BC cell lines were purchased from American Type Culture Collection and were validated by single locus short tandem repeat (STR) typing (Bio-Synthesis, Inc.) prior to its use.

Isolation of human primary breast cancer cells. Nineteen histologically confirmed BC tumors were collected at the Hospital Clinic in Barcelona under the approval of the institutional review board. Tumor tissue was processed to obtain single cells by digestion with a mixture of collagenase and hyaluronidase, as previously described (43), and the cells were frozen in FBS with 10% DMSO until further analysis.

Time-course studies. *In cell lines:* To determine the effects of SP treatment on HER2, EGFR, p42/44 MAPK and Akt activation cells treated at the indicated times with SP 100 nM, washed, and rapidly frozen until protein extraction. *In primary cultures:* Tumor cells were thawed, filtered to ensure single-cell suspensions, and kept in Mammary Epithelial Basal Medium (MEBM®, Lonza, Cologne) supplemented with growth factors for 3 hours at 37°C. Each single-cell suspension was split into 3 groups: control, SP 6 min and SP 10 min. They were serum starved for 2 hours, and then treated with PBS or SP 100 nM at 37°C. After treatment, the cells were washed, fixed in PFA 4%, and resuspended in PBS with glycerol 10%. A Cytospin slide centrifuge was used to concentrate and fix the samples on glass slides until further immunodetection of activated HER2.

***In vivo* experiments.** Animal experiments were performed in accordance with the regulations of our institution's ethics commission, following the guidelines established by the regional authorities of Catalonia, Spain. The specific procedures are outlined in the Supplementary Methods on line.

Western blot. Briefly, cells or tumor pieces were lysed in ice-cold radioimmunoprecipitation assay buffer (RIPA). Protein quantification and detection was performed as described elsewhere applying the primary antibodies at the dilutions indicated in the Supplementary Methods on line.

Immunofluorescence. Cell lines and cytopin slides containing the cells from the primary cultures were immunostained with a combination of different antibodies as explained in the Supplementary Methods online. The slides were counterstained with DAPI (Roche Diagnostic, Germany) for 15 minutes and mounted with a coverslip using ProLong® Gold antifade reagent (Invitrogen, Molecular Probes, CA).

Fluorescence microscopy and image acquisition. Images were acquired using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). In the images of the primary cultures, the mean fluorescence (pHER2 staining) (Integrated density/Area) of selected cells (those pan-CK positive) was quantified using a custom macro in the FIJI software (a distribution of ImageJ-Wayne Rasband, NIH, USA) as explained in detail in the Supplementary Methods online.

Tumor subtype classification. Tumor subtype definitions in this study were as follows: luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+, HER2+), HER2+ (ER-, PR-, HER2+), basal-like (ER-, PR-, HER2-, CK5/6+, and/or EGFR+), and unclassified (negative for all markers), as previously described (45).

Immunohistochemistry procedure and evaluation. Immunohistochemistry for NK-1R, NK-2R, SP, COX2, or SMA was performed following standard protocols as indicated in the Supplementary Methods online. For the semi-quantitative analysis, an immunoreactive score (IRS) was derived by multiplying the percentage of positive cells (PP) by the staining intensity (SI). The SI was determined as: 0 for no staining; 1+ for weak staining; 2+ for moderate staining; 3+ for strong

staining and 4+ for very strong staining. We classified the IRS into 4 groups: 0 (negative staining), 1 (staining score >0 and <100), 2 (staining score >100 and <200), 3 (staining score >200).

Statistical analysis. Detailed statistic methods used to compare differences between groups can be found in the Supplementary Methods online.

RESULTS

NK-1R and SP are highly expressed in human primary tumors of the HER2+ subtype, and circulating SP levels are increased in BC patients

The expression levels of NK-1R, NK-2R and SP were scored as both continuous and ordinal variables (for further details see the Methods section) (Supplementary Table S1 and S2), and both statistical analyses showed similar correlations with clinical characteristics. NK-1R, NK-2R, and SP were expressed by luminal cells from normal breast tissue (Supplementary Fig. S1), and overexpressed in tumors (Fig. 1A), in agreement with previous reports (19). In tumors, NK-1R, NK-2R, and SP expression was detected in 94%, 86% and 52% of the 318 samples analyzed, respectively (Fig. 1A-C). With few exceptions, we did not find significant intratumor heterogeneity for the expression of these proteins; when present they were expressed in the majority of the cells within each tumor. Expression of either NK-1R or SP was positively associated with tumor histopathologic characteristics related to poor prognosis. For example, NK-1R expression levels were associated with estrogen receptor (ER) and progesterone receptor (PR) negativity, and p53 overexpression. SP expression was associated with a high histological grade, ER and PR negativity, and a high Ki67 proliferation index. In contrast, NK-2R expression was associated with low histologic grade, ER and PR positivity, and no p53 overexpression (Supplementary Tables S1 and S2). Interestingly, we found that NK-1R and SP expression were significantly higher in tumors of the HER2+ subtype, when the expression levels were considered as either continuous or ordinal variables (Fig. 1D-I), and the same positive association was found in tumors with HER2 gene amplification (Supplementary Tables S1 and S2). In contrast, NK-2R expression was lowest in this particular tumor subtype (Fig. 1E and 1H).

We next determined the SP concentration in the serum of 139 BC patients and 92 healthy controls by ELISA. The levels of SP were significantly higher in BC patients than in healthy controls, although we did not observe any association with a particular tumor subtype or any other clinicopathologic characteristic (Fig. 1J and Supplementary Table S3).

Human tachykinins modulate EGFR and HER2 receptor activation in BC cells

Considering that the higher expression of NK-1R and SP in HER2+ tumors could be the consequence of functional co-operation between the two signaling systems, we next explored the effects of SP on a panel of BC cell lines with (MDA-MB-453, SK-BR-3, BT-474) and without (MDA-MB-231, MCF7) amplification or overexpression of the *ERBB2* gene (23), and with overexpression of the *EGFR* gene (MDA-MB-468) (Fig. 2A). We detected expression of the three main tachykinin receptors, *TACR1*, *TACR2* and *TACR3*, and expression of *TAC1* (which codifies for SP) by qPCR in all the cell lines (Fig. 2B). We further confirmed the expression of NK-1R and SP by immunofluorescence in all the cell lines (Fig. 2C). Through time-course studies, we observed that SP quickly induced the phosphorylation of HER2 Tyr1248 (specific tyrosine residue that serves as docking site for the adaptor molecules that will activate the Ras-ERK pathways (24-26)) within the first 6 to 10 minutes after stimulation in all the cell lines (Fig. 2D; the densitometric quantification is shown in Supplementary Fig. S2A). The activation of HER2 within this time frame was consistently observed in all the replicates performed, although the exact time-point of maximum activation varied. The kinetics of HER2 receptor activation was dose-dependent, showing faster activation with increasing concentrations of SP (Supplementary Fig. S3). SP treatment also induced the phosphorylation of EGFR Tyr1068 (ligand-activated) (27) and EGFR Tyr845 (Src-activated) (28), as well as the p42/44 MAPK and Akt pathways (Fig. 2D and Supplementary Fig. S2A).

By immunofluorescence we also observed an increase in the levels of phospho-HER2 Y1248 in the cell membrane of MDA-MB-453 cells (HER2+) and an increase in the formation of endocytic

vesicles containing activated EGFR in the MDA-MB-468 cell line (EGFR+) (29) (Supplementary Fig. S2B) after SP treatment.

The MAPK is a common downstream pathway triggered by both HER2 and NK-1R activation (7, 30, 31). Then, to confirm that the phosphorylation of HER2 caused by SP treatment was indeed activating HER2 receptor we determined the capability of SP to activate the MAPK pathway in the presence of HER2 inhibitors. Using the MDA-MB-453 cell line we observed that the inhibition of HER2 with either Lapatinib or AG825 decreased the capability of SP to activate the p42/44-MAPK (Supplementary Fig. S4A and B), suggesting that the activation of MAPK by SP partially depends on the transactivation of HER2.

SP transactivates HER2 in human primary tumor cells

Since HER2 is a target for therapy in BC patients, we next studied the clinical relevance of SP-mediated HER2 activation. We determined the activation of phospho-HER2 Y1248 after SP treatment for 6 and 10 minutes in 1896 single cells obtained from 19 BC primary cultures of different cancer subtypes that were classified by immunohistochemistry as having some degree of expression of HER2 (Supplementary Fig. S5 and Supplementary Table S4). We used multicolor immunofluorescence and confocal microscopy to analyze and quantify the levels of phospho-HER2 Y1248 in pan-cytokeratin positive cells (to select only epithelial cells) (Fig. 3A). The basal levels of phospho-HER2 Y1248 were higher in the control samples derived from HER2+ and luminal B tumors than in the luminal A or triple negative tumors (Fig. 3B and C). Although overall we only observed a statistically significant increase in phospho-HER2 Y1248 by SP in cells derived from tumors of the HER2+ and triple negative subtypes (Fig. 3D), there was some inter-group variation in the tumors of the luminal A subtype. In fact, in 7 out of 10 luminal A tumors, SP activated HER2, in agreement with the results obtained with BC cell lines (Supplementary Fig. S6). We did not observe activation of HER2 in the cells derived from luminal B tumors at the time points tested. There could be several reasons for the lack of HER2 activation in this subgroup; for instance, due to low levels of

NK-1R expression in luminal B compared to HER2+ tumors (Fig. 1D); or because of a lack of additional signaling mediators involved in the transactivation process with differential intracellular signaling.

SP/NK-1R autocrine/paracrine signaling in BC cells elicits a constitutive transactivation of HER2 and EGF receptors

Since NK-1R is the high-affinity receptor for SP, we next decided to investigate its contribution to SP-mediated HER2 transactivation. The MDA-MB-231 cell line was transfected with the pcDNA3.1(+)-*TACR1* vector, enriched with NK-1R+ cells by fluorescence-activated cell sorting (Supplementary Fig. S7A), and the expression of NK-1R confirmed by qPCR (Supplementary Fig. S7B). Upon SP stimulation, the levels of pHER2 Y1248 and p-p42/44-MAPK were significantly increased in the cells transfected with pcDNA3.1(+)-*TACR1* compared to mock cells (Fig. 4A and Supplementary Fig. S7C), indicating that NK-1R is involved in SP-induced HER2 transactivation. The inhibition of *TACR1* by siRNA in the SK-BR-3 and BT-474 cell lines caused a significant decrease in the basal levels of pHER2 (Fig. 4B). Consistent with this finding, the inhibition of NK-1R activity by the chemical inhibitor L-733,060 for 24 hours markedly reduced pHER2 Y1248 in the SK-BR-3, BT-474 and MDA-MB-453 cells and pEGFR Y1068 in the MDA-MB-468 cell line (Fig. 4C). This suggests that tumor cell oncogene addiction to NK-1R could rely in part on NK-1R-mediated effects on HER2 and EGFR basal activity. We also observed that signal transduction through phospho-p42/44-MAPK in BT-474 and/or phospho-Akt in SK-BR-3, BT-474, and MDA-MB-468 was also inhibited in cells treated with L-733,060 (Fig. 4C). Moreover, the blockade of NK-1R, NK-2R and NK-3R in the SKBR3 cell line caused almost a complete inhibition of HER2 phosphorylation and a significant decrease in the Akt survival signaling pathway (Fig. 4D); thereby further confirming the tachykinergic system to be a modulator of the steady state (or basal activation) of HER2.

Besides the well-known role of several GPCR ligands as activators of EGFR and HER2 (7, 9, 32), their contribution to the basal activity of RTKs has not been intensely studied. For this reason, we next determined whether GPCR-mediated signaling modulates the activity of HER2 in BC cells. We observed that the inhibition of pertussis toxin-sensitive G-proteins strongly decreased the pHER2 Y1248 in all the cell lines, and total HER2 in the SK-BR-3 and BT474 cells. In some cases a decrease in p42/44-MAPK and Akt were also observed (Fig. 4E and Supplementary Fig. S7D). All together these data suggests that G-protein signaling in BC cells directly modulates HER2 activation.

Chronic overexposure to SP *in vivo* has mitogenic activity and enhances HER2 activation

To further study the effects of overexposure to SP on tumor growth *in vivo*, we subcutaneously implanted mini osmotic pumps challenged to continuously deliver 500 nM of SP for 28 days in SCID mice wearing MDA-MB-231 (HER2-) or MDA-MB-453-derived (HER2+) xenograft tumors. We aimed to determine whether long-term SP exposure, simulating a scenario of chronic inflammation, could favor the outgrowth of tumor cells with different properties and thus influence the progression of tumors with different phenotype: HER2- or HER2+.

Chronic SP delivery for 28 days did not affect the tumor growth or the final tumor weight in any of the tumor models (Fig. 5A and B). However, we observed a significant increase of total HER2 in the SP treated tumors by Western blot in both models (Fig. 5C) and by immunohistochemistry (Fig. 5D) in the MDA-MB-453. In the MDA-MB-231-derived xenografts we also detected a weak expression of HER2 in the treated tumors, compared with the control tumors that were totally negative. The MAPK pathway was also highly expressed in the MDA-MB-231 SP-treated tumors. In addition, in both tumor models there was a significant increase in the number of mitotic bodies (Supplementary Fig. S8A and B) probably due to the known mitogenic activity of SP (33, 34). These data suggests that the continuous presence of SP in a tumor, like in a situation of chronic inflammation, could enhance the expression of HER2 in tumor cells with different phenotypes.

The NK-1R antagonist L-733,060 inhibits tumor growth *in vivo* in HER2 and EGFR expressing tumors

We next decided to study the effects of NK-1R inhibition on the *in vivo* growth of the BC cell lines MDA-MB-453 (HER2+), MDA-MB-468 (EGFR+), and MDA-MB-231 (HER2- and EGFR-). The tumor growth was significantly decreased only in MDA-MB-453 and MDA-MB-468-derived xenograft tumors treated with L-733,060 (5mg/kg three times weekly) (Fig. 5E and F), but not in the MDA-MB-231 tumors (Fig. 5G), suggesting that the anti-tumor effects of NK-1R inhibition *in vivo* depend on the modulatory properties of NK-1R signaling on HER2 and EGFR activity. Final tumor weight was significantly reduced by L-733,060 only in the HER2+ model derived from MDA-MB-453, and slightly decreased in the MDA-MB-468 cells (Fig. 5H and I). On the other hand, we did not observe significant effect on the growth or final tumor weight of the MDA-MB-231-derived tumors (Fig. 5G and J). At the protein level, there were no significant differences in HER2 or EGFR, suggesting that NK-1R inhibition does not select for particular cell subpopulations; and the treatment with L-733,060 only decreased p42/44-MAPK and Akt in the MDA-MB-231-derived tumors (Fig. 5K-M). In addition, we noticed a slight decrease in the expression of the COX2 pro-inflammatory mediator as a consequence of NK-1R inhibition in all the tumor models, and a significant decrease in alpha-SMA (smooth muscle actin) positive cells, suggesting that the blockade of NK-1R modulates both tumor and stromal properties (Supplementary Fig. S9A-C).

Chronic SP treatment alters cellular responses to therapies targeting HER2 and EGFR

Since the hyperactivation of ERBB signaling by proinflammatory components may influence cellular responses to targeted therapies, we next studied the effects of short or chronic exposure to SP on the cellular responses to HER2 and EGFR inhibitors in the MDA-MB-453 (HER2+) or MDA-MB-468 (EGFR+) cell lines. To develop a model of SP overexposure, we kept the different cell lines growing in the absence (control group) or presence of SP (100 nM daily) for over 5 months. The cell lines chronically treated with SP showed increased levels of total and activated HER2 and EGFR,

and in some cases a higher activation of the p42/44-MAPK and Akt pathways (Supplementary Fig. S10A-D).

Next, we studied the changes in IC₅₀ values (inhibitory concentration 50%) for the EGFR tyrosine kinase inhibitor AG1478, the HER2 tyrosine kinase inhibitor AG825, and the dual EGFR/HER2 tyrosine kinase inhibitor Lapatinib upon acute (72 hours) or chronic (5 months) SP treatment. A short exposure to 100 nM or 500 nM of SP had little or no effect on the IC₅₀ value in any cell line, while only the response of the MDA-MB-453 cells to AG825 upon addition of 100 nM SP and that of the MDA-MB-468 cells to AG1478 upon 500 nM SP stimulation were significantly affected (Fig. 6A and B and Supplementary Table S5). However, chronic treatment with SP for up to 5 months induced a significant increase in the viability of the MDA-MB-453 and MDA-MB-468 cell lines after being treated with AG1478, AG825 and Lapatinib (Fig. 6A and B and Supplementary Table S5), as reflected by the differences in the IC₅₀ values. These results indicate that chronic exposure to SP affects the response to EGFR and HER2-targeted therapies.

Combination of the NK-1R antagonist L-733,060 with anti-HER2 therapies synergistically inhibits the proliferation of BC cells

We next used MTT assays and applied combination index (CI) equations (35) to determine if the inhibition of NK-1R activity could enhance the effects of EGFR or HER2 inhibitors. Treatment with L-733,060 synergized with the cytotoxic effects of AG825 (CI= 0.5686) and Lapatinib (CI= 0.653) in the MDA-MB-453 cell line and also with Lapatinib in the MDA-MB-468 cells (CI= 0.4494), although the combination of L-733,060 with AG1478 (CI= 1.7642) in the MDA-MB-468 cell line indicated an antagonist effect at the doses used (Fig. 6C and Supplementary Table S5). These results strongly support the idea that the modulatory properties of NK-1R on ERBB receptors could be exploited to increase the effectiveness of targeted therapies for the treatment of BC by concomitant inhibition of NK-1R and ERBB receptors.

DISCUSSION

Although the oncogenic addiction to HER2 signaling is therapeutically exploited for BC treatment, the disappointing clinical responses observed in a significant percentage of patients suggests that additional mechanisms regulating the HER2 pathway may be used to fine tune the cancer cell responses to anti-HER2 therapies. The work presented here highlights a previously unidentified role of SP/NK-1R autocrine/paracrine constitutive signaling as a modulator of the basal activation of HER2 and EGFR in BC; a mechanism with several clinical implications.

The transmodulation of RTKs by SP and other neuropeptides and proinflammatory mediators (36, 37) can serve as a mechanism for RTK activation in a ligand-independent way (7). The fact that the steady states of HER2 and EGFR depend upon the activity of additional signaling pathways implies that these instigator pathways could be therapeutically used to down-regulate the activation of RTKs. For example, our laboratory has previously shown that SP inhibition decreases EGFR and HER2 activity and induces apoptosis, and that SP blockade is effective in inducing cell death even in cell lines that are resistant to the EGFR/HER2 inhibitors Lapatinib and Trastuzumab (17). Likewise, in this work we have shown that the chemical inhibition of NK-1R, alone or in combination with the inhibition of NK-2R and NK-3R, or the silencing of *TACR1* gene by siRNA strongly decreases HER2 activity in all the cell lines studied, as well as the NK-1R antagonist L-733,060 reduces tumor growth in SCID mice harboring HER2⁺ or EGFR⁺ tumor cells. Although we did not reach high levels of *TACR1* inhibition to avoid the effects of massive cell death, even *TACR1* gene silencing by 50%-70% showed effects on the steady state of HER2, and given the diversity of GPCRs expression in cancer cells, it is probable that this finding may be applied to a huge variety of ligands. It should be noted that a vast number of receptors involved in tumor progression, such as some cytokine and chemokine receptors, fall into the category of GPCRs, implying that steady G-protein activation is always present in cancer cells. In this context, we observed that the inhibition of G-protein-mediated signaling decreases HER2 activity and expression, supporting the idea that the dual blockade of activation and transactivation pathways could enhance the therapeutic response to particular targeted

therapies. Our results also imply that the lack of target dependency in some anti-HER2 therapies could be due in part to NK-1R transactivation mechanisms in those tumors that express the NK-1R receptor. In fact, we observed that an overexposure to SP *in vivo* increases HER2 expression in HER2+ or HER2- xenograft tumors, and that this could be a relevant finding to consider in relation to those tumors initially classified as HER2 negative; the constant signaling of SP in tumor cells could favor a long-term selection of cells with increase levels of HER2. Although in our model we did not observe an increase in tumor volume despite HER2 increase, it is likely that in a longer period of time this constant activation of HER2 would translate in increased tumor volume, as it can be expected from situations of chronic inflammation in humans. Furthermore, *in vitro* experiments demonstrate that chronic exposure to SP also increases HER2 and/or EGFR expression and activation in the cell lines studied. Moreover, NK-1R inhibition synergizes with anti-EGFR and anti-HER2 therapies thereby significantly reducing cell survival.

The modulation of RTK activation by NK-1R also suggests oncogenic co-operation between these two types of receptors during tumor progression. As we observed in human primary tumors, NK-1R and SP are highly expressed in those of the HER2+ subtype, and SP can activate HER2 in primary cultures derived from human tumors with and without HER2 overexpression. In addition to HER2 overexpression, our current results identify NK-1R and SP as two new markers associated with other clinicopathologic characteristics related to a poor prognosis such as p53 overexpression, ER negativity expression, high proliferation, and a high histological grade (38, 39).

In addition, the relevance of SP signaling in BC could be context dependent. For instance, the specificity of NK-1R signal transduction depends on the formation of scaffolding protein complexes after its activation (40). In fact, the co-expression of EGFR and/or HER2 in the same cancer cell could provide SP with additional signal transduction adaptors, thus diversifying its effects. In a similar way, the apoptotic effects of NK-1R inhibition could depend on its ability to block the oncogenic dependence on HER2 and EGFR, as we observed *in vivo* where only HER2+ or EGFR+ tumors were affected after NK-1R inhibition. Moreover, as we showed in this work, not all SP

receptors are related to the activation of the same tumor promoting mechanism; for example NK-2R receptor showed a correlation with ER and PR positive tumors in contrast to NK-1R, which is associated with ER and PR negative tumors. Finally, SP activation of the MAPK pathway has been shown to be RTK dependent (40) or independent (41), suggesting a cell-type specific modulation of SP signaling.

In conclusion, the results presented here imply another mechanism by which inflammation, via SP, could enhance tumor aggressiveness. Furthermore, the interplay between multiple signaling pathways, such as those controlled by the receptors NK-1R and HER2, implies that to achieve a complete therapeutic response with therapies designed to target RTK, both mechanisms (activation and transactivation) may be inhibited, as we shown for NK-1R and HER2 in the present work.

ACKNOWLEDGMENTS

We thank the technical support of Maria Calvo and Anna Bosch at the Confocal Facility of the University of Barcelona; Isabel Crespo, from the Citomics unit of the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS); Olga Collazo, Laia Vinyals Mireia Pares, Anna Lopez, and Estel Enreig from the Laboratory of Molecular Oncology. Also, Blanca Farrus for her help with the collection of blood samples; Ferran Torres and Abiguel Torrents from the Biostatistics and Data Management Platform Department at the Hospital Clinic for the assistance with the statistical analysis; the Tumor Bank of the Hospital Clínic and the Tumor Bank Web of Catalonia. This work was supported by a grant from the Fondo de Investigación Sanitaria (PI08022) (P.G.), Instituto de Salud Carlos III-Subdirección General de Evaluación y Fomento de Investigación, Fondo Europeo de Desarrollo Regional, Unión Europea, Una manera de hacer Europa; by a grant from the Fundación Cellex (P.G. and V.A.), by Redes Temáticas de Investigación en Cáncer (RTICC, RD07/0020/2014) (P.G.), by the University of Barcelona, School of Medicine (P.F-N), and by the Juan de la Cierva Fellowship program (G.F.). The work was carried out in part at the Esther Koplowitz Centre, Barcelona.

FOOTNOTES

Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-74.
2. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature*. 2008;454:436-44.
3. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet*. 2001;357:539-45.
4. Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis*. 2009;30:1073-81.
5. Hofseth LJ, Khan MA, Ambrose M, Nikolayeva O, Xu-Welliver M, Kartalou M, et al. The adaptive imbalance in base excision-repair enzymes generates microsatellite instability in chronic inflammation. *J Clin Invest*. 2003;112:1887-94.
6. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell*. 2009;139:693-706.
7. Almendro V, Garcia-Recio S, Gascon P. Tyrosine kinase receptor transactivation associated to G protein-coupled receptors. *Curr Drug Targets*. 2010;11:1169-80.
8. Dorsam RT, Gutkind JS. G-protein-coupled receptors and cancer. *Nat Rev Cancer*. 2007;7:79-94.
9. Daub H, Weiss FU, Wallasch C, Ullrich A. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature*. 1996;379:557-60.
10. Delcourt N, Bockaert J, Marin P. GPCR-jacking: from a new route in RTK signalling to a new concept in GPCR activation. *Trends Pharmacol Sci*. 2007;28:602-7.

11. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J.* 2000;19:3159-67.
12. Stern DF. Tyrosine kinase signalling in breast cancer: ErbB family receptor tyrosine kinases. *Breast Cancer Res.* 2000;2:176-83.
13. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. *Nature.* 2000;406:747-52.
14. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A.* 2001;98:10869-74.
15. Palma C. Tachykinins and their receptors in human malignancies. *Curr Drug Targets.* 2006;7:1043-52.
16. Murthy RG, Reddy BY, Ruggiero JE, Rameshwar P. Tachykinins and hematopoietic stem cell functions: implications in clinical disorders and tissue regeneration. *Front Biosci.* 2007;12:4779-87.
17. Mayordomo C, Garcia-Recio S, Ametller E, Fernandez-Nogueira P, Pastor-Arroyo EM, Vinyals L, et al. Targeting of Substance P induces cancer cell death and decreases the steady state of EGFR and Her2. *J Cell Physiol.* 2012;227:1358-66.
18. Hong HS, Lee J, Lee E, Kwon YS, Ahn W, Jiang MH, et al. A new role of substance P as an injury-inducible messenger for mobilization of CD29(+) stromal-like cells. *Nat Med.* 2009;15:425-35.
19. Singh D, Joshi DD, Hameed M, Qian J, Gascon P, Maloof PB, et al. Increased expression of preprotachykinin-I and neurokinin receptors in human breast cancer cells: implications for bone marrow metastasis. *Proc Natl Acad Sci U S A.* 2000;97:388-93.
20. Patacchini R, Lecci A, Holzer P, Maggi CA. Newly discovered tachykinins raise new questions about their peripheral roles and the tachykinin nomenclature. *Trends Pharmacol Sci.* 2004;25:1-3.

21. Zhang Y, Lu L, Furlonger C, Wu GE, Paige CJ. Hemokinin is a hematopoietic-specific tachykinin that regulates B lymphopoiesis. *Nat Immunol.* 2000;1:392-7.
22. Maggi CA. The mammalian tachykinin receptors. *Gen Pharmacol.* 1995;26:911-44.
23. Kury FD, Schneeberger C, Sliutz G, Kubista E, Salzer H, Medl M, et al. Determination of HER-2/neu amplification and expression in tumor tissue and cultured cells using a simple, phenol free method for nucleic acid isolation. *Oncogene.* 1990;5:1403-8.
24. Dankort D, Jeyabalan N, Jones N, Dumont DJ, Muller WJ. Multiple ErbB-2/Neu Phosphorylation Sites Mediate Transformation through Distinct Effector Proteins. *J Biol Chem.* 2001;276:38921-8.
25. Montgomery RB, Makary E, Schiffman K, Goodell V, Disis ML. Endogenous anti-HER2 antibodies block HER2 phosphorylation and signaling through extracellular signal-regulated kinase. *Cancer Res.* 2005;65:650-6.
26. Ben-Levy R, Paterson HF, Marshall CJ, Yarden Y. A single autophosphorylation site confers oncogenicity to the Neu/ErbB-2 receptor and enables coupling to the MAP kinase pathway. *EMBO J.* 1994;13:3302-11.
27. Munoz M, Rosso M, Covenas R. The NK-1 Receptor: A New Target in Cancer Therapy. *Curr Drug Targets.* 2011.
28. Tice DA, Biscardi JS, Nickles AL, Parsons SJ. Mechanism of biological synergy between cellular Src and epidermal growth factor receptor. *Proc Natl Acad Sci U S A.* 1999;96:1415-20.
29. Sorkin A, Goh LK. Endocytosis and intracellular trafficking of ErbBs. *Exp Cell Res.* 2008;314:3093-106.
30. Pierce KL, Luttrell LM, Lefkowitz RJ. New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. *Oncogene.* 2001;20:1532-9.
31. Della Rocca GJ, van Biesen T, Daaka Y, Luttrell DK, Luttrell LM, Lefkowitz RJ. Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. *Convergence*

of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. *J Biol Chem.* 1997;272:19125-32.

32. Arora P, Cuevas BD, Russo A, Johnson GL, Trejo J. Persistent transactivation of EGFR and ErbB2/HER2 by protease-activated receptor-1 promotes breast carcinoma cell invasion. *Oncogene.* 2008;27:4434-45.

33. Nilsson J, von Euler AM, Dalsgaard CJ. Stimulation of connective tissue cell growth by substance P and substance K. *Nature.* 1985;315:61-3.

34. Payan DG, Brewster DR, Goetzl EJ. Specific stimulation of human T lymphocytes by substance P. *J Immunol.* 1983;131:1613-5.

35. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul.* 1984;22:27-55.

36. Rivas MA, Tkach M, Beguelin W, Proietti CJ, Rosemblyt C, Charreau EH, et al. Transactivation of ErbB-2 induced by tumor necrosis factor alpha promotes NF-kappaB activation and breast cancer cell proliferation. *Breast Cancer Res Treat.* 2010;122:111-24.

37. Cabioglu N, Summy J, Miller C, Parikh NU, Sahin AA, Tuzlali S, et al. CXCL-12/stromal cell-derived factor-1alpha transactivates HER2-neu in breast cancer cells by a novel pathway involving Src kinase activation. *Cancer Res.* 2005;65:6493-7.

38. Hensel M, Schneeweiss A, Sinn HP, Egerer G, Solomayer E, Haas R, et al. P53 is the strongest predictor of survival in high-risk primary breast cancer patients undergoing high-dose chemotherapy with autologous blood stem cell support. *Int J Cancer.* 2002;100:290-6.

39. Malamou-Mitsi V, Gogas H, Dafni U, Bourli A, Fillipidis T, Sotiropoulou M, et al. Evaluation of the prognostic and predictive value of p53 and Bcl-2 in breast cancer patients participating in a randomized study with dose-dense sequential adjuvant chemotherapy. *Ann Oncol.* 2006;17:1504-11.

40. DeFea KA, Vaughn ZD, O'Bryan EM, Nishijima D, Dery O, Bunnett NW. The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta -arrestin-dependent scaffolding complex. *Proc Natl Acad Sci U S A.* 2000;97:11086-91.

41. Yamaguchi K, Kugimiya T, Miyazaki T. Substance P receptor in U373 MG human astrocytoma cells activates mitogen-activated protein kinases ERK1/2 through Src. *Brain Tumor Pathol.* 2005;22:1-8.

FIGURE LEGENDS

Figure 1. Expression of NK-1R, NK-2R and SP and systemic levels of SP. Representative immunohistochemistry images for the expression of (A) NK-1R, (B) NK-2R and (C) SP proteins in tumors of different subtypes. Scale bar represents 50 microns. D-F, Bar plots showing the percentage of cases with negative, low, medium and high expression (expression score 0 to 3, respectively) of NK-1R, NK-2R and SP. G-I, Box plots showing the distribution of the expression of each marker evaluated as a continuous variable. Significant differences by ANOVA are indicated as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. J, Serum SP levels. Significant differences by Wilcoxon two sample test are indicated as * $P < 0.05$. The boxes show the 25th to 75th percentile, and the whiskers extend to the 5th and 95th percentiles. The yellow diamonds indicate the mean. *LumA*, luminal A; *LumB*, luminal B; *Un*, undetermined.

Figure 2. Transmodulation of HER2 and EGFR by SP in human BC cell lines. A, Heatmap depicting the relative qPCR expression levels for *EGFR*, *ERBB2*, *ERBB3* and *ERBB4* genes and for (B) the *TAC1*, *TACR1*, *TACR2* and *TACR3* genes in different BC cell lines. The expression levels are represented as normalized values of $2^{-\Delta\Delta Ct}$. C, Immunofluorescence showing the expression of SP and NK-1R in different BC cell lines. Scale bar represents 50 microns. D, Representative images of

Western blots showing the phosphorylation (denoted by p- throughout the manuscript) of HER2 (Y1248), EGFR (Y1048 and Y845), p42/44-MAPK and Akt at 0, 1, 2, 4, 6, 8, 10, 15 and 30 minutes after SP 100 nM stimulation in different BC cell lines. Tubulin was used to ensure the equal protein loading. The quantification of the blots is shown in Supplementary Fig. S2.

Figure 3. SP-mediated transactivation of HER2 in primary cultures derived from human BC samples. A, Schematic outline of the experimental design used to study the effects of SP on HER2 activation in short-term BC primary cultures. B, Estimated least squares means (LSM) of basal phosphorylated HER2 Y1248 fluorescence intensity in tumor subtypes (control point). C, Representative immunofluorescence images showing the expression of pHER2 Y1248 and pan-cytokeratin antigens in human BC cells treated with SP. Scale bar represents 20 microns. D, Quantification of the immunofluorescence signal for pHER2 Y1248; values are expressed as estimated LSM of phosphorylated HER2. The effects of SP on each subgroup are shown as control (white dot), SP 6 minutes (light green dot) and SP 10 minutes (dark green dot) for each sample. Statistical differences of LSM are indicated as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Error bars represent 95% confidence interval.

Figure 4. NK-1R signaling modulates EGFR and HER2 activity in BC cells. Representative images of Western blots corresponding to experiments showing: A, the contribution of NK-1R to the phosphorylation of HER2 and p42/44-MAPK in the MDA-MB-231 cell line transfected with pcDNA3.1(+)-*TACR1* or the empty vector and treated with SP 100 nM; B, the effects of *TACR1* inhibition by siRNA on the steady state of pHER2 in different BC cell lines. NK-1R expression is shown as a control for siRNA efficiency; C, the effects of L-733,060 20 μ M (SKBR3), 20 μ M (BT-474), 30 μ M (MDA-MB-453)) on the phosphorylated and total levels of HER2, EGFR, p42/44-MAPK and Akt in different BC cell lines; D, the effects of the triple inhibition of NK-1R, NK-2R and NK-3R with L-733,060 (20 μ M), MEN 10376 (30 μ M) and SB 218795 (20 μ M) respectively, on

the phosphorylation of HER2, p42/44-MAPK and Akt in the SKBR3 cell line; E, the effects of the G-protein inhibitor pertussis toxin (PTX) on the phosphorylated and total levels of HER2, p42/44-MAPK and Akt in different BC cell lines. The plots accompanying each panel show the densitometric quantification of the Western blots relative to the expression of tubulin, which was used to ensure equal protein loading. All the Western blots and the quantitative data are for a minimum of 3 replicates and are presented as mean + S.D. and compared by *t*-test (two-tailed) as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 5. Modulation of tumor growth *in vivo* by SP/NK-1R. A, Volume of MDA-MB-231 and MDA-MB-453 xenograft tumors treated with saline or SP for 27 days (mean \pm s.e.m); B, final tumor weight. Sample size (n) is indicated. C, Western blot of MDA-MB-231 and MDA-MB-453 xenografts, and box plots showing the densitometric quantification (right panels, tubulin was used to ensure equal protein loading) for the proteins indicated. D, Representative images for the immunohistochemical detection of HER2. Scale bar represents 50 microns. Volume of (E) MDA-MB-453, (F) MDA-MB-468 and (G) MDA-MB-231 xenograft tumors treated with the NK-1R inhibitor L-733,060 (mean \pm s.e.m) and (H, I, J) final tumor weight, respectively. Western blot of (K) MDA-MB-453, (L) MDA-MB-468, and (M) MDA-MB-231 xenografts and box plots showing the densitometric quantification (right panel, tubulin was used to ensure equal protein loading) for the proteins indicated. The boxes show the 25th to 75th percentile, and the whiskers extend to the 5th and 95th percentiles. Yellow diamonds in each plot indicate the mean. Data was compared by *t*-test (two-tailed). Significant *P* values are indicated as * $P < 0.05$, and ** $P < 0.01$.

Figure 6. The NK1 antagonist L-733,060 synergizes with anti-HER2 therapies. MTT assays comparing IC50 for the drugs indicated. Colored circles represent IC50 \pm 95% confidence interval for each treatment in (A) MDA-MB-453 and (B) MDA-MB-468 upon short (72 hours) or chronic (5 months) SP exposure. Curve fitting and statistical analysis was performed by *F* test (extra sum of

squares). Significant P values are indicated as * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. C, Combination assay comparing the effect of NK-1R antagonist L-733,060 with the combination of AG825, AG1478 and Lapatinib. Diamonds represent CI of drug interactions.

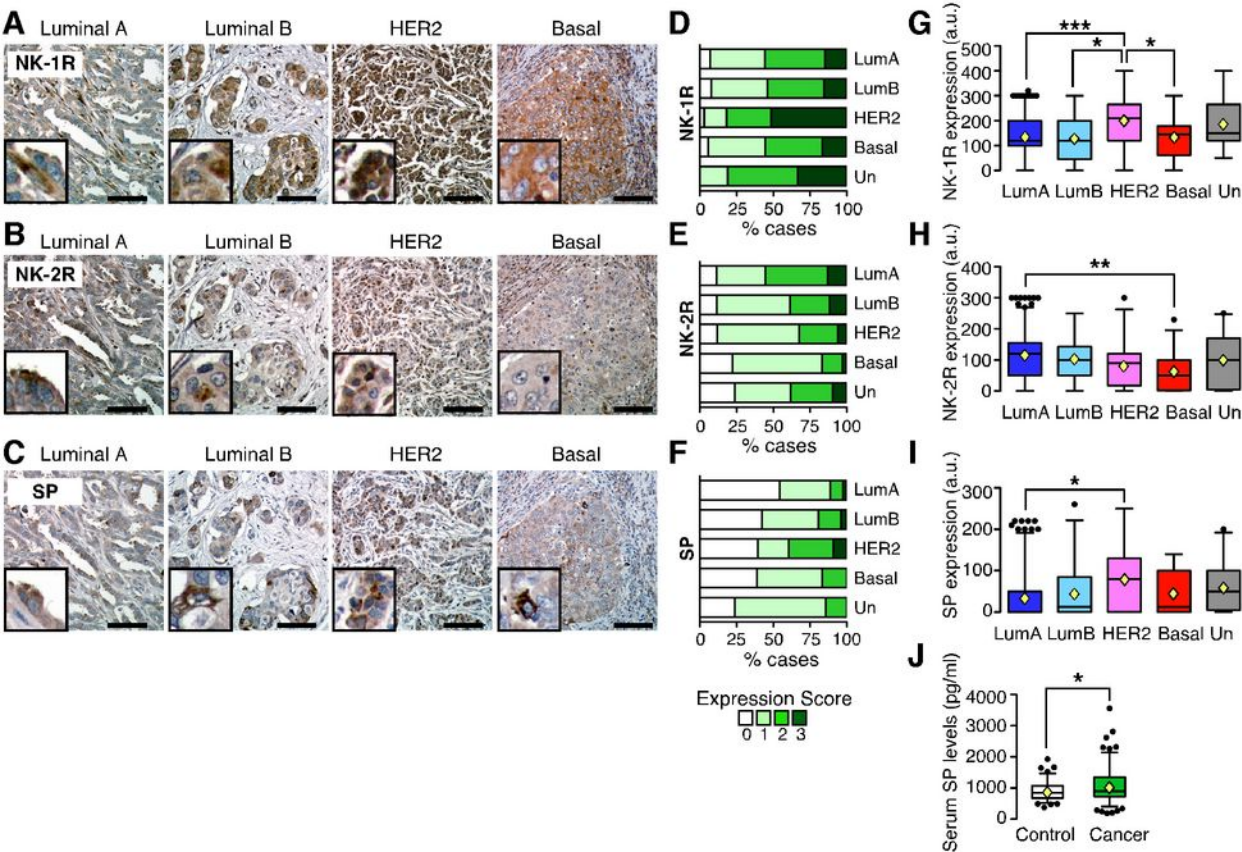
Figure 1

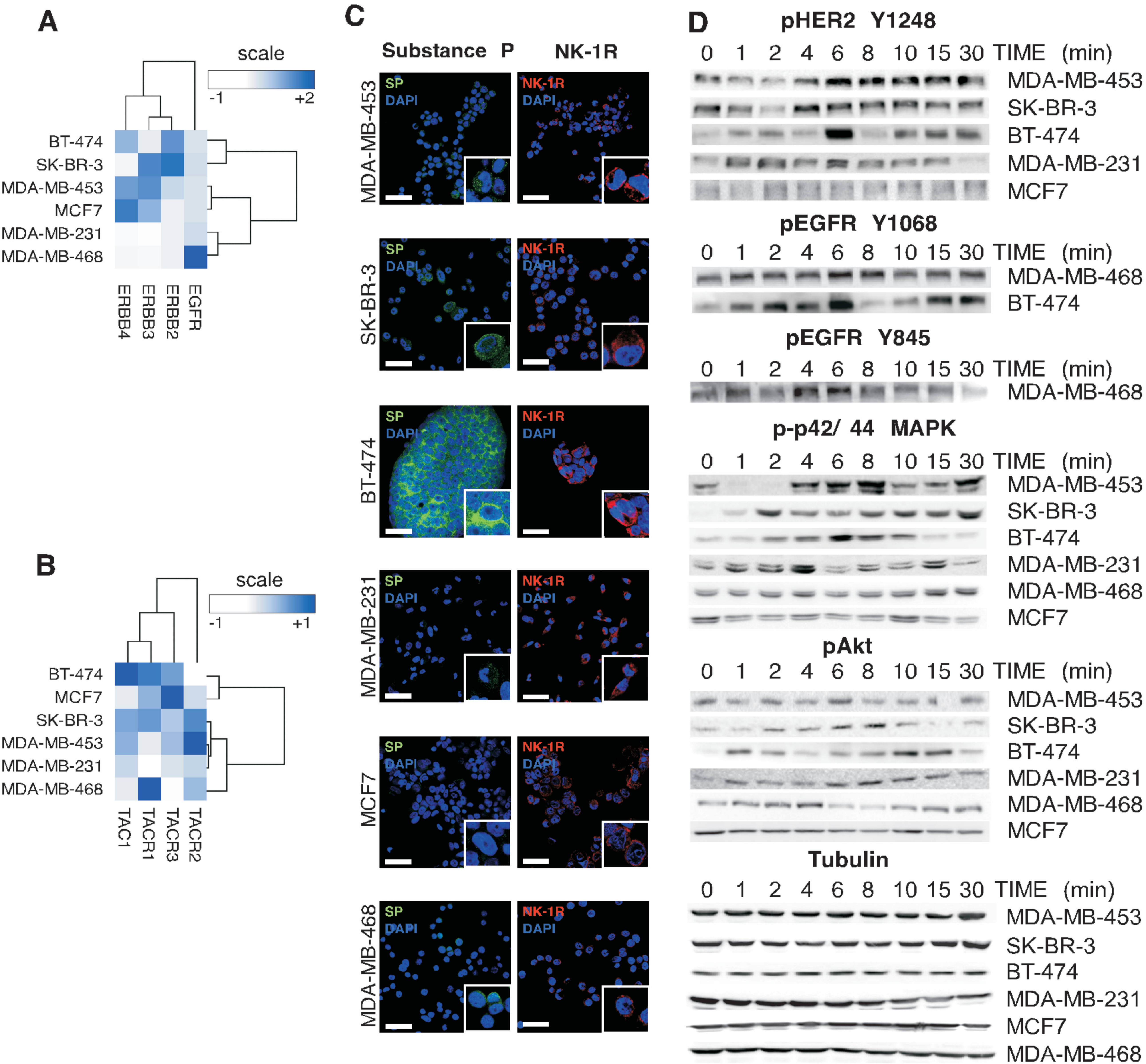
Figure 2

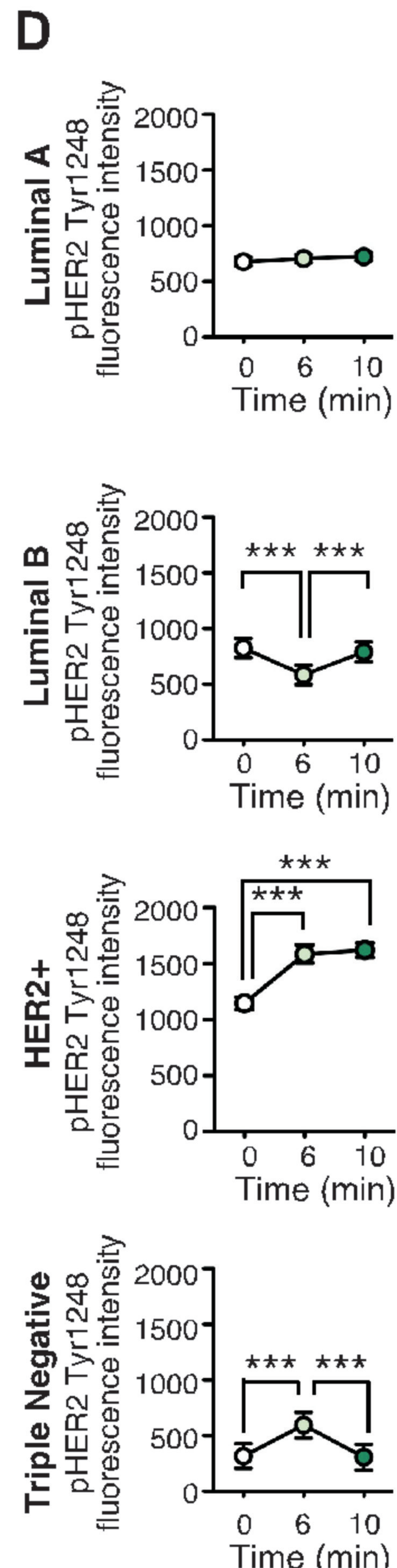
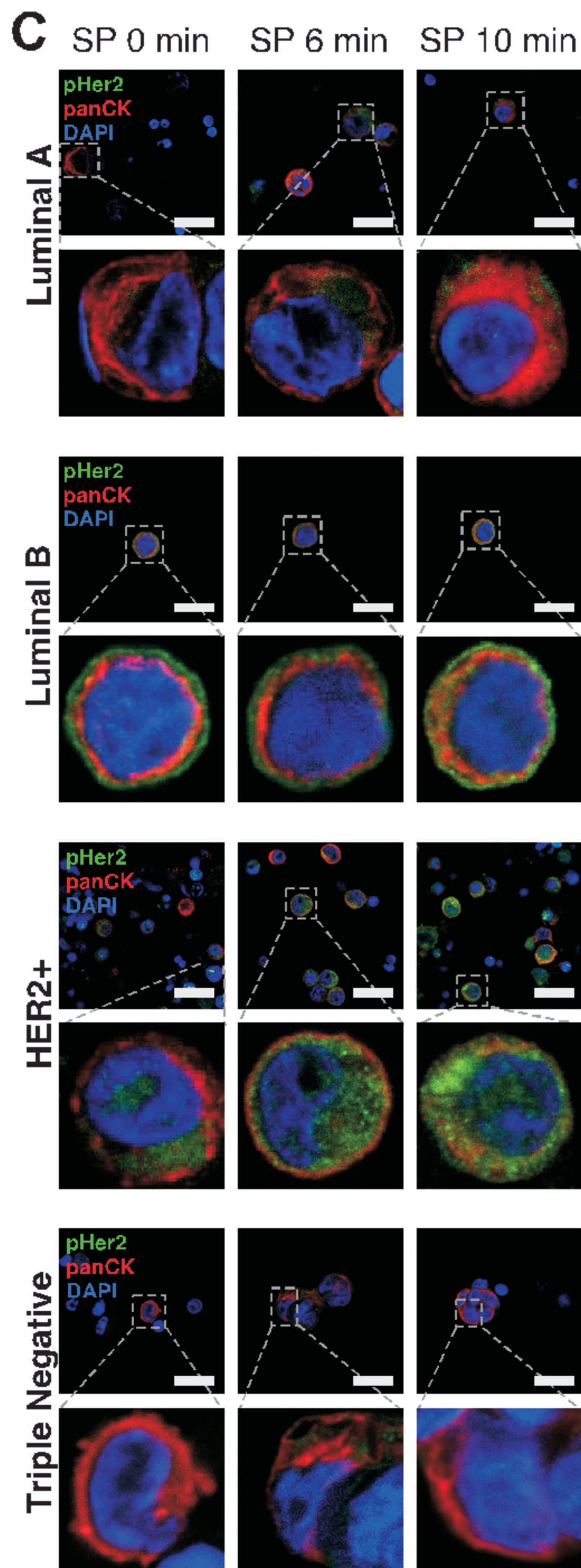
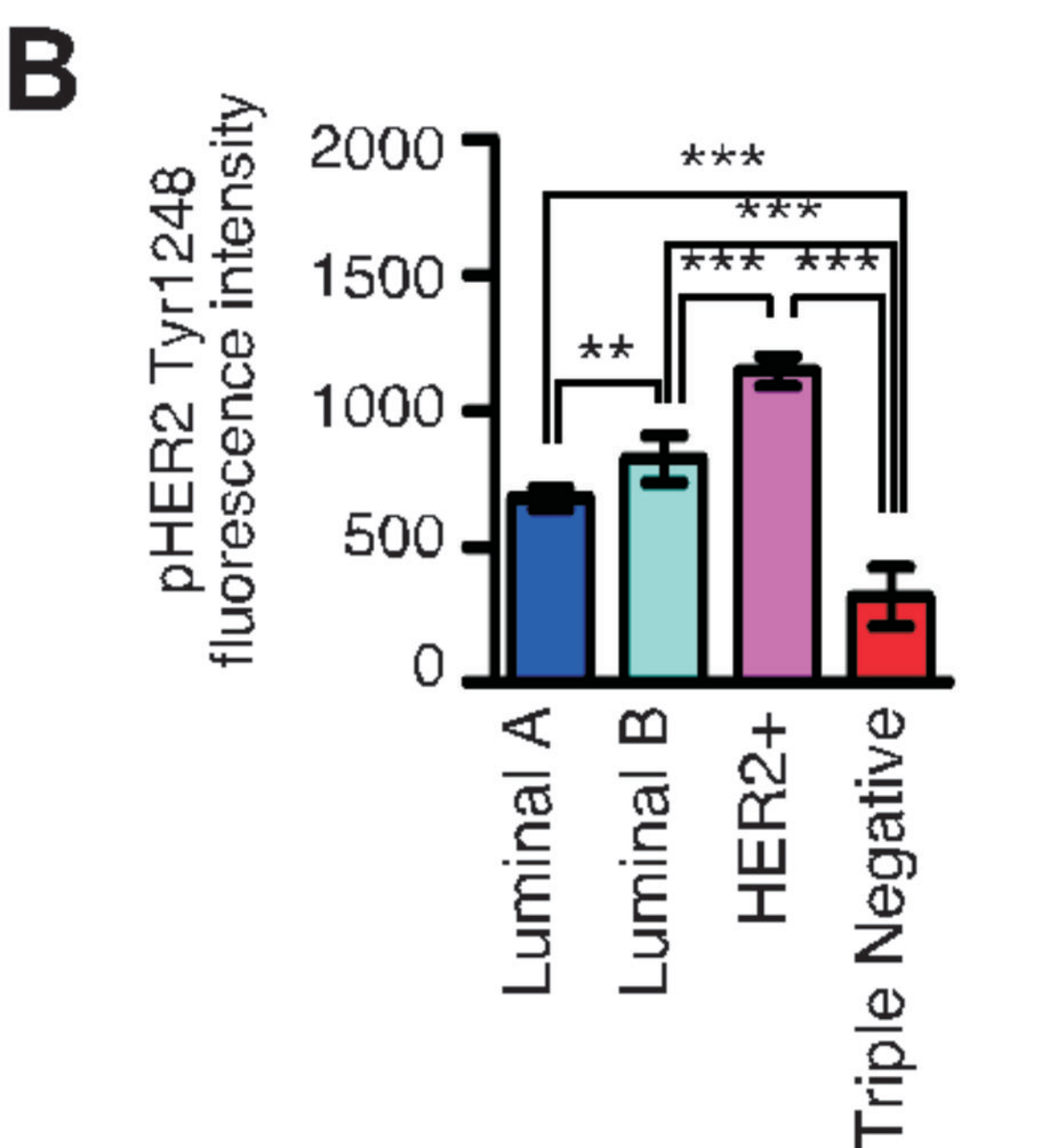
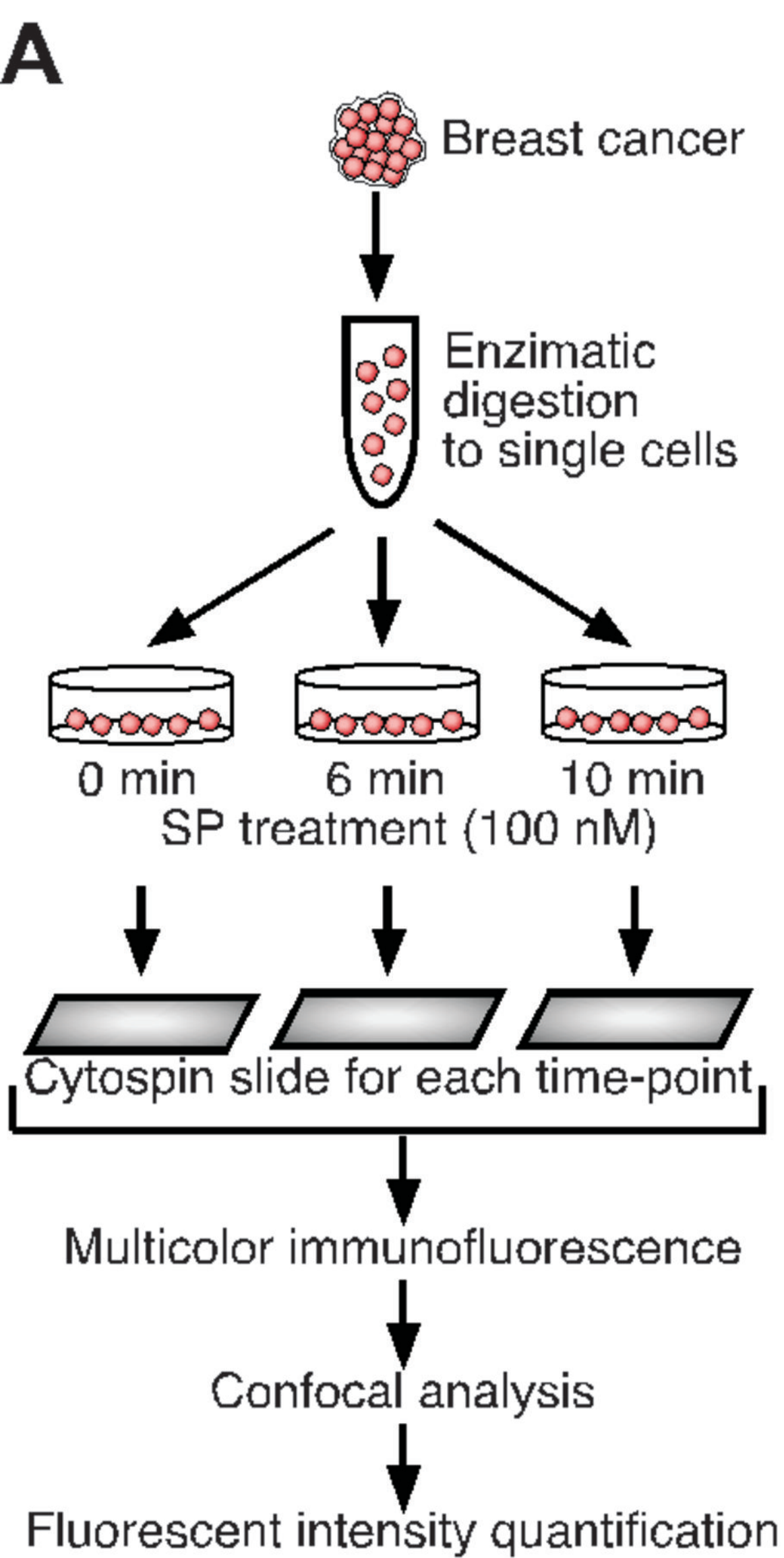
Figure 3

Figure 4

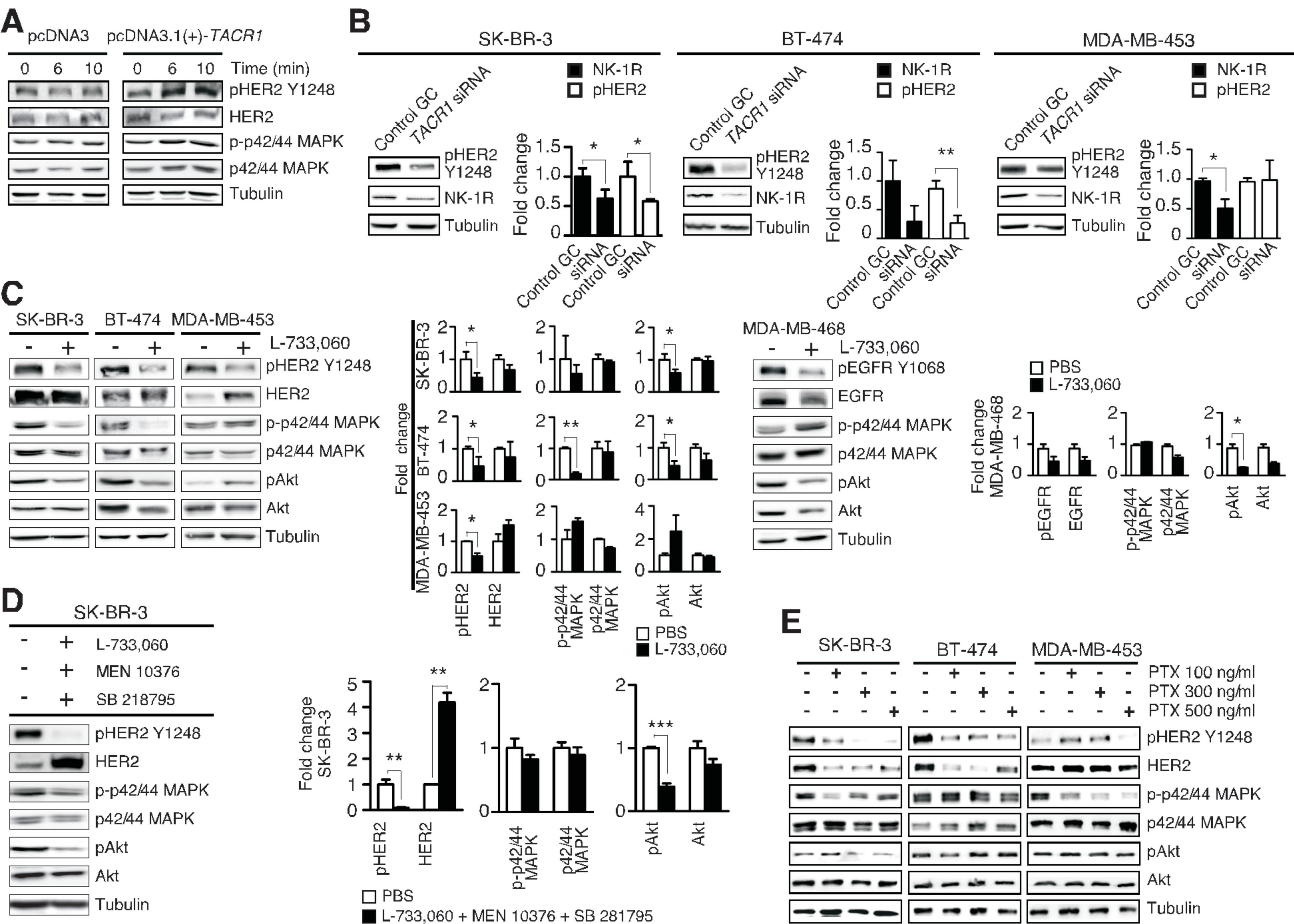


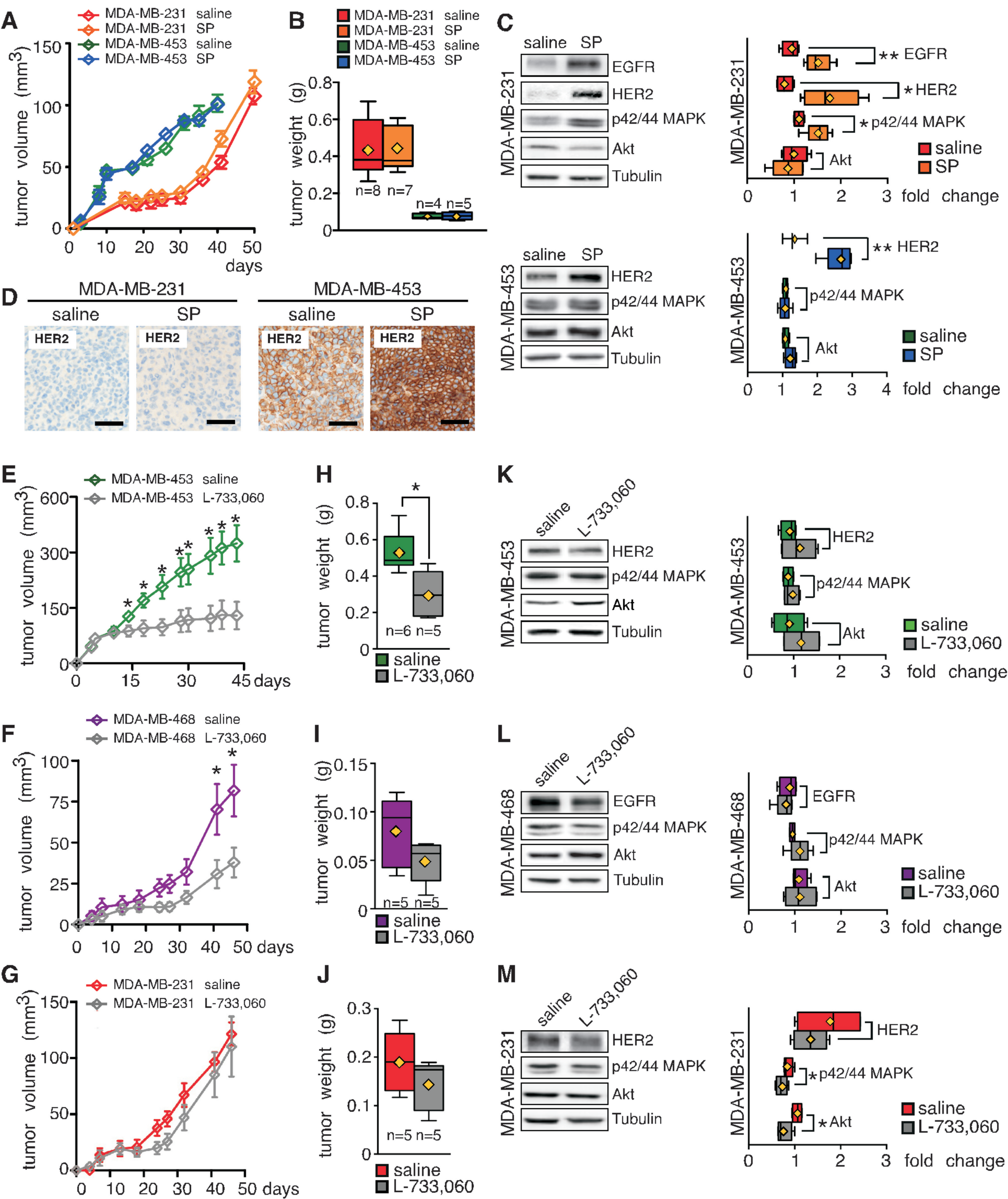
Figure 5

Figure 6