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Trastuzumab inhibits pituitary tumor cell growth modulating the TGFB/SMAD2/3 pathway

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ABSTRACT

In pituitary adenomas, early recurrences and resistance to conventional pharmacotherapies are common, but the mechanisms involved are still not understood. The high expression of epidermal growth factor receptor 2 (HER2)/extracellular signal-regulated kinase (ERK1/2) signal observed in human pituitary adenomas, together with the low levels of the antimitogenic transforming growth factor beta receptor 2 (TBR2), encouraged us to evaluate the effect of the specific HER2 inhibition with trastuzumab on experimental pituitary tumor cell growth and its effect on the antiproliferative response to TGFB1. Trastuzumab decreased the pituitary tumor growth as well as the expression of ERK1/2 and the cell cycle regulators cyclin D1 and CDK4. The HER2/ERK1/2 pathway is an attractive therapeutic target, but its intricate relations with other signaling modulators still need to be unraveled. Thus, we investigated possible cross-talk with TGFB signaling, which has not yet been studied in pituitary tumors. In tumoral GH3 cells, co-incubation with trastuzumab and TGFB1 significantly decreased cell proliferation, an effect accompanied by a reduction in ERK1/2 phosphorylation, an increase of SMAD2/3 activation. In addition, through immunoprecipitation assays, a diminution of SMAD2/3-ERK1/2 and an increase SMAD2/3-TGFBR1 interactions were observed when cells were co-incubated with Trastuzumab and TGFB1. These findings indicate that blocking HER2 by trastuzumab inhibited pituitary tumor growth and modulated HER2/ERK1/2 signaling and consequently the anti-mitogenic TGFB1/TBRs/SMADs cascade. The imbalance between HER2 and TGFBRs expression observed in human adenomas and the response to trastuzumab on experimental tumor growth, may make the HER2/ERK1/2 pathway an attractive target for future pituitary adenoma therapy.

INTRODUCTION

Pituitary adenomas occur in around 10-15% of all intracranial neoplasms, but only a few of these are symptomatic according to estimations from various clinical studies (Asa and Ezzat 1998; Kaltsas, et al. 2005; Tomita and Gates 1999). Although the vast majority are benign, between 25 and 55% of pituitary adenomas are invasive, with some exhibiting clinically aggressive behavior (Ironside 2003; Kaltsas and Grossman 1998). The initial treatment of pituitary adenomas is based on protocols designed for each tumor type. Dopamine agonists have become the initial treatment for most patients with prolactin-secreting tumors, whereas for growth hormone□producing tumors surgical resection is the first-line therapy, while somatostatin analogs are used in patients with large tumors. However, 20 % of patients are refractory to these medical treatments (Di Ieva, et al. 2014).

In normal pituitary tissue, the stimulatory and inhibitory growth signals are finely regulated, with alterations in these being directly or indirectly associated with tumorigenesis. The transforming growth factor beta 1 (TGFB1) and epidermal growth factor (EGF) have opposite functions but play essential roles in tumor development by converting them into attractive therapeutic targets (Yarden and Sliwkowski 2001). The human EGF receptor family consists of four members (HER/ERBB1-4) (Roskoski 2014), with the HER receptors having of an extracellular domain, a single hydrophobic transmembrane segment, and an intracellular domain with protein kinase activity (Dworakowska, et al. 2009). Dimerization of the HER receptors leads to the induction of kinase activity, which as a result a number of tyrosine residues at the C terminal end of the HER molecules become phosphorylated. The two major signaling pathways activated by HER receptors are the MEK/ERK and PI3K-AKT pathways (Yarden and Sliwkowski 2001), which both participate in cell proliferation, angiogenesis, cell adhesion, cell motility and tumorigenesis (Seshacharyulu, et al. 2012). Certain components of these pathways may be activated/inactivated by mutations or

epigenetic silencing, and dysregulation of the components of these cascades can contribute to resistance to other pathway inhibitors and chemotherapeutic drug resistance (McCubrey, et al. 2012). In addition, the MEK/ERK and PI3K/AKT cascades are often activated by genetic alterations in upstream signaling molecules such as receptor tyrosine kinases. In this way, several malignancies are associated with the mutation or increased expression of members of the HER family, including lung, breast, stomach, colorectal, head, neck, and pancreatic carcinomas, as well as glioblastomas (Roskoski 2014). HER2 is a more potent oncoprotein than the other HERs, and thus blocking their action might inhibit a myriad of mitogenic pathways affecting the HER2-expressing tumor cells (Onishi, et al. 2016). Although several new strategies are currently being developed trastuzumab (a drug designed to block the receptor HER2) has been the first to attain clinical use, mainly for the treatment of metastatic breast cancer (Baselga, et al. 1996; Cobleigh, et al. 1999).

The expression of HER2 has been reported in pituitary tumors (Ezzat, et al. 1997; Nose-Alberti, et al. 1998; Roncaroli, et al. 2003), including prolactinomas (Vlotides, et al. 2009), and it has been demonstrated that overexpression of constitutively active HER2 markedly induces PRL expression and secretion as well as cell growth in rat GH3 lactotroph tumor cells, effects that are blocked by Lapatinib, a dual tyrosine kinase inhibitor of both epidermal growth factor receptors HER1 and HER2 (Fukuoka, et al. 2011). For signaling pathways activated by HER2, there are studies showing increases in MEK/ERK activity in pituitary tumors (Cakir and Grossman 2009). However, although the MEK/ERK signaling pathway plays a central role in the regulation of cell proliferation, differentiation and survival, its exact functional relevance in complex signaling network, pituitary tumorigenesis and resistance to conventional medical treatments is not fully understood, but it may enable the development of attractive novel strategies for treating common tumors.

It is well known that the MEK/ERK is not a unidirectional cascade of protein kinases, but forms a complex signaling network with many interactions including inhibitory cascades. Recently, we demonstrated that the TGFB1-antimitogenic effect in pituitary tumor cells was attenuated by the MEK/ERK1/2 pathway via modulating SMAD2/3 phosphorylation (Petiti, et al. 2015). TGFB1 signaling is mediated by TGFB type 1 receptor (TBR1) and TBR2. Upon TGFB binding, the TBR2 receptors activate the TBR1 receptors, inducing phosphorylation of mothers against the decapentaplegic homologs SMAD2 and SMAD3 which form trimers with Smad4 that translocate into the nucleus, where they regulate the expression of genes that control cell cycle progression (Kang, et al. 2009).

One of the principal obstacles to the development of new antitumoral therapies is that the inhibition of a unique signaling pathway essential for the cell survival is a pharmacological strategy made ineffective due to the tumoral cells utilizing alternative cascades and promoting a paradoxical enrichment of resistant cells. In this sense, the study of the crosstalk between the signals from HER and TBRs in pituitary tumors might permit a better understanding of the altered balance between the positive and negative regulators that control the pituitary tumor growth which may condition the therapeutic response. The HER/ERK1/2 pathway is an attractive therapeutic target as an alternative medical therapy for pituitary tumors. In order to safely choose the candidate drugs, their intricate relations, positive and negative feedback loops and signaling modulators must be thoroughly understood. In the present study, our objectives were to determine the effect of the specific HER2 receptor inhibitor trastuzumab on pituitary tumor growth, and to evaluate its impact on the antiproliferative response to TGFB1.

MATERIALS AND METHODS

Human pituitary tissues and tumors

The study group included 20 patients diagnosed with pituitary adenomas from Hospital Córdoba, Sanatorio Allende or Clínica Reina Fabiola, Córdoba, Argentina in the period 2004-2015. Pituitary tumors were obtained from consented patients who had not been treated with radiotherapy by trans-sphenoidal surgery after full endocrine preoperative evaluation. The fresh human pituitary adenoma tissue at the time of surgery was obtained from 12 functioning adenomas (4 PRL-secreting, 6 GH-secreting and 2 ACTH-secreting tumors) and 8 non-functioning adenomas. The samples were processed for immunohistochemical analysis (Table 1 and Table 2). In addition, a group of tumor samples were processed for western blot. Non-tumor human adenohypophyses (n=6) were obtained from autopsies from patients with no evidence of endocrine abnormality and examined histologically. The present project was approved by the Ethics Committee (Repis N° 37/2014).

Animal model

Three-month-old Fischer-344 male rats were bred and housed at the Animal Research Facility of the INICSA-CONICET-School of Medicine, National University of Córdoba, under controlled temperature (21±3 °C) and lighting conditions (14 h light:10 h darkness cycle), with free access to commercial rodent food and tap water. Forty-three rats were randomly assigned to four groups: Group C (n=13): intact animals as control; Group E (n=13): intact animals treated with estradiol benzoate pellets (30 mg, Sigma–Aldrich, St. Louis, MO, USA), as described by Sahores et al. (Sahores, et al. 2013), for 45 days to induce a PRL pituitary tumor development (Asa and Ezzat 2002); Group T (n=4): animals treated with trastuzumab 6 mg/kg (Herceptin, Roche) during 15 days; Group E+T (n=13): during the

last 15 days before the end of the estradiol treatment, the rats received trastuzumab. The treatments with trastuzumab consisted of two intraperitoneal injections given once a week following protocols previously reported (Eryilmaz, et al. 2015; Guler, et al. 2009; Hendry, et al. 2016).

All animals were treated in agreement with the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (1996), and experiments were approved by the Institutional Animal Care Committee of the School of Medicine, National University of Córdoba.

Immunohistochemistry

Paraffin-embedded pituitary glands were processed by immunohistochemistry for HER2, pHER2, TBR1, TBR2, and Ki67. The sections were blocked and incubated ON with anti-HER2 (1:300: Dako, Glostrup, Denmark), anti-pHER2 (1:300; Santa Cruz Biotechnology, Dallas, TX, USA), anti-TBR1 (1:200; Santa Cruz Biotechnology, Dallas, TX, USA), anti-TBR2 (1:200; Santa Cruz Biotechnology, Dallas, TX, USA) or anti-Ki67 (1:75; BD Biosciences, San Jose, CA, USA) at 4°C. The sections were then incubated with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) and streptavidin (Dako, Glostrup, Denmark). The slides from three animals of each experimental condition were photographed in randomly chosen fields at 400X. The quantification of the expressions was assessed by subsequent manual counting of positive and negative cells per highpower visual field, with more than 2000-immunoreactive cells being examined.

181 *Detection of apoptosis by DNA nick-end labeling*

182 Nick-end labeling was detected using the TUNEL technique, as previously reported
183 (Palmeri, et al. 2009), following the manufacturer's protocol (In Situ Cell Death Detection
184 Kit; Roche). The slides were observed using a Zeiss Axiostar plus microscope at 400x.

185

186 *Cell culture*

187 The rat GH3 lactosomatotroph pituitary tumor cell line (ATCC®-BAA-1926^{TM}) was
188 used as an *in vitro* pituitary tumoral model (McIntyre, et al. 2004). The cells were cultured in
189 Ham's F-12 Nutrient Mixture medium supplemented with fetal bovine and horse serum, and
190 antibiotics. After 3 days of culture and with a confluence of 70%, the cells were submitted to
191 different experimental protocols. Cell cultures were treated with TGFB1 (4 ng/ml, Sigma; St
192 Louis,USA), Trastuzumab (100ug/mL) or the combination of both factors for 30 min or 24 h.

193 *Flow cytometry staining for detection of Ki67*

194 To analyze GH3 proliferation by Ki67 detection, the cells were detached using TrypLE™
195 Express (Gibco, NY, USA), washed twice with PBS and fixed overnight with ice-cold 70%
196 ethanol. After washing, cells were incubated with anti-Ki67 (1:75; BD Biosciences, San Jose,
197 CA, USA) at 37°C for 45 min, followed by incubation with secondary antibody Alexa-Fluor
198 488 (1:1000; Invitrogen, Carlsbad, California, USA) for 1h at 37°C. Finally, GH3 cells were
199 suspended in PBS and run on a flow cytometer (FACSCanto™ II, BD Biosciences). The data
200 obtained was analyzed by FlowJo V10 software (Tree Star,Inc, Ashland, OR, USA).

201

202 *Immunocytochemical detection of BrdU uptake*

203 GH3 cells at the DNA-synthesizing stage were identified by the immunocytochemical
204 detection of BrdU. After of stimulation with the different reagents, BrdU (200nM) was added
205 for an additional 4h. The cells attached to coverslips were fixed in 4% formaldehyde in PBS

for 2h at room temperature (RT) and BrdU incorporation detection was performed as described by Ferraris and co-workers (Ferraris, et al. 2014)

Immunoprecipitation

Protein extraction of tumoral pituitary cells (1 mg of protein) was carried out according to previous protocols (Sosa Ldel, et al. 2013), which was then subjected to immunoprecipitation using specific anti-pSMAD2/3 (5µg/mL, Santa Cruz Biotechnology, Dallas, TX, USA) or Trastuzumab (5µg/mL). The immune complexes were adsorbed and precipitated using Protein A-Sepharose beads (Sigma–Aldrich, St. Louis, MO, USA), washed 3 times with lysis buffer and denatured by boiling for 5 min in the sample buffer. Parallel immunoprecipitations were performed using a non-immune goat serum, which verified the specificity of the bands detected by western blotting using anti-TBR1 (1:200), anti-pSMAD2/3 (1:1000 Cell Signaling, Massachusetts, USA), and anti-pERK1/2 (1:1000 Cell Signaling, Massachusetts, USA)

Analysis of cell-surface proteins by biotinylation

The cell-surface proteins were labeled according manufacture protocol (Pierce™ Cell Surface Protein Isolation Kit (Prod#89881). Briefly, GH3 and MCF7 cell cultures were washed three times with ice-cold PBS buffer, pH 8, and then incubated with membrane impermeable sulfo-NHS-biotin at a final concentration of 0.3 mg/ml in PBS, pH 8, at 4 °C for 20 min. After biotinylation, the cultures were washed three times with ice-cold PBS, and harvested with lysis buffer containing 1.25% Igepal CA-630, 1 mM EDTA, 2 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin (all inhibitors were from Sigma, St. Louis, MO). The cellular extracts were sonicated and incubated with avidin-agarose beads (Pierce, Rockford, IL) for 2 h at 4 °C. Then, the extracts were centrifuged at 500g for 10 min at 4 °C, the

supernatant (200 µl) was separated and the pellet was washed three times with 1 ml of lysis buffer. The proteins from the supernatant fractions were run in 7.5% acrylamide gel. Both pellet and supernatant fractions were analyzed by Western blot using specific antibodies.

Western blot analysis

Protein extracts (30 µg) were separated in a polyacrylamide gel (Sigma–Aldrich, St. Louis, MO, USA), transferred to a nitrocellulose membrane, and nonspecific binding blocked with PBS-5% non-fat dried milk at RT. The membranes were then rinsed and incubated overnight with anti-HER2 (1:1000), anti-pHER2 (1:350), anti-TBR1 (1:300), anti-TBR2 (1:300), anti-Cyclin D1 (1:300, Santa Cruz Biotechnology, Dallas, TX, USA), anti-CDK4 (1:400, Abcam, Cambridge, UK), anti-pSMAD2/3 (1:1000 Cell Signaling, Massachusetts, USA), anti-SMAD2/3 (1:1000, Cell Signaling, Massachusetts, USA), anti-TERK1 (1:500, Santa Cruz Biotechnology, Dallas, TX, USA), anti-pERK1/2 (1:1000, Cell Signaling, Massachusetts, USA) or anti-b-Actin (1:4000; Sigma Aldrich, St. Louis, MO, USA).

The blots were incubated with HPRT-conjugated bovine anti-goat (1:2500; Santa Cruz Biotechnology, Dallas, TX, USA), goat anti-mouse (1:2500, Jackson ImmunoResearch, West Grove, PA, USA) or goat anti-rabbit secondary antibodies (1:5000, BioRad, Hercules, California, USA). The membranes were thoroughly rinsed in TBS 0.1% Tween 20, and the HPRT-coupled secondary antibody was revealed with enhanced chemiluminescence Western blotting detection reagents (GE Healthcare, Little Chalfont, UK). Emitted light was captured on Hyperfilm (GE Healthcare, Little Chalfont, UK).

Confocal laser scanning microscopy

Pituitary cells were fixed in 4% formaldehyde, permeabilized in 0.25% Triton X-100 in PBS, blocked for 1 hour in PBS 3% BSA, and incubated with anti-HER2 (1:200) for 1 h.

Then, cells were incubated with Alexa Fluor 488 antirabbit antibody (1:2000; Invitrogen, Carlsbad, California, USA) for 1 h. Images were obtained using the inverted confocal laser scanning microscope FluoView FV 1200 (Olympus, Center Valley, PA). Serial z-axis sections were collected with an X60 objective and analysis of the confocal microscopy images was carried out using FV10-ASW 1.6 Viewer software.

Immunogold electron microscopy (EM)

The subcellular localization of the TGFB receptors in pituitaries from rats was examined by an ultrastructural immunocytochemical technique using protocols previously standardized in our laboratory (Petiti, et al. 2009). The grids were incubated with anti-TBR1 (1:300) or anti-TBR2 (1:300) overnight at 4°C, and then with anti-mouse or antirabbit secondary antibodies conjugated to 15 nm (Electron Microscopy Science, Hatfield Pennsylvania, USA) colloidal gold particles (1:30). The following controls were performed: 1) replacement of primary antiserum with PBS 1% BSA; and 2) replacement of primary antiserum with diluted pre-immune serum followed by the secondary antibody. Then, the sections were examined in a Zeiss LEO 906-E transmission EM (TEM) (Zeiss, Oberkochen, Germany) and photographed with a megaview III camera (Olympus, Center Valley, PA).

Correlative Light and Electron Microscopy (CLEM)

CLEM was carried out on ultrathin cryo-sections by applying the Tokuyasu technique as described by Oorschot (Oorschot, et al. 2014). Briefly, GH3 cells were fixed in 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and 0.2 % glutaraldehyde (Electron Microscopy Science, Hatfield, Pennsylvania, USA) for 2 h, embedded in 12 % gelatin, infiltrated with 2.3 M sucrose (Sigma-Aldrich, St. Louis, MO, USA) overnight at 48 °C, frozen in liquid nitrogen and sectioned at -120°C (100 nm cryosections) with a cryo-

ultramicrotome RMC PowerTome-XL (RMC Boeckeler, Tucson Arizona, USA). Flat ribbons of 100nm thick cryo-sections, collected with 1.15 M sucrose and 1% methylcellulose (Sigma-Aldrich, St. Louis, MO, USA), were transferred on formvar-coated 100-µm mesh hexagonal nickel grids (Electron Microscopy Science, Hatfield, Pennsylvania, USA).

For immunolabeling, samples were incubated on 2% gelatin in PBS for 20 min at 37°C, blocked, incubated with anti-HER2 1:50 ON at 4°C, rinsed and incubated with anti-rabbit Alexa-Fluor 594 (Invitrogen, Carlsbad, California, USA) for 1h at 37°C in the presence of 40,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA). For immunogold labelling, grids were incubated with gold conjugated protein-A 1:15 at 37 °C for 1h.

For fluorescence light microscopy (FLM), grids layered with a 200nm coat of 2% methylcellulose were mounted between a microscope slide and coverslip with 50% glycerol in water. For TEM, grids were unmounted, washed in Milli-Q water and incubated for 5 min in 0.4% uranyl acetate and 1.8% methylcellulose. Fluorescence images were obtained using a FluoView FV 1200 microscope and EM images using a Zeiss LEO 906-E TEM. The analysis was carried out with ImageJ software.

Double-Immunohistochemistry on pituitary cryosections

To assess co-expression of Ki67/PRL, Ki67/GH, Ki67/ACTH and Trastuzumab-HER2/PRL, additional sets of pituitaries from different experimental conditions were used for confocal microscopy. Pituitary semi-thin cryo-sections were obtained by the Tokuyasu technique as described above. Then, the pituitary slices were incubated ON with anti-PRL (1/3000), anti-GH (1/3000), anti-ACTH (1/1000), anti-Ki67 (1/50) or Trastuzumab (1/1000) at 4ºC. These slices were washed and incubated with FITC anti-human, Alexa 594 anti-rabbit or Alexa 488 anti-mouse secondary antibodies (1:3000; Dako, Invitrogen) for 1 h, and with

DAPI for 10min. The images were then obtained using a FluoView FV 1200 microscope. The analysis of the images was carried out using ImageJ software.

Computational structural analysis

The crystal structures and amino acid sequences of the extracellular region of rat sHER2 (code:1n8y) and human sHER2 (code1: n8z) (Cho, et al. 2003) were downloaded from the Protein Data Bank (www.rcsb.org) and superposition of the structures was calculated with the program ProFit (http://www.bioinf.org.uk/software/profit/). Sequence alignment was performed with ClustalX (Larkin, et al. 2007), molecular images were created with VMD (Humphrey, et al. 1996), and the effect of change of the amino acid sequence on binding affinity was calculated with MutaBind (Li, et al. 2016).

Statistical analysis

The experimental points represented the mean ± SEM of 3 replicates measured in 3 independent cell cultures. A statistical analysis was carried out using the t-test or ANOVA followed by the Fisher test using the InfoStat program. Significance levels were chosen as $P < 0.05$.

RESULTS

Expression of HER2 and TBRs in human pituitary adenomas

To investigate the protein expression of HER2 and TBRs in normal pituitaries (n=6) and in different types of human pituitary adenoma samples (table 1, n=20), we examined these receptors by immunohistochemistry (IHC, n=12) or western blot (WB, n=8) in non-functioning and functioning tumors. As shown in table 2, HER2 expression was present in

three out of four prolactinomas with different percentages of positive cells (10%, 44% and 27%); and also in one out of three and one out of four in GH-secreting and non-functioning tumors respectively, with a high percentage of HER2+ cells (79% and 98%) and strong staining (+++) observed, which was similar to the percentage present in the ACTH-secreting adenoma (80%). Ductal human breast carcinoma tissue samples were used as immunoreactive controls (Figure 1A).

It has been previously reported that the phosphorylation status of HER2 protein provides more accurate information on the clinical outcome of patients undergoing trastuzumab treatment (Hudelist, et al. 2006). We analyzed the expression of pHER2 by IHC in seven pituitary adenomas including two PRL, GH-secreting and non-functioning ones, which were positive or negative for HER2, and also in an ACTH-secreting pituitary adenoma (table 2). The pHER2 was detected in the GH, ACTH-secreting and non-functioning pituitary adenomas that were highly positive for HER2, while the prolactinomas did not express pHER2. An interesting finding was the detection of pHER2 in the HER2 negative non-functioning adenoma (table 2 and Figure 1A).

With respect to the expression of the TGFB receptors, we observed a varied expression in the different pituitary tumors. The quantification of the different receptors in non-tumor pituitaries is showed in table 2.

In parallel with the IHC analyzes, we determined the HER2 expression in eight other patients with pituitary adenomas (four GH secreting and four non-functioning adenomas) and 5 non-tumor pituitaries using WB, revealing a marked expression in pituitary tumor samples compared to non-tumor pituitaries (Figure 1B). None of the pituitary tumor sample were observed to be positive to pHER2 (ptyr-1248 HER2) (Figure 1B).

With respect to the TGFB receptors, we observed a reduced expression of TBR2 in pituitary tumors compared to non-tumor pituitaries (Figure 1B), while no differences were detected for TBR1.

In addition, in order to investigate HER/ERK1/2 signaling, we determined the activation of ERK1/2 in human pituitary adenomas by WB. A significant increase in the phosphorylation of ERK1/2 was observed in the pituitary tumors analyzed compared to non-tumor tissue (Figure 1B).

Trastuzumab effect on rat pituitary tumors in vivo

To evaluate *in vivo* effects of HER2 inhibition on pituitary tumor growth, we used experimental PRL pituitary tumors, which were treated with trastuzumab. First, we determined whether trastuzumab, as the primary antibody (Bussolati, et al. 2005; Glazyrin, et al. 2007), could bind the HER2 receptor in different human samples as well as in extracts from the rodent model (Estrogen-treated Fisher rat) and rat GH3 cells. As shown in figure 2, trastuzumab recognized the HER2 receptor in the human tumors and in different rat samples, as was confirmed by the presence of a band at 185 kDa, which corresponded to the molecular weight of HER2 from total extract samples (Figure 2A) and purified cell surface proteins samples (Figure 2B). With the aim of further strengthening these results, we investigated the trastuzumab/HER2 interactions in rat GH3 cells and in human breast tumor sample with HER2 amplification by immunoprecipitation assays. The HER2 receptor was precipitated in an immune complex with trastuzumab as primary antibody, and then immunoblotting with the anti-HER2 antibody was performed (Figure 2C). As was expected, Trastuzumab interacted with human HER2 and, also to a lesser intensity with rat HER2, indicating a possible trastuzumab/rat HER2 interaction in GH3 cells. In addition, comparing human and rat HER2 sequences by computational analysis, three different amino acids (P571S, P572S

and F573S) have been identified in the binding interface. Out of the three amino acid differences present, only P572S was predicted to be deleterious, but with a low confidence (Supplementary Table 1 and Figure 1). The other two alterations were predicted to reduce the binding affinity, but not to be deleterious. These results show a highly conserved binding interface with three points of amino acid differences, which could predict a reduced affinity of Trastuzumab by rat HER2 compared to human sHER2, in line with the immunoprecipitation assays.

In addition, we performed IHC using trastuzumab as the primary antibody for HER2 detection on cryosections from rat pituitary tumor, and observed immunofluorescence in the plasma membrane and cytoplasm in rat pituitary tumor cells. Moreover, we realized double-immunofluorescence staining for HER2 (using trastuzumab) and PRL, which revealed PRL positive cells with HER2 labelling delineating the plasma membrane, as can be observed in the figure 2D.

Next, we analyzed HER2 and TBR expression in experimental pituitary tumors exposed to the HER2 inhibitor by IHC with the HER2 quantification indicating an increase of expression in the E and E+T groups with respect to the non-estrogen(C) and control–trastazumab (T) groups. In addition, the quantification of both TGFB receptors (TBR1 and TBR2), showed a decrease in E and E+T compared to the C and T groups (Figure 2E).

To investigate further the results obtained by conventional IHC, we examined the subcellular localization of both TBRs in experimental pituitary tumors treated with trastuzumab by means of TEM immunogold. In the pituitary tumors without trastuzumab administration, the subcellular distribution of TBR1 and TBR2 was similar, as identified by gold particles in the plasma membrane and cytoplasm. In some cells, the cytoplasmic presence of both receptors was associated with an endoplasmic reticulum (Figure 2F).

However, the administration of the HER2 inhibitor did not induce any changes in the subcellular localization of either TGFR receptor.

Having demonstrated that the rat pituitary tumors expressed HER2, we now tested the effect of HER2 blockade on pituitary weight and cell proliferation, with cells which were immunoreactive to the mitogenic marker Ki67 in sections from three different levels of pituitary glands of each experimental group being quantified as shown in Figure 3A-B. The pituitary tumor proliferation decreased by about 50% (average percentages C: 1.5%; T: 1.3%; E: 18.75%; and E+T: 8.3%) after treatment with trastuzumab for two weeks. In addition, we performed double-immunostaining of PRL, GH or ACTH hormones with Ki67 in the Estrogen-treated rat group. As expected, the majority of cells identified in this experimental group were PRL+, with some of these being co-labelled with Ki67 and a few GH/Ki67 positive cells being detected. However, the ACTH cells were negative to Ki67, as shown in the Figure 3C.

In order to elucidate whether the cell proliferation decrease observed after trastuzumab treatment could be associated with an increase in apoptosis, we used the TUNEL assay as this is useful for examining DNA fragmentation *in situ*, and apoptosis of pituitary cells was evaluated over histological samples of each experimental group. As shown in the Figure 3D, no changes were observed in the number of TUNEL positive cells in the different experimental groups.

Trastuzumab effect on the protein expression of cell-cycle regulators

The activation of the HER2 receptor promotes cell proliferation through the stimulation of proteins involved in the control of the cell cycle progression. After observing that the inhibition of HER2 decreased the proliferation of pituitary tumor cells *in vivo*, we investigated whether the trastuzumab effects on the expression of ERK1/2, Cyclin D1 and

CDK4. As shown in Figure 3E-G, the treatment with trastuzumab significantly reduced the expression of all the mitogenic proteins analyzed, compared to the pituitary tumors from rats without treatment with the HER2 inhibitor, in agreement with the blockade observed in the cell proliferation in Figure 2.

Trastuzumab effect on TGFB1 signaling in vitro

Having analyzed the key role of HER2 on the pituitary tumor cell proliferation, we investigated whether the trastuzumab treatment could modulate the anti-mitogenic effect of TGFB1. To carry this out, we used GH3 lactosomatotroph pituitary tumor cells and first determined the expression of HER2 by confocal microscopy (Figure 4A) and CLEM (Figure 4B), which allowed the simultaneous observation of a given subcellular structure. As shown in figure 4A and B, the receptor was localized at the plasma membrane, similar to the location found in colon cancer CaCO2 cells used as a positive control.

To investigate whether blocking HER2 regulated TGFB1-induced cell proliferation inhibition, we quantified the levels of Ki67 by flow cytometry in GH3 cells exposed to TGFB1 in the presence of trastuzumab. It was observed that the treatment with TGFB1 or trastuzumab significantly decreased the cell proliferation, an effect that was potentiated when the GH3 cells were co-incubated with TGFB1 and trastuzumab (Figure 4B). In parallel we performed new assays using the bromodeoxyuridine (BrdU) technique, which is commonly used to measure cell proliferation. Similar results were obtained, thus confirming those adquired by Ki67 measurements (Figure 4C).

After demonstrating the inhibition of cell proliferation by co-incubation with trastuzumab and TGFB1, we decided to study whether this effect was associated with activation of SMAD2/3. A significant increase in p-Smad2/3 expression occurred when GH3 cells were incubated with trastuzumab in the presence of TGFB1 (Figure 4D), suggesting that

activation of the canonical signal TGFB1/SMAD2/3 was inhibited by the signaling pathways activated by HER2.

To evaluate the effect of trastuzumab and TGFB1 on ERK1/2 activation, the GH3 cells were treated with the HER2 inhibitor for 30 min in the presence or absence of TGFB1, and their phosphorylated states were determined by WB. As shown in Figure 4E, the analysis of pERK1/2 did not reveal any changes in the ERK1/2 pathway activation after TGFB1 stimulation. However, treatment with trastuzumab co-incubated with TGFB1 for 30 min induced a significant decrease in the phosphorylation of ERK1/2.

In order to try to obtain more evidence of crosstalk between HER2/ERK1/2 and TBR/SMAD2/3, we analyzed the interaction of SMAD2/3 with ERK1/2 using immunoprecipitation studies on GH3 cells treated with trastuzumab in the presence of TGFβ1. The interaction of p-SMAD2/3-p-ERK1/2 decreased when the GH3 cells were incubated with trastuzumab in the presence of TGFβ1, compared with the treatment with TGFβ1 alone, effects that may be associated with the reduction in p-ERK1/2 and the increase of p-Smad2/3 observed in Figure 4F.

A possible mechanism to regulate SMAD2/3 phosphorylation is by controlling the pool of Smad2/3 available for TGFβ signaling. The high phosphorylation of SMAD2/3 in the presence of trastuzumab (Figure 4D) and the interaction between SMAD2/3 with ERK1/2 (Figure 4F) led us to hypothesize that these MAPK altered the SMAD2/3 phosphorylation induced by TβRI. Thus, we tested the interaction between TBR1 and SMAD2/3 by immunoprecipitation assays in GH3 treated with trastuzumab in the presence of TGFB1, an increase in TBR1-SMAD2/3 association was observed when the cells were co-incubated with the inhibitor of the HER2/ERK1/2 pathway and TGFB1.

The activation of SMAD2/3 can be regulated by controlling the pool of Smad2/3 available for TGFB signaling. Thus, we studied whether blocking HER2 would alter the interaction of SMAD2/3 with TBR1 by using immunoprecipitation in the GH3 cells treated with trastuzumab or TGFB1. As shown in Figure 4F, the TGFB1 or HER2 inhibitor significantly increased interaction between SMAD2/3 and TBR1, effects that were potentiated in GH3 cells incubated with TGFB1 in the presence of trastuzumab. These results suggest that the HER2/ERK1/2 pathway inhibited SMAD2/3 phosphorylation by interfering with its association with the TGFB receptor.

DISCUSSION

In the present study, we demonstrated that inhibition of the HER2 receptor by trastuzumab decreased cell proliferation of pituitary tumor cells through blocking the ERK1/2 pathways and facilitating the activation of antimitogenic TGFB/SMADs signaling.

There are a wide variety of treatment options available to manage pituitary adenomas, such as pharmacotherapy with dopamine, as well as, somatostatin analogs, surgery, radiotherapy and chemotherapy. However, aggressive pituitary adenomas are notoriously difficult to manage due to their size, invasiveness, speed of growth and high frequency of recurrence (Buchfelder 2009). There is no clear definition of aggressive adenomas, but such tumors are generally considered (from the clinical point of view) to be those corresponding to a massive invasion of surrounding tissue, with features of rapid growth, large size, a tendency to recur rapidly, resistance to conventional treatments (including radiotherapy), and in some patients, a fatal outcome (Buchfelder 2009; Lloyd 2004). Specific biomarkers have not yet been identified that can distinguish between clinically aggressive and non-aggressive pituitary adenomas, although the antigen Ki67 proliferation index might be of use. The study

of aggressiveness in pituitary tumors is of crucial importance for improving the management of patients by enhancing prognostic predictions and the effectiveness of treatments. In this sense, it is clear the need for the development of an alternative strategy for recurrent invasive adenomas or those that are resistant to conventional therapies.

We have demonstrated here the presence of HER2 in human non-functioning and in PRL, GH and ACTH secreting pituitary tumors. HER2 expression was previously found to be in 31% of all pituitary tumors, 43% of non-functioning ones and 24% of functioning adenomas (Cooper, et al. 2011). In different studies, HER2 expression has been confirmed in 24% of GH-secreting, 26% of PRL-secreting, and 32% of GH/PRL adenomas, as well as in 3% of the ACTH-secreting adenomas tested (Botelho, et al. 2006; Chaidarun, et al. 1994; Ezzat et al. 1997; Nose-Alberti et al. 1998; Vlotides et al. 2009). The higher HER2 expression and proliferation index recorded in invasive adenomas compared to non-invasive ones suggests a worse prognosis in adenohypophyseal neoplasia (Nose-Alberti et al. 1998), making this receptor an attractive therapeutic target as an alternative medical therapy for pituitary tumors. The presence of intact HER2 signaling has been reported in tumors from breast cancer patients who were found to be HER2 negative by IHC. This subgroup of patients was excluded from trastuzumab treatment, although these patients may have responded to the drug (Wulfkuhle, et al. 2012). On analyzing the activation of HER2, we detected pHER2 expression in pituitary adenomas positive for HER2, interestingly in a HER2 negative non-functioning adenoma by IHC. Nevertheless, the pHER2 expression could not be observed by WB, probably due to the differences in the sample processing that enables a better exposition of the antigen in IHC. The immuno detection of pHER2 could be clinically relevant, as it has been reported that some breast cancer patients with negative HER2 tumors present have benefited from trastuzumab (Paik, et al. 2008).

Considering that the HER2 effect and signaling are mediated by the MEK/ERK1/2 cascade, we determined the activation of ERK1/2 in human pituitary tumors, with an increase in the phosphorylation of ERK1/2 in the pituitary tumors being observed compared to non-tumor tissues. These results are in agreement with a previous report that demonstrated a rise in phosphorylation, and hence, activation of MEK1/2 and ERK1/2 in all pituitary adenoma subtypes compared to normal pituitaries (Dworakowska et al. 2009). In rat prolactinoma tumor cells, treatment with the EGFR inhibitor gefitinib suppressed proliferation and ERK1/2 phosphorylation, thereby demonstrating the involvement of EGFR/ERK signaling in pituitary tumor growth (Vlotides, et al. 2008). To obtain a deeper understanding of the role of the HER2/ERK pathway in pituitary tumor growth, it would be interesting to study possible crosstalk with TGFB signaling, which may help to improve the therapeutic strategy against HER2.

TGFB signaling in the development of pituitary tumors is still not fully understood and the subject of much controversy. In normal epithelial cells, TGFB1 acts as a potent tumor suppressor and prevents incipient tumors from progression to malignancy. However, due to the subsequent inactivation of TGFB signaling or key target genes, tumor cells lose their TGFB1 tumor-suppressive responses (Massague 2008). Considering that tumor cells can evade the suppressive effects of TGFB through inactivation of core components of the pathway (such as TGFB receptors), we examined the protein expression of TGFB receptors in normal and tumoral human pituitaries, with reduced TBR2 levels being observed in the tumor tissues compared to non-tumoral pituitaries. These results are in agreement with previous reports that described immunoreactivity for TBR2 expression in 26 out of 48 cases of human pituitary adenomas (Fujiwara, et al. 1995) and a down-regulation of the TGFB/SMAD signaling cascade in dopamine-resistant prolactinomas compared to normal human anterior pituitaries (Li, et al. 2015). Our results on TBRs obtained from human

samples suggest that TGFB signaling may be restrained in pituitary adenomas and might be correlated with tumor growth. Consequently, recovering the capability of TGFB signaling to suppress tumor development could be a promising therapeutic strategy for pituitary tumors.

Pituitary tumors are often unresponsive to therapy, and even when an initial response is achieved, their recurrence is common. Bearing in mind the presence of HER2 in human pituitary tumors, we investigated the effect of specific HER2 inhibition with trastuzumab on pituitary tumor cell growth by employing two experimental approaches; an *in vivo* pituitary tumor model using Fisher 344 rats and *in vitro* experiments performed with rat GH3 lactosomatotroph pituitary tumor cell line. In this study, it was demonstrated that the human-specific HER2 monoclonal antibody (trastuzumab) recognized rat-derived HER2 in both the rodent model and GH3 cells. The analysis of cell-surface proteins by biotinylation and the theorical model of trastuzumab/HER2 interaction showed differences between human and rat binding. The experimental PRL pituitary tumor presented a significant HER2 expression, in contrast to decreased TBR1 and TBR2 receptors. The HER2 inhibitor treatment decreased the pituitary tumor weight, cell proliferation, without inducing apoptosis, and the levels of mitogenic MAPK-ERK1/2 signaling. In addition, trastuzumab administration for two weeks in estrogen-treated rats inhibited the expression of CDK4 and cyclin D1, key regulators of the cell cycle progression from G1 to the S phase (Sherr and Roberts 1995). The trastuzumab treatment in non-estrogen treated rats did not change any of the parameters analyzed with respect to intact animals, suggesting that the trastuzumab effect takes place when there is a previous PRL pituitary tumor, which was associated with the high HER2 expression recorded in the E group.

It has been reported in transgenic mice models that CDK4 is required for both the physiological and tumorigenic control of cell cycle progression in the pituitary (Gillam, et al. 2015). During the G1 phase, CDK4 is activated by cyclin D1 (Sherr and Roberts 1995),

570 which has been previously observed to be overexpressed in 22 of 45 human pituitary tumors
571 analyzed (Simpson, et al. 2001). It has been reported that lactotroph cells of CDK4-deficient
572 mice did not proliferate in response to estrogen administration (Moons, et al. 2002). In the
573 present study, the noticeable increase of CDK4 expression observed in the E group suggests
574 the contribution of both proteins (cyclin D1 and CDK4) in the regulation of tumor pituitary
575 proliferation. Thus, these results provide evidence that the inhibition effect of trastuzumab on
576 pituitary tumor growth *in vivo* can be mediated by blocking the G1/S-specific ERK1/2/cyclin
577 D1/CDK4 pathway.

578 Although the blocking of HER2, an inhibitory effect on pituitary tumor growth has
579 been previously described by using lapatinib (a dual tyrosine kinase inhibitor of both EGF
580 receptors HER1 and HER2 (Fukuoka et al. 2011), this is the first study testing the specific
581 HER2 inhibitor trastuzumab on pituitary tumor cells, which is a widely promising agent for
582 molecular targeting therapy against breast cancer and more recently tested in patients with
583 HER2-positive colorectal cancer (Carter, et al. 1992; Ross, et al. 2009; Sartore-Bianchi, et al.
584 2016). Considering the relevance of EGF receptors in pituitary tumor progression, the EGFR
585 antagonist lapatinib in conjunction with a dopamine agonist was evaluated in patients with
586 dopamine analog-resistant prolactinomas and demonstrated a beneficial effect of the EGFR
587 inhibitor in the clinical outcome, including reduced prolactin levels and tumor shrinkage
588 (Cooper, et al. 2014). In a related study performed on patients with advanced breast cancer, it
589 was demonstrated that the lower pathological complete response rates in the lapatinib treated
590 group might be explained by a reduced capability of the tyrosine-kinase-inhibitor to block the
591 HER2 pathway compared with the trastuzumab antibody (Untch, et al. 2012). These reports,
592 in addition to our results, suggest that the study of the specific inhibition of HER2 by
593 trastuzumab in pituitary tumors should be expanded, in view of the attractive therapeutic
594 target HER2 in pituitary adenomas. It would be relevant to determine the trastuzumab/HER2

595 binding in human pituitary cells, which may predict the efficacy of the treatment in pre-
596 clinical human studies. In the present study, considering the presence of HER2 positive cells
597 in three out of four human prolactinomas, and also in the experimental pituitary tumor used,
598 the principal target for trastuzumab treatment could be PRL producing tumors. However,
599 taking into account the high percentage (79%) and strong staining (+++) of HER2 expression
600 in one out of three GH-producing tumors, and also the presence of Ki67/GH positive cells in
601 the estrogen-treated rat group, we suggest that trastuzumab might have an effect on GH
602 secreting tumors. Future work should expand the number of cases using appropriate
603 experimental pituitary tumor models.

604 Having demonstrated the anti-proliferative effect of trastuzumab in pituitary tumor
605 growth, we investigated possible cross-talk with TGFB signaling, which has not yet been
606 studied in pituitary tumors. Trastuzumab treatment in the presence of TGFB1 decreased GH3
607 cell proliferation significantly, an effect accompanied by a reduction in ERK1/2
608 phosphorylation, an increase in SMAD2/3 activation suggesting a negative role of the
609 HER2/ERK1/2 pathway on the activation of SMAD2/3 in pituitary tumor cells. Although
610 SMADs are activated by TβRI-mediated phosphorylation, their activity and stability are
611 further regulated by downstream kinases of other signaling pathways (Matsuzaki 2011). In a
612 previous report we demonstrated that the activation of ERK1/2 by EGF blocked SMAD2/3
613 phosphorylation in pituitary tumor cells, and we also described a physical interaction between
614 SMAD2/3 and ERK1/2, which interfered with the association between SMAD2/3 and TBR1
615 (Petiti et al. 2015). Here, we tested the interaction of SMAD2/3 with ERK1/2 and TBR1 by
616 immunoprecipitation assays in rat GH3 cells treated with trastuzumab in the presence of
617 TGFB1, and observed a decrease in SMAD2/3-ERK1/2 and an increase in SMAD2/3-TBR1
618 associations. These results suggest that the reduction of pERK1/2 in cells treated with
619 trastuzumab in the presence of TGFB1 could be associated with a decrease in SMAD2/3-

ERK1/2 interaction, which might allow the SMAD2/3 pool to become available for activation by TBRI and consequently decrease the cell proliferation. Thus, the anti-proliferative effect of trastuzumab in pituitary tumor cells may be mediated by a blockade of the HER2/ERK1/2 pathway and a consequent stimulation of the anti-mitogenic TGFB/TBR1/SMAD2/3 cascade.

In conclusion, in the present study, using *in vivo* and *in vitro* models, we demonstrated that blocking HER2 by trastuzumab inhibited pituitary tumor growth and modulated HER2/ERK1/2 signaling and consequently the anti-mitogenic TGFB/TBR/SMAD signaling (Figure 5). Furthermore, the high expression of HER2 and ERK1/2 in contrast with the low levels of TBR2 detected in hu man pituitary adenomas may have been responsible, at least in part, for the limited antiproliferative response to TGFB1detected in pituitary tumor cells (Sarkar, et al. 1998). This imbalance between proliferative and anti-proliferative signals in pituitary tumor cells has also been previously described in breast cancer, with HER2-induced tumorigenesis in an *in vivo* model resulting in the loss of the TGFB signaling pathway and a lack of SMAD2 activation, accompanied by a reduction of TBR1 expression (Landis, et al. 2005).

The loss of tissue homeostasis by alteration in HER2 and TBRs signaling, the frequent resistance to therapy and the early recurrence of aggressive pituitary tumors led us to investigate new pharmacological strategies. The inhibition of HER2/ERK1/2 pathway is attractive, but undoubtedly new studies are now needed that consider a more integrated vision that involves intricate relations with other signaling pathways.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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LEGENDS

Figure 1:

HER2 and TBRs expression in human pituitary adenomas. (A) Representative micrographs of the immunohistochemical analysis of HER2, pHER2, TBR1 and TBR2 expression in human non-tumor and pituitary tumor samples. HER2 positive (M+) and negative (M-) breast tumor samples were used as controls. Scale bar: 20μm. Representative western blot images of (B) HER2 and pHER2, (C) TBR1, TBR2, pERK1/2 and T-ERK1/2 expression in non-tumor pituitaries and pituitary adenomas. PRL: PRL-secreting, GH: GH-secreting, ACTH: ACTH-secreting and NF: non-secreting pituitary tumors.

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HER2 and TBR expression in rat pituitary tumors. (A) Western blot detection of HER2 in total extracts from rat pituitary tumors (F1 and F2) and rat GH3 pituitary tumor cells using trastuzumab as the primary antibody. Human HER2+ (M+) breast tumor, CaCo2 and MCF7 lysates were used as positive controls an human HER2- (M-) breast tumor as negative control. (B) Analysis of cell-surface proteins by biotinylation. Trastuzumab recognized the HER2 receptor in GH3 and MCF7 pellet samples, as confirmed by the presence of a band at 185 kDa, corresponding to the molecular weight of HER2, without any expression in the supernatant fractions as expected. The same result was observed using anti-HER2 as primary antibody. The expression of NFkB was used as negative control of cell surface proteins, with its presence being observed only in the supernatant fractions, indicating the purity of the isolation. (C) Trastuzumab/HER2 interactions in rat GH3 cells and in human breast tumor sample with HER2 amplification by immunoprecipitation assays. The HER2 receptor was precipitated in an immune complex with trastuzumab as primary antibody, and then anti-

HER2 antibody was used for the immunoblotting. (D) Immunofluorescence staining for HER2 and PRL on cryo-sections of a rat pituitary tumor. On the left panel, white arrowheads indicate HER2 expression at the plasma membrane, while double positive (HER2-PRL) cells are shown on the right panel. Scale bar: 20um. (E) Representative micrographs and quantification of immunohistochemistry staining for HER2, TBR1 and TBR2 in the following groups: control [C], trastuzumab [T], estrogen-induced rat pituitary tumors [E] and estrogen-induced rat pituitary tumors treated with trastuzumab (6 mg/kg) for the last 15 days of estrogen exposure [E+T]. Scale bar: 20 μm. *** $p<0.001$ E vs. C. (F) Immuno-gold labelling for TBR1 and TBR2 on E and E+T groups. Arrows show the localization of TBR1 and TBR2 at the plasma membrane and the endoplasmic reticulum (ER).

Figure 3:

Effect of trastuzumab treatment on rat experimental pituitary tumors. (A) Pituitary gland weight (mg) and the corresponding representative photographs from C, T, E and E+T groups. *$p<0.05$ E vs. C or T and ^$p<0.05$ E+T vs. E. (B) Representative micrographs and quantitative analysis of immunohistochemistry staining for Ki67 as a proliferation marker. Scale bar 20um. *$p<0.05$ E vs. C or T and ^$p<0.05$ E+T vs. E. (C) Double-immunofluorescence staining for Ki67 (green) and PRL, GH or ACTH (red) in pituitary ultrathin cryo-sections from the E group. Scale bar 10um. Double-positive cells are indicated with an asterisk (*). (D) TUNEL assay from C, T, E and E+T groups. Arrowheads indicate apoptotic cells. Negative control [C-]: pituitary gland section without corresponding primary antibody. Positive control [C+]: pituitary gland section treated with DNAase I. Scale bar 20um. Western blot analysis of proliferation and cell cycle-regulator proteins. Representative panels and densitometric analysis of (E) pERK1/2-ERK1/2, (F) Cyclin D1, (G) CDK4 protein expression. * $p<0.05$ E vs. C or T and ^ $p<0.05$ E+T vs. E.

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Effect of trastuzumab on the antiproliferative response to TGFB1. (A) Representative images of immunofluorescence labelling for HER2 in GH3 and CaCO2 (positive control) cell lines. White arrowheads indicate the expression of the receptor at the plasma membrane. Scale bar 10um. Correlative Light and Electron Microscopy (CLEM): [1] Immunofluorescence images for HER2 detection in ultrathin cryo-sections of GH3 cells (scale bar 10um). [2] CLEM overlay of TEM and the corresponding FLM image. [3] TEM images for the whole section and, at higher magnification, the immuno-gold labelling for selected regions (a, b) shown by white boxes. Gold particles are indicated by black arrows. Cell proliferation analysis in GH3 cells, which were treated with TGFB1 (4 ng/ml), Trastuzumab (Trast, 100 mM) or the combination of both factors for 24 h. (B) Representative flow cytometry histograms and quantitative analysis of the proliferation-associated protein Ki67. (C) Representative micrographs and quantification of BrdU uptake. *$p<0.05$ TGFB1 or Trast vs. C, and ^$p<0.05$ TGFB1+Trast vs. TGFB1 or Trast. TGFB1 and MAPK-ERK1/2 signaling pathway analysis. Western blot panels and relative quantification of (D) pSMAD2/3-TSMAD2/3 and (E) pERK1/2-ERK1/2 expression from GH3 cell cultures treated with TGFB1, Trastuzumab or their combination for 30 min. (F) Immunoprecipitation (IP) of pSMAD2/3 and pERK1/2 and TBRI immunodetection in total cell extracts of GH3 cell cultures, untreated and treated for 30 min with Trastuzumab, TGFB1 or their combination. Input: TBRI or pERK1/2 or pSMAD2/3 antibody recognized the antigen in the total cell culture lysate.

Figure 5.

Model for the cross talk of HER2/ERK1/2 with TGFβ1/SMAD pathways in pituitary GH3 cells. Blocking HER2 by trastuzumab inhibits pituitary tumor growth and modulates

HER2/ERK1/2 signaling, and consequently the anti-mitogenic TGFB/TBR/SMAD signaling. The HER2/ERK1/2 pathway impinges on the TGFβ1/SMAD2/3 signaling, thereby modulating SMAD2/3 phosphorylation. The association of SMAD2/3-ERK1/2 inhibits the TβRI induced- SMAD2/3 activation, thus counteracting the antimitogenic effect of TGFβ1.

Supplementary Figure 1. (A) Sequence alignment of the C-terminal of rat and human sHER2. The residues in the binding interface with Trastuzumab are marked with hyphens. An amino acid was defined as interfacial if its Cα was within 9A of any Cα of Trastuzumab. (B) Superposition of rat (pdb: 1n8y, blue) and human (pdb:1n8z, green) structures of sHER2. The Trastuzumab molecule is shown on the surface in red and grey. The Cα atoms of sHER2 at the binding interface are shown as spheres.

TABLE 1: Clinicopathologic and immunohistochemical characteristics of human pituitary adenomas

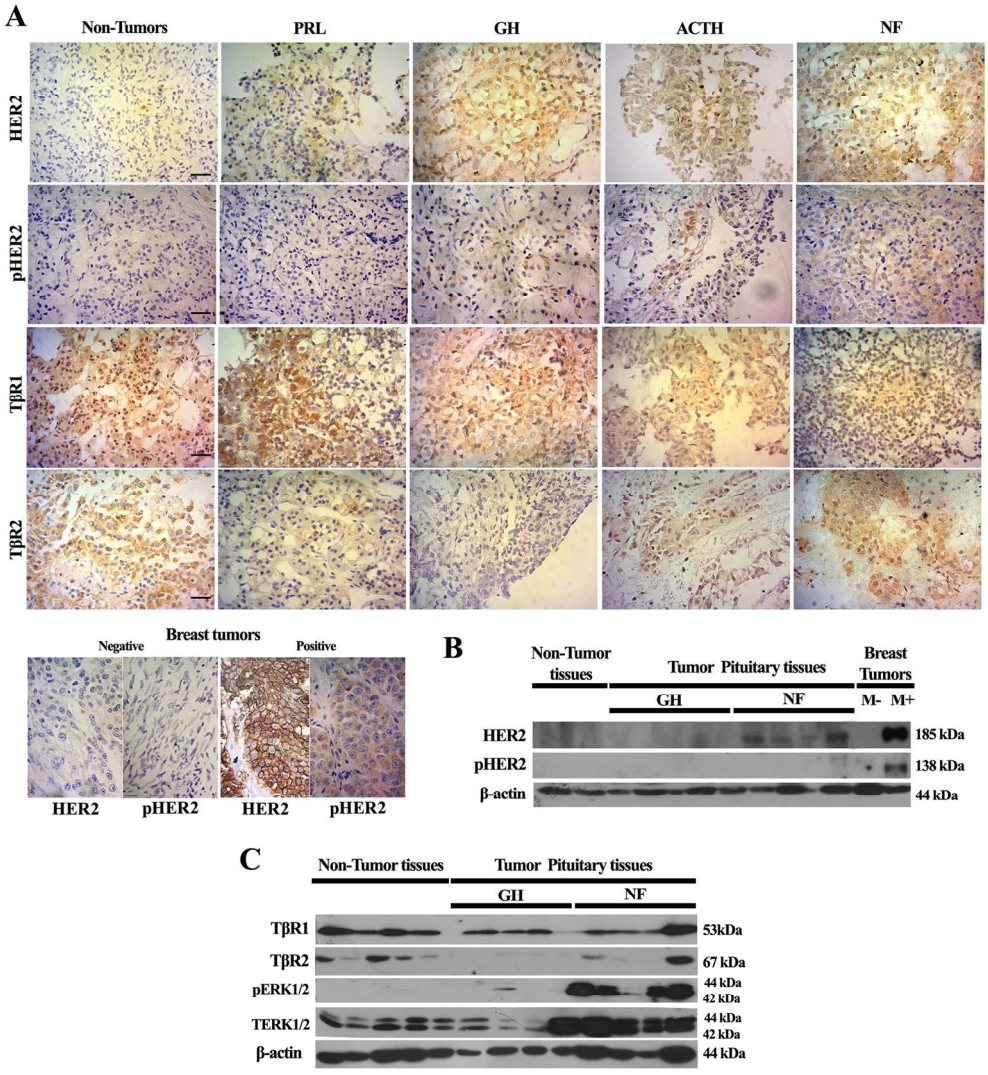
Case	Gender/age	Clinicopathologic classification	IHC	Ki67 %
1	F/20	Functioning	PRL	1
2	F/38	Functioning	PRL	1
3	F/31	Functioning	PRL	1
4	F/26	Functioning	PRL	1
5	F/58	Functioning	GH	3
6	F/37	Functioning	GH	2
7	F/57	Functioning	GH	2
8	F/27	Functioning	GH	1
9	M/31	Functioning	GH	1
10	F/64	Functioning	GH	N.D.
11	F/32	Functioning	ACTH	5
12	F/62	Functioning	ACTH	2
13	M/63	Non-functioning	Negative	5
14	M/44	Non-functioning	Negative	1
15	F/45	Non-functioning	Negative	3
16	M/58	Non-functioning	Negative	3
17	F/68	Non-functioning	Negative	2
18	M/56	Non-functioning	Negative	5
19	F/62	Non-functioning	Negative	1
20	M/50	Non-functioning	Negative	1

M: male, F: female, GH: Growth hormone; ACTH: Adenocorticotroph hormone.; PRL: Prolactin; N/D: no data

TABLE 2: Immunohistochemical characteristics of human pituitary samples

Case	Gender/age	Clinicopathologic classification	IHC	Ki67 %	HER2%	pHER2	TBRI%	TBRII%
1	F/20	Functioning	PRL	1	10++	0	100	100
2	F/38	Functioning	PRL	1	44+	N/D	100	94
3	F/31	Functioning	PRL	1	27+	N/D	100	0
4	F/26	Functioning	PRL	1	0	0	100	1
5	F/58	Functioning	GH	3	79+++	36.1	51	100
6	F/37	Functioning	GH	2	0	0	52	40
7	F/57	Functioning	GH	2	0	N/D	94	0
8	F/32	Functioning	ACTH	5	80+++	59.1	40	72.8
9	M/63	Non-functioning	Negative	5	0	10.5	16	N/D
10	M/44	Non-functioning	Negative	1	98+++	49.5	26	100
11	F/45	Non-functioning	Negative	3	0	N/D	11	0
12	M/58	Non-functioning	Negative	3	0	N/D	0	80
13	F/54	Non-tumor tissue	N/A	N/A	0	N/D	100	80
14	M/35	Non-tumor tissue	N/A	N/A	0	N/D	28	80
15	M/57	Non-tumor tissue	N/A	N/A	5+	0	60	90

M: male, F: female, GH: Growth hormone; ACTH: Adenocorticotroph hormone; PRL: Prolactin. N/D: no data.
Staining score: +=low, ++=moderate, +++= strong.



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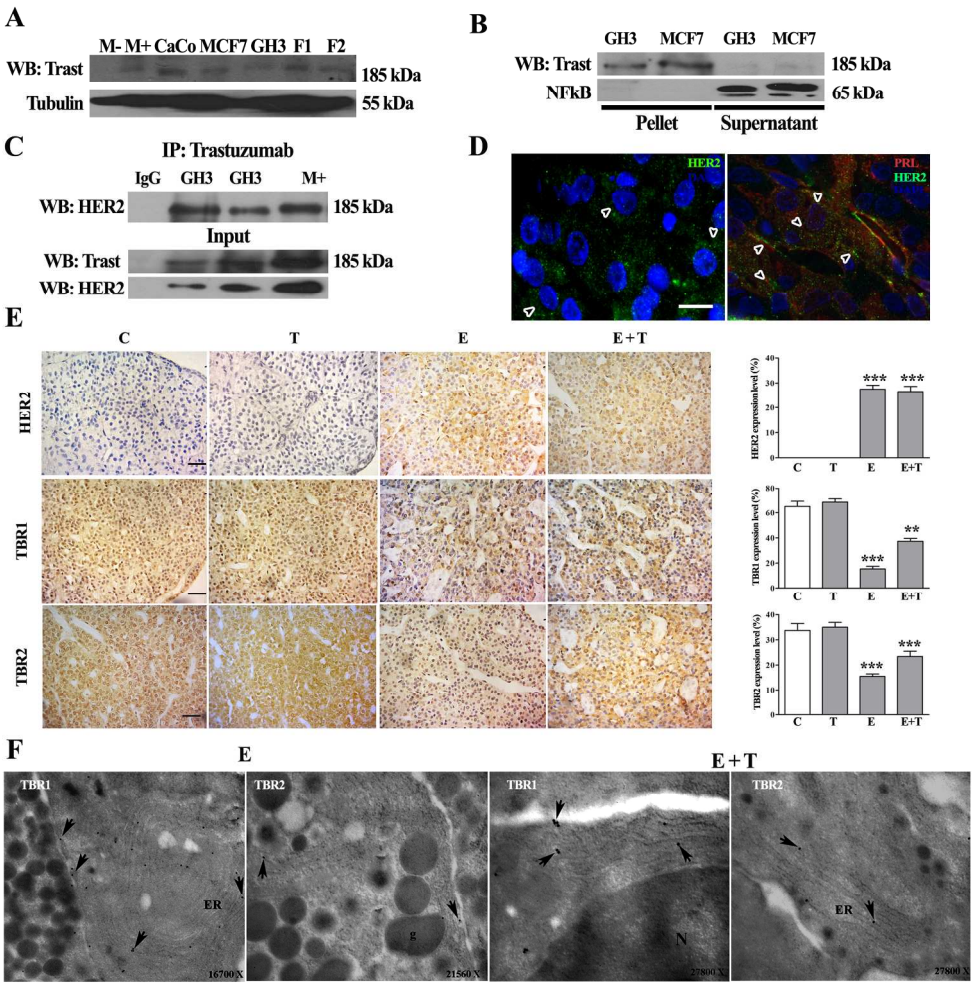

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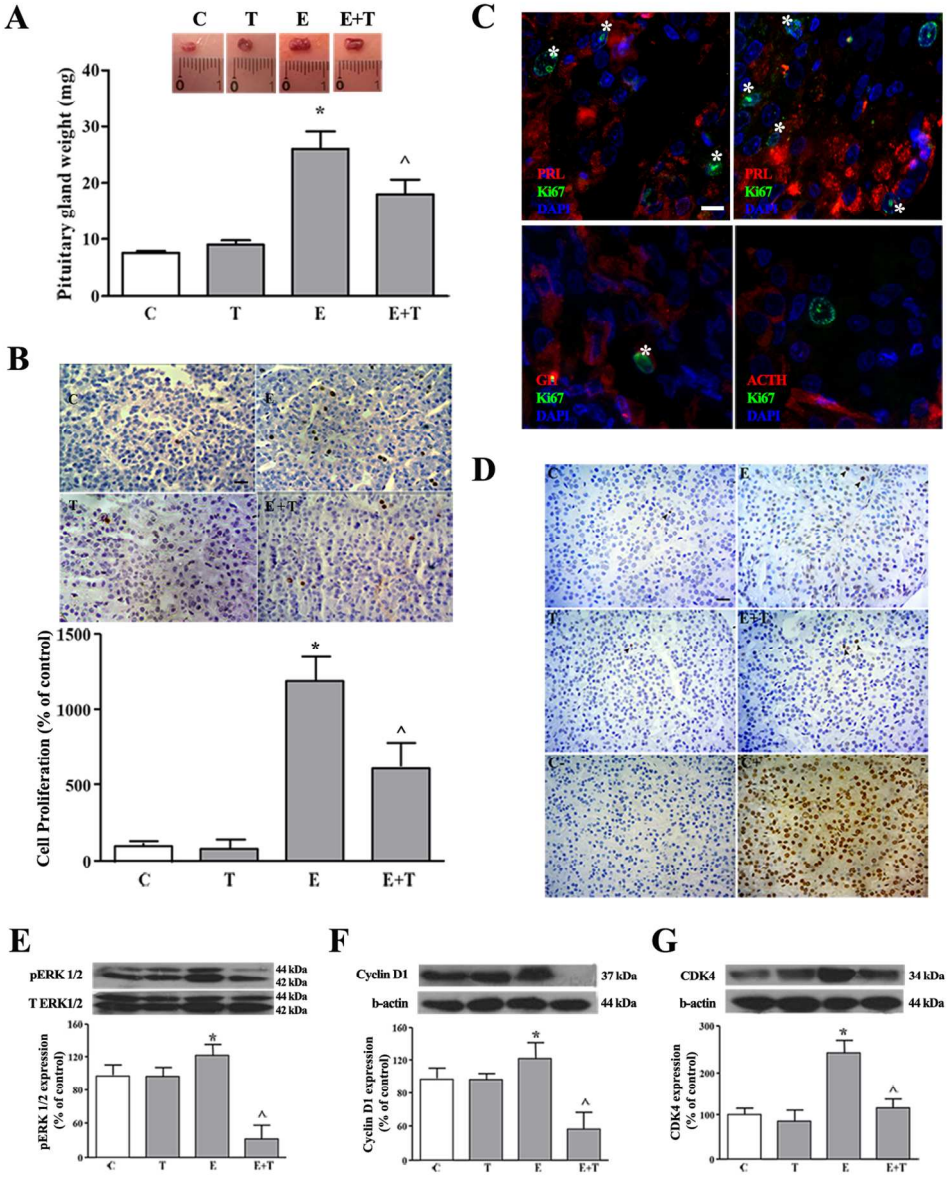

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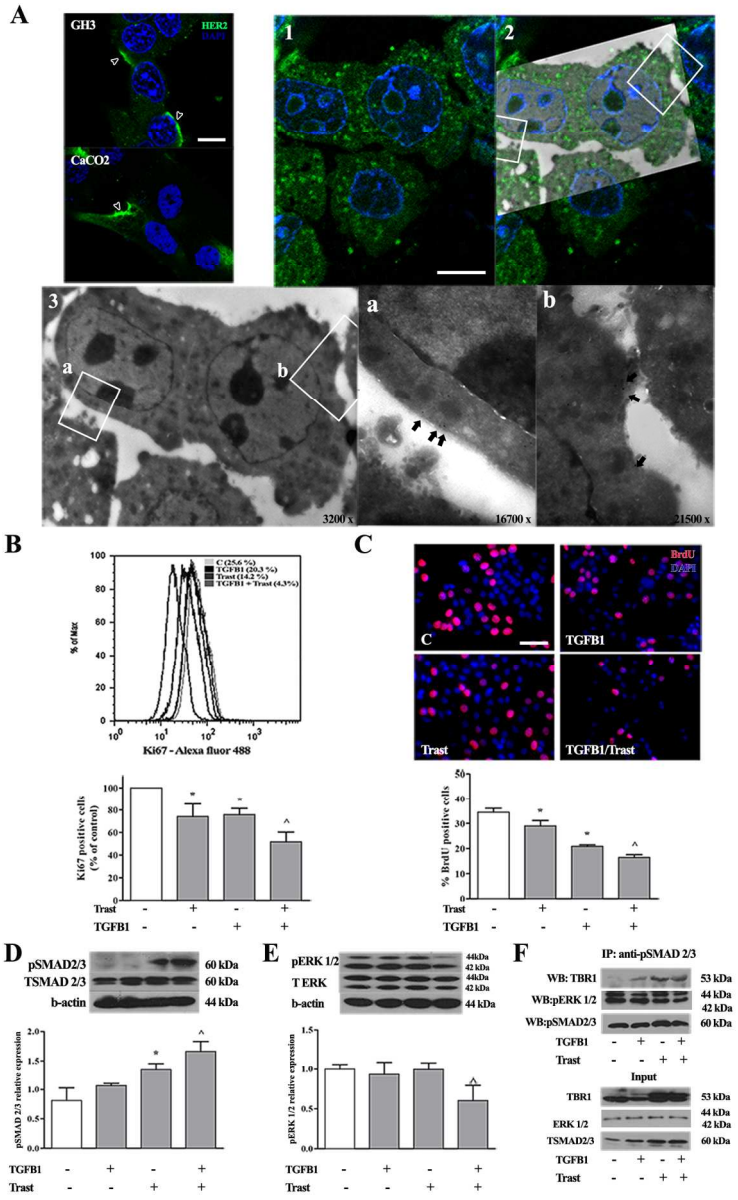

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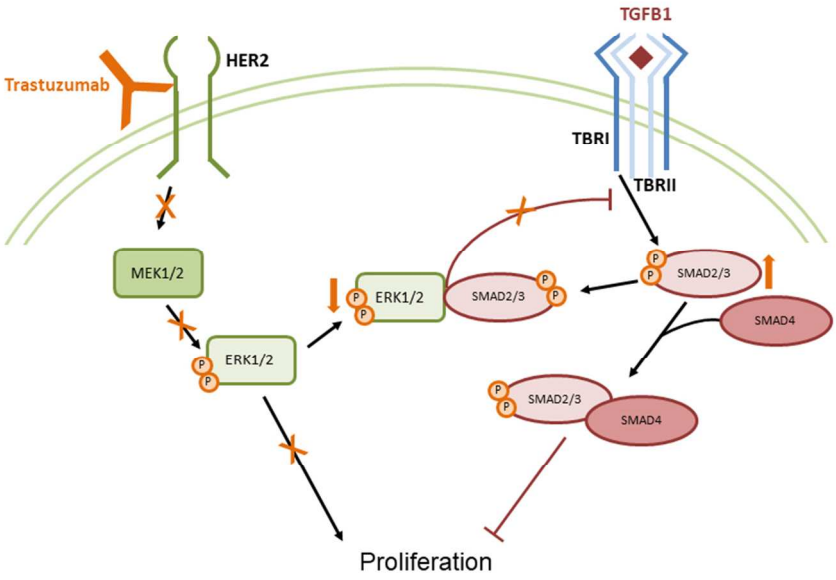

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