SUPPLEMENTAL DATA

Deregulation of the Pit-1 transcription factor in human breast cancer cells promotes tumor growth and metastasis

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SUPPLEMENTAL METHODS

Reagents and cell lines. The pRSV-hPit-1, the pcDNA3-Snail, and the pGL3-Snail promoter vectors were kindly provided by Dr. Castrillo (Centro de Biología Molecular Severo Ochoa, Madrid, Spain), Dr. Nieto (Instituto de Neurociencias, Alicante, Spain), and Dr. Garcia de Herreros (Unidad de Biología Celular y Molecular, Universidad Pompeu Fabra, Barcelona, Spain), respectively. The polyclonal anti-HA antibody was a gift from Dr. Pandiella (Centro de Investigacion del Cancer, Salamanca, Spain). The pSingle-tTS-shRNA vector and MCF-7 Tet-Off cells were purchased from Clontech. MCF-7 and MDA-MB-231 (human breast adenocarcinoma cell lines) were obtained from the European Collection of Cell Cultures (Salisbury, Wilts., UK).

Cell culture. Stock cultures were grown in 90-mm Petri dishes in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2mM L-glutamine in an air-CO_2 (95:5) atmosphere at 37ºC. Confluent cells were washed twice with phosphate-buffered saline and harvested by a brief incubation with trypsin-EDTA solution (Sigma Aldrich) in PBS.

Plasmids and transfections. Twelve to 24 h before transient transfection, 10^6 cells per well were seeded in 90-mm Petri dishes and allowed to attach overnight. MCF-7 and/or MDA-MB-231 cells were: a) transfected with the pRSV-hPit-1 expression vector, or the pcDNA3-

Snail expression vector, or the pRc/RSV and the pcDNA3 empty vectors (employed as a controls), using FuGene (Roche Molecular Biochemicals); b) transfected with 20 nM Pit-1 siRNA-1, Pit-1 siRNA-2, Snail siRNA, or scrambled siRNA (employed as a control), using jetSI (jetSI^{TM} siRNA transfection reagent, Polyplus-transfection) as previously described (1). Primer sequences of Pit-1 siRNA-1 were as follows: site 1, 5'-AACCCCTTGTCTTTACAAGTTCCTGTCTC-3' (antisense), site-2, 5'-AATTAAGTTAGGATACACCCACCTGTCTC-3' (antisense), and site-3, 5'-AATTGAATCTCGAGAAAGAAGCCTGTCTC-3' (antisense). Primer sequences of Pit-1 siRNA-2 were as follows: site-1, 5'-AATGTTGCTGTAGACATCACACCTGTCTC-3′ (antisense), site-2, 5′-AAGCTTTCAGTTTGCATGCATCCTGTCTC- 3′ (antisense), and site-3, 5′ - AACTTTCTCCAGATTCAGTTCCCTGTCTC- 3′ (antisense). Gene sequence of mis-sense control (universal scrambled siRNA, Ambion) was 5'-AAGCTTCATAAGGCGCATAGC-3'. Primer sequences of Snail siRNA were as follows: site-1, 5′-AATATTTGCAGTTGAAGGCCTCCTGTCTC-3′ (antisense), site-2, 5′-AAACAGAACCAGAAAATGGTCCCTGTCTC- 3′ (antisense), and site-3, 5′ - AAAGTCCCTTTTCAGACATTGCCTGTCTC- 3′ (antisense); c) co-transfected with the pRSV-hPit-1 or the pRc/RSV empty vector (employed as a control), and one of the pGL3-Snail constructs (pGL3B-Snail$_{-869/+59}$, pGL3B-Snail$_{-514/+59}$, pGL3B-Snail$_{-144/+59}$, pGL3B-Snail$_{-144/-23,}$ or pGL3B-hSnail$_{-869/+59}$mut), and the Rous Sarcoma Virus β-galactosidase (pRSV-gal), using JetPei^{TM} (Polyplus transfection). The proximal promoter regions of the human Snail gene (-144/+59, pGL3B-Snail$_{-144/+59}$, and -144/-23, pGL3B-Snail$_{-144/-23}$) were synthesized by PCR using the oligonucleotides: forward: 5′-CCCAAGCTTCGCCGATTCCGCCAGCAG-3′ and reverse: 5′- CCCGGTACCTGCGGGAGTGGCCTT - 3′, or 5′-CCCGGTACCATGCAGCAGCGCCGCCAACT-3′, respectively. The PCR product was subcloned into the Xho I and Hind III site of the pGL3 Basic plasmid. The pGL3B-Snail$_{-869/+59}$ vector was mutated in a Pit-1 consensus binding site, positioned between -5 bp and +4

from the transcription start site of the human Snail gene. Site-directed mutagenesis was performed with the QuikChange kit (Stratagene) under the conditions recommended by the manufacturer. The mutagenized oligonucleotide primer was as follows (mutagenized bases on the sense strand identified by lowercase letters): 5'-GAGTTGGCGGCGCTGCTGCcccgAcTGCGCCGCGGCACGGCCTA-3′. The newly constructed mutant plasmid was designated pGL3B-hSnail$_{-869/+59}$mut.

A hemagglutinin (HA) epitope tag (YPYDVPDYA) was introduced into the Hind III-Nco I sites of the pRSV-hPit-1 overexpression vector, yielding pRSV-HA-hPit-1 vector. The nucleotide sequences of the cloned inserts were confirmed by sequencing. In luciferase reporter assays, the cells were harvested in buffer (5X lysis buffer; Promega), and luciferase activity was then measured. β-galactosidase activity was measured at 420 nm using *o*-nitrophenyl-β-D-galactopyranoside as substrate. Cells were incubated for 48 h or 72 h, as indicated below, and protein, RNA and DNA extraction (for Western blot, PCR, ChIP, or reporter assays) were then performed.

The G1/S-phase cell cycle transition after Pit-1 overexpression or after Pit-1 knockdown was examined by bromodeoxyuridine (BrdU) incorporation. MCF-7 cells were seeded in 24-well plates with cover slides and allowed to attach overnight. Cells were co-transfected with the pEPuro construct (that confers puromycin resistance) and the pRSV-HA-Pit-1 vector or the Pit-1 siRNA (20 nM), for 48 h. MCF-7 cells were selected (1 μg/ml of puromycin) at 24 h. BrdU (10 μM) was added to the DMEM medium one hour before fixation with ethanol overnight. Fixed cells were subsequently treated with 4 N HCl for 20 min and incubated with a mouse monoclonal anti-BrdU antibody (clone 3D4; 1:100; BD Biosciences Pharmingen) and an anti-HA-tag rabbit polyclonal antibody (1:100). The cells were then labelled with DAPI, with Cy2-conjugated rabbit anti-mouse, and Cy3-conjugated goat anti-rabbit IgG secondary

antibodies (for BrdU and HA, respectively, Jackson ImmunoResearch). Cells with bright nuclear staining for BrdU were defined as BrdU positive cells.

Stable transfection of the MCF-7 Tet-Off cells (Clontech) with the pTRE2 control vector and the pTRE2-hPit-1 overexpression vector was performed by electroporation with the Nucleofector apparatus (Amaxa biosystems) using the VCA-1003 (Cell line Nucleofector Kit V, Amaxa biosystems). Stable transfectants were obtained as described below. The pTRE2-hPit-1 vector was obtained by digestion of the pRSV-hPit-1 plasmid with the Hind III and Not I restriction enzymes (to obtain the coding region of the human Pit-1 gene), and subcloned into the pcDNA3 vector. This vector was then digested with Hind III and Xba I, and the human Pit-1 coding sequence was inserted into the pTRE2 vector. To produce a stable knockdown of Pit-1 in MDA-MB-231 cells, we used the Knockout^{TM} Single Vector Inducible RNAi System (Clontech). Construction of the pSingle-tTS-shRNA-hPit-1 vector was performed according to the manufacturer's instructions. Sequence of the Pit-1 shRNA oligonucleotides was as follows: a) sense 5'-CACCAATGTGATGTCTACA-3'; b) antisense 5'-TGTAGACATCACATTGGTG-3'. The oligonucleotides were subcloned into the Xho I and the Hind III restriction sites of the pSingle-tTS-shRNA vector. Stable transfectants of both MCF-7 Tet-Off, and MDA-MD-231 cells were isolated as single colonies following selection in G418 (500 μg/ml).

Western blot analysis and antibodies. MCF-7 and MDA-MB-231 cells were lysed at 4ºC in 300 μl of lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 5 mM EGTA; 1.5 mM $MgCl_2$; 1% SDS; 10% glycerol; 1% Triton X-100; 10 mM sodium orthovanadate; 4 mM PMSF, and 50 μg/ml aprotinin). The cell lysate was then centrifuged at 14,000 x g for 5 min at 4ºC, the resulting supernatant was collected, and protein concentration determined by the Bradford method. Western blotting was carried

out as described elsewhere (1, 2). Briefly, 70 μg of total protein were subjected to SDS-PAGE electrophoresis. Proteins were transferred to a nitrocellulose membrane, blocked, and immunolabeled overnight at 4ºC with a primary antibody (see below), washed three times with PBS-Tween-20, and incubated with the appropriate secondary antibody for 1 hour. The signal was detected with the ECL™ Western blotting analysis system (GE Healthcare), and visualized by placing the blot in contact with standard X-ray film, as per the manufacturer's instructions.

Antibodies used

Antigen	**Source**	**Application**
Bcl-2 (N-19)	Santa Cruz Biotech	WB
CK (clon AE1/AE3)	Dako	IHC
CK19 (clon RKC108)	Dako	IHC
Active caspase 3 (ab2302)	Abcam	IHC
CK7 (clon OV-TL12/30)	Dako	IHC
Cyclin D1 (clon 7213G)	Santa Cruz Biotech	WB
E-cadherin (clon 36)	BD Biosciences	WB
E-cadherin (clon NCH-38)	Dako	IHC
Ki-67 (clon MIB-1)	Dako	IHC
MMP-1 (RB-9225)	Thermo Lab	WB
Cleaved PARP	Cell Signaling	WB
Pit-1 (X-7)	Santa Cruz Biotech	WB, IHC, ChIP
Snail (ab17732)	Abcam	WB
Snail (clon EC3)	Dr. Garcia de Herreros (3)	IHC
Vimentin (clon V9)	Dako/Thermo Scientific	IHC, WB
β-catenin (clon 1)	Dako	IHC
β-catenin (clon 14)	BD Transduction	WB
α-BrdU (clon 3D4)	BD Biosciences	IFS
Hemagglutinin (HA)	Dr. Pandiella	IFS
β-actin (clon AC-74)	Sigma-Aldrich	WB

Apoptosis assay. Apoptosis assay after 48 h of transfection MCF-7 cells (3×10^5 cells/well) was carried out by flow cytometry (FACScan, BD Biosciences), using propidium iodide (PI), FICT Annexin V (BD Biosciences) staining, and TUNEL assay (*In Situ* Cell Death Detection Kit, Fluorescein, Roche Applied Science). For PI analysis, cells were harvested, fixed with 70% cold ethanol for 30 minutes, and washed with PBS. Cells were then incubated with ribonuclease (100 μg/ml), and PI (50 μg/ml) for 30 minutes in darkness. For FITC Annexin V staining, MCF-7 cells were harvested,

washed twice with PBS, and resuspended in 1X binding buffer (0.1 M Hepes (pH 7.4), 1.4 M NaCl, and 25 mM $CaCl_2$). 5µl of FITC Annexin V was added and incubated for 15 min at room temperature in darkness. Finally, 400 µl of 1X binding buffer was added to each tube, and analyzed. The *In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science) was employed according to the instructions of the manufacturer.

Soft agar assay. MCF-7 Tet-Off cells or MDA-MB-231 cells (5×10^4) stably transfected with either pTRE2 control vector or pTRE2-hPit-1 overexpression vector, and the pSingle-tTS-shRNA-hPit-1 vector, were seeded on 35-mm plates containing DMEM 2X, 10% FBS with 1.8% agarose in the bottom layer, and 0.7% agarose in DMEM 2X in the top layer, and maintained at 37ºC in an incubator. Doxycyclin (1 µg/ml) was added to the MDA-MB-231 cells every 72 h. Cultures were maintained during eight or twelve days for MCF-7 Tet-Off or MDA-MB-231 cells, respectively. After these days, cells were stained with crystal violet (0.005%, Sigma-Aldrich) for 1 hour, and counted. Colonies that grew beyond 50 µm in diameter were scored as positive. Each experiment was done in triplicate.

Wound healing. MCF-7 cells transfected with the pRSV-hPit-1 overexpression vector or with the pRc/RSV control vector were seeded in T6-well culture dishes at a density of 15×10^4 cells per well. A wound was incised 24 h later in the central area of the confluent culture, which was incubated for a further 24 h after careful washing to remove detached cells and addition of fresh medium. Cultures were observed at 0, 12, and 24 h and phase-contrast pictures were taken on the wounded area using an inverted microscope.

Cell Migration and Invasion Assay. Assays were performed in BD BioCoat Matrigel invasion chambers according to the manufacturer's instructions (BD Biosciences). Uncoated porous filters (8 µm pore size) were used for estimating cell migration, and filters precoated with Matrigel were used for examining cell invasion.

MCF-7 cells were transfected with pRc/RSV or pRSV-hPit-1, while MDA-MB-231 cells were transfected with scrambled siRNA or Pit-1 siRNA, 26 hours before experimentation. Cells were detached from the plates, and the cell suspension was placed into the upper chamber in 0.5 ml of DMEM serum-free medium (5 X 10^4 cells per filter). DMEM medium supplemented with 10% FBS was placed in the lower chamber as a chemoattractant. After incubation for 22 hours, cells that had migrated to the lower surface of the filters were fixed in methanol for 2 min at room temperature, stained using crystal violet for 2 min, visualized and counted. Values for cell migration or invasion were expressed as the mean number of cells per microscopic field over four fields per one filter for duplicate experiments. Experiments were repeated three times.

RNA isolation, RT-PCR, and quantitative RT-PCR. Total RNA was isolated from the cell lines using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA synthesis, and RT-PCR were performed as described elsewhere (2). Reactions of quantitative RT-PCR were done using iQ SYBR Green Supermix (Bio-Rad) on iCycler equipment (7500 PCR Systems, Applied Biosystems). The samples for Pit-1, Snail and 18S were denatured at 94ºC for 10 sec, annealed at 58ºC for 10 sec and extended at 72ºC for 10 sec, for a total of 40 cycles. The samples were quantified using the Sequence Detection Software 1.4 (Applied Biosystems), with 18S as normalization control.

ChIP Assay. ChIP assays were performed using the protocol of Upstate as described previously (4). Diluted soluble chromatin fractions were immunoprecipitated with 1 μg polyclonal anti-Pit-1 antibody (Santa Cruz Biotechnology) or control human IgG (Sigma Aldrich). The DNA extracted was then dissolved in 30 μl of H_2O. Five microliters of assayed DNA sample and 5 μl of input/start material were used in each

50-µl reaction. The PCR was run for 60 sec at 95ºC, 58ºC, and 72ºC within each cycle, for a total of 35 cycles.

Primers

Gene	**Primers**
Pit-1	F: 5′-GTGTCTACCAGTCTCCAACC-3′ R: 5′-ACTTTTCCGCCTGAGTTCCT-3′
Snail	F: 5′-GGTTCTTCTGCGCTACTGCT-3′ R: 5′-TAGGGCTGCTGGAAGGTAAA-3′.
18S	F: 5'-GTAACCCGTTGAACCCCATT-3' R: 5'-CCATCCAATCGCTA GTAGCG-3'
Snail promoter (A)	F: 5'-CCCTTGATAATTCCTCACTTCC-3' R: 5'-GGGTAGCTTCTGGTCCAGTG-3'
Snail promoter (B)	F: 5′-CTTCGTCTGTCTCCCTCACTG-3′ R: 5′-ACATCACTGGGGAGGAAGC-3′
Snail promoter (C)	F: 5′-CTCGTCAATGCCACGCTCT-3′ R: 5′-GCCGCCAACTCCCTTAAGTA-3′,
Snail promoter (D)	F: 5′-CCCGGAGTACTTAAGGGAGTT-3′ R: 5′-CATCCTGTGACTCGATCCTG-3′

Patient selection, breast cancer samples, and immunohistochemistry. This study is comprised of 110 women with a histologically confirmed diagnosis of early invasive breast cancer (without distant metastasis at time of initial diagnosis) and treated at Fundación Hospital de Jove of Gijón (Spain), between 1990 and 2003. We selected women with the following inclusion criteria: invasive ductal carcinoma, and a minimum of five years of follow-up for those women without tumor recurrence. The exclusion criteria were the following: metastatic disease at presentation, prior history of any kind of malignant tumor, bilateral breast cancer at presentation, having received any type of neoadjuvant therapy, development of loco-regional recurrence during the follow-up period and development of a second primary cancer. From the patients fulfilling these criteria, we randomly selected a sample size of 110 patients divided into four different groups with similar size and stratified with regard to nodal status and to the

development of metastatic disease, which were the key measure variables of the study. Thus, we included an important number of events in both node-positive and node-negative patient subgroups (half of the cases that developed distant metastases during the follow-up period are included in each subgroup) in order to guarantee the statistical power of the survival analysis. Patient characteristics included in the two main groups, with or without distant metastases, are listed in Table S1. Desmoplastic reaction was defined as the pervasive growth of dense fibrous tissue around the tumor. The fibrous peritumoral stroma undergoes proliferation of newly formed fibroblasts in an edematous, myxomatous or highly collagenized matrix. A tumor was considered positive for peritumoral inflamammation if we observed a dense chronic inflammatory infiltrate rich in plasma cells and lymphocytes in at least 50% of peritumoral area. Staining for ERs and PgRs was scored according to the method described by Allred et al. (5). Patients underwent either modified radical mastectomy or wide resection with axillary lymphadenectomy when necessary. Postoperative radiotherapy was given to 38 patients (65.5%). The criteria for systemic adjuvant therapy were as follows: i) node-negative patients with ER and /or PgR positive tumors received tamoxifen (20 mg per day during five years); ii) node-negative patients with ER and PgR negative tumors received six cycles of intravenous CMF (cyclophosphamide, methotrexate and 5-fluorouracil) every 3 weeks, if their tumors were either larger than one centimeter, moderately or poorly differentiated, or if patients were younger than 35 years old; iii) node-positive patients received six cycles of intravenous FEC (5-fluorouracil, epirubicin and cyclophosphamide) every 3 weeks, plus sequential tamoxifen if they had ER and/or PgRpositive tumors. Overall, 39 patients received chemotherapy, 35 patients received tamoxifen, and 16 patients received both types of systemic therapy. Women were treated according to the guidelines used in our institution. The study adhered to national regulations and was approved by our institution Ethics and Investigation Committee.

The end-point was distant metastatic relapse. The median follow-up period in patients without metastases was 88 months, and 45 months in patients with metastases. Breast carcinoma tissue samples were obtained at the time of surgery. Prior informed consent was obtained from patients. Out of these 110 samples, 65 were also evaluated for Snail immonopositivity. Routinely fixed (overnight in 10% buffered formalin), paraffin-embedded tumor samples stored in our pathology laboratories were used. Histopathologically representative tumor areas without necrosis were defined on haematoxylin and eosin-stained sections. Serial 5-μm sections were consecutively cut with a microtome (Leica Microsystems) and transferred to adhesive-coated slides. Imunohistochemistry was done on these sections using a TechMate TM50 autostainer (Dako). Antibodies for Pit-1 and Snail were obtained from Santa Cruz Biotechnology and from Dr. Garcia de Herreros, respectively. We used human pituitary gland (obtained from autopsy) as positive control for Pit-1. Tissue sections were deparaffinized in xylene, and then rehydrated in decreasing concentrations of ethanol (100%, 96%, 80%, 70%, then water). To enhance antigen retrieval, sections were microwave-treated (H2800 Microwave Processor, EBSciences) in citrate buffer (Target Retrieval Solution, Dako) at 99ºC for 1 min. Endogenous peroxidase activity was blocked by incubating the slides in peroxidase-blocking solution (Dako) for 5 min. The EnVision Detection Kit (Dako) was used as the staining detection system. Sections were counterstained with hematoxilin, dehydrated with ethanol, and permanently coverslipped. For each antibody preparation studied, the location of immunoreactivity, percentage of reactive area and intensity were determined. All the cases were semiquantified for each protein-stained area. An image analysis system with the Olympus BX51 microscope and soft analysis (analySIS®, Soft imaging system) were used as follows: tumor sections were stained with antibodies according to the method explained above and counterstained with haematoxylin. There were different optical

thresholds for both stains. Each slide was scanned with a 400X power objective and selected four fields per case searching for the protein-reactive areas. The computer program selected and traced a line around antibody-reactive areas (higher optical threshold: red spots), with the remaining, non-stained areas (haematoxylin-stained tissue with lower optical threshold) standing out as a blue background. Any field had an area ratio of stained (red) versus non-stained (blue). To evaluate immunostaining intensity we used a numeric score ranging from 0 to 3, reflecting the intensity as follows: 0, no reactivity; 1, weak reactivity; 2, moderate reactivity; and 3, intense reactivity. Using an Excel spreadsheet, the score of one field was obtained by multiplying the intensity score (I) by the percentage of reactivity area (PA) and the results were added together (total score: I x PA). In addition, for each tumor the mean score of the four fields evaluated was calculated.

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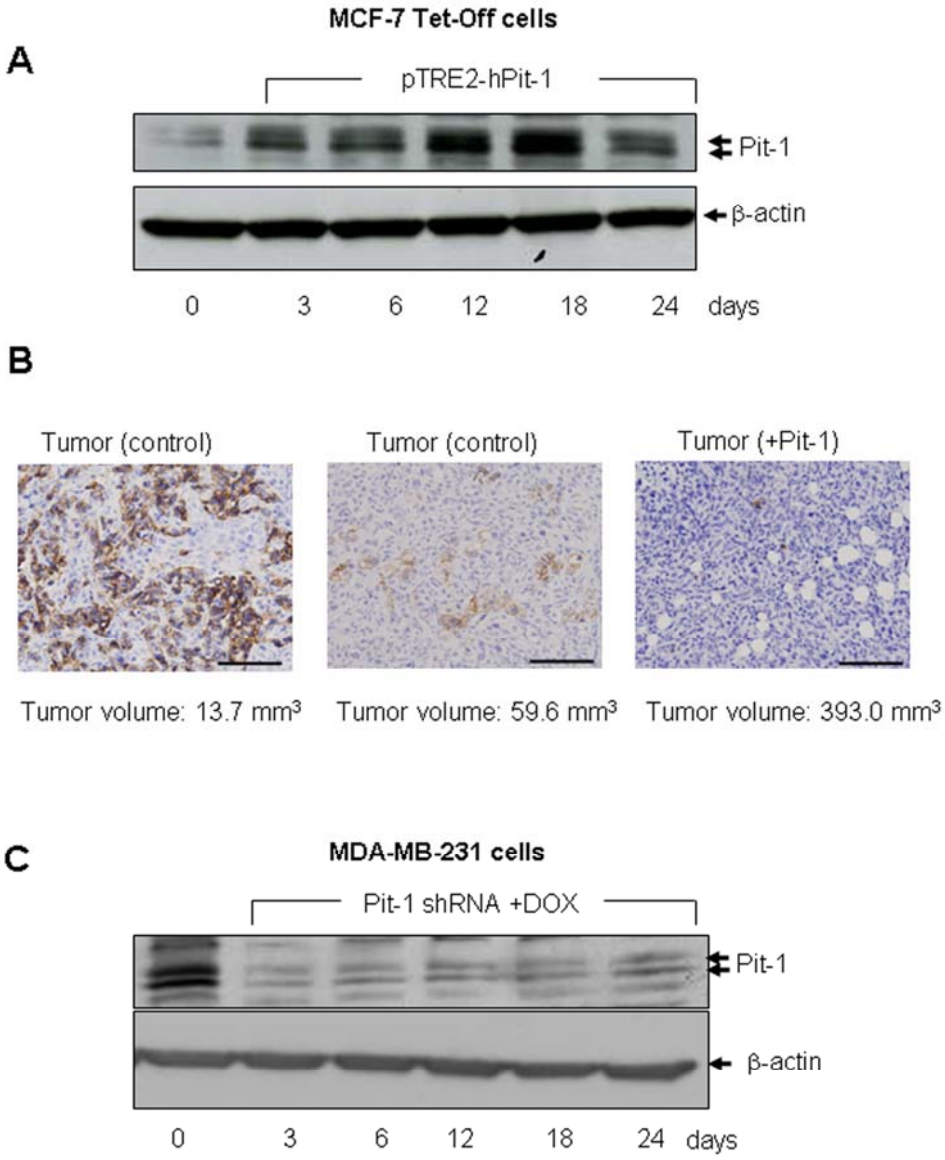
SUPPLEMENTAL FIGURES LEGENDS

Supplemental Figure 1

(A) Western blot of Pit-1 expression at 0, 3, 6, 12, 18, and 24 days of culture of MCF-7 cells stably transfected with the pTRE2-hPit-1 overexpression vector. **(B)** E-Cadherin expression is related to tumor size. Representative example of E-cadherin immunostaining in two control SCID mice with different size tumors (induced by subcutaneous injection of MCF-7 cells), and in an SCID mouse injected with MCF-7 cells stably transfected with the pTRE2-hPit-1 overexpression vector (+Pit-1) during 20 days. Scale bar, 100 mm. **(C)** Western blot of Pit-1 expresion at 0, 3, 6, 12, 18, and 24 days in MDA-MB-231 cells stably transfected with the pSingle-tTS-Pit-1 shRNA vector and treated with doxycyclin.

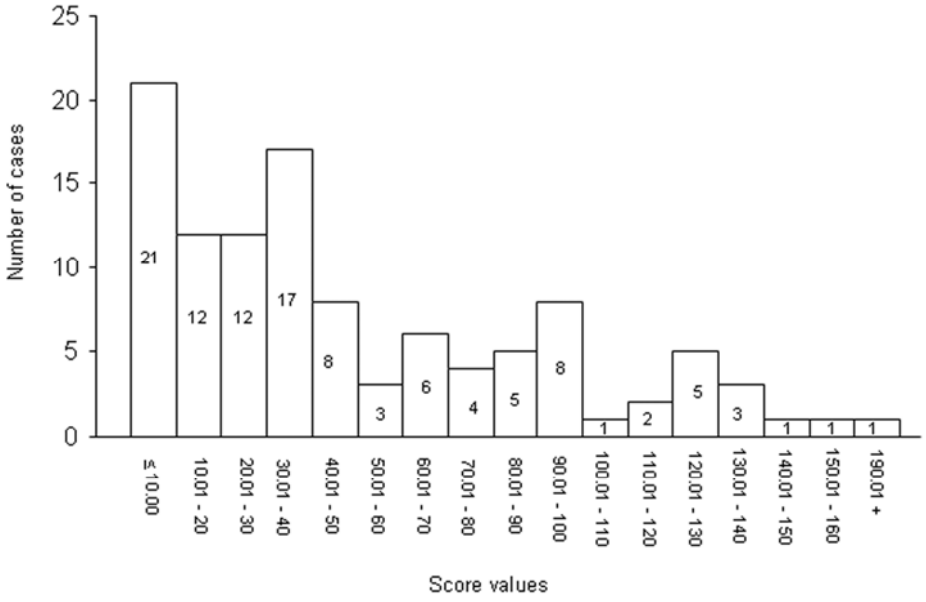
Supplemental Figure 2

Distribution of score values obtained by immunohistochemical staining of Pit-1 in 110 ductal invasive carcinomas of the breast.



Supplemental Figure 1

(A) Western blot of Pit-1 expression at 0, 3, 6, 12, 18, and 24 days of culture of MCF-7 Tet-Off cells stably transfected with the pTRE2-hPit-1 overexpression vector. **(B)** E-Cadherin expression is related to tumor size. Representative example of E-cadherin immunostaining in two control SCID mice with different size tumors (induced by subcutaneous injection of MCF-7 cells), and in an SCID mouse injected with MCF-7 cells stably transfected with the pTRE2-hPit-1 overexpression vector (+Pit-1) during 20 days. Scale bar, 100 mm. **(C)** Western blot of Pit-1 expresion at 0, 3, 6, 12, 18, and 24 days in MDA-MB-231 cells stably transfected with the pSingle-tTS-Pit-1 shRNA vector and treated with doxycyclin.



Supplemental Figure 2
Distribution of score values obtained by immunohistochemical staining of Pit-1 in 110 ductal invasive carcinomas of the breast.

Supplemental Table 1. Basal characteristics of 110 patients with invasive ductal carcinoma of the breast.

CHARACTERISTICS	WITHOUT RECURRENCE	WITH RECURRENCE
	N (%)	N (%)
TOTAL CASES	52 (47.2)	58 (52.8)
AGE (YEARS)		
<57	25 (48.1)	30 (51.7)
>57	27 (51.9)	28 (48.3)
MENOPAUSAL STATUS		
PREMENOPAUSAL	17 (32.7)	17 (29.3)
POSTMENOPAUSAL	35 (67.3)	41 (70.7)
TUMOR SIZE		
T1	27 (51.9)	25 (43.1)
T2	25 (48.1)	33 (56.9)
NODAL STATUS		
N-	27 (51.9)	25 (43.1)
N+	25 (48.1)	33 (56.9)
HISTOLOGICAL GRADE		
WELL DIF.	19 (36.5)	13 (22.4)
MOD DIF.	27 (51.9)	26 (44.8)
POORLY DIF.	6 (11.5)	19 (32.8)
NOTTINGHAM PRONOSTIC INDEX		
<3.4	25 (48.1)	14 (24.1)
3.4-5.4	21 (40.4)	32 (55.2)
>5.4	6 (11.5)	12 (20.7)
ESTROGEN RECEPTORS		
NEGATIVE	15 (32.6)	31 (56.4)
POSITIVE	31 (67.4)	24 (43.6)
PROGESTERONE RECEPTORS		
NEGATIVE	20 (43.5)	36 (65.5)
POSITIVE	26 (56.5)	19 (34.5)
DESMOPLASIA		
NEGATIVE	21 (40.4)	15 (25.9)
POSITIVE	31 (59.6)	43 (74.1)
PERITUMORAL INFLAMMATION		
NEGATIVE	30 (62.5)	33 (56.9)
POSITIVE	18 (37.5)	25 (43.1)
TUMOR ADVANCE		
EXPANSIVE	27 (55.1)	22 (37.9)
INFILTRATE	22 (44.9)	36 (62.1)
PERINEURAL INVASION		
NEGATIVE	47 (90.4)	53 (91.4)
POSITIVE	5 (9.6)	5 (8.6)
LYMPHOVASCULAR INVASION		
NEGATIVE	30 (57.7)	39 (67.2)
POSITIVE	22 (42.3)	19 (32.8)
TUMOR NECROSIS		
NEGATIVE	44 (89.8)	51 (87.9)
FOCAL	4 (8.2)	6 (10.3)
EXTENSIVE	1 (2)	1 (1.7)
MITOSIS		
<10	32 (65.3)	32 (55.2)
>10	17 (34.7)	26 (44.8)
ADJUVANT RADIOTHERAPY		
NO	31 (59.6)	23 (39.6)
YES	21 (40.3)	35 (60.3)
ADJUVANT SYSTEMIC THERAPY		
CHEMOTHERAPY	25 (43.1)	14 (26.9)
ADJUVANT TAMOXIFEN	14 (24.1)	21 (40.4)
CHEMOTHERAPY+SEQUENTIAL TAMOXIFEN	6 (10.3)	10 (19.2)
NO TREATMENT	13 (22.4)	7 (13.5)

Supplemental Table 2. Relationship between Pit-1 immunostaining and clinic-pathological characteristics in 110 patients with invasive ductal carcinoma of the breast.

CHARACTERISTICS	Nº CASES	PIT-1(score values)	p
TOTAL CASES	110	35.4 (0-194.5)	n.s
AGE (YEARS)			
<57	55	32.5 (0-156.7)	n.s
>57	55	36.3 (0-194.5)	n.s
MENOPAUSAL STATUS			
PREMENOPAUSAL	34	46.3 (0-156.7)	n.s
POSTMENOPAUSAL	76	33.6 (0-194.5)	n.s
TUMOR SIZE			
T1	52	33.8 (0-143.1)	n.s
T2	58	36.6 (0-194.5)	n.s
NODAL STATUS			
N+	58	39.7 (0-194.5)	n.s
N-	52	32.6 (0-135.6)	n.s.
HISTOLOGICAL GRADE			
WELL DIF.	32	32.3 (0-135.6)	n.s
MOD DIF.	53	34.4 (0-194.5)	n.s
POORLY DIF.	25	46.2 (0-129.7)	n.s
NOTTINGHAM PRONOSTIC INDEX			
<3.4	39	35.7 (0-135.6)	n.s
3.4-5.4	53	40.3 (0-194.5)	n.s
>5.4	18	22.9 (0-143.2)	n.s
ESTROGEN RECEPTORS			
NEGATIVE	46	36.7 (0-156.7)	n.s
POSITIVE	55	33.1 (0-143.2)	n.s
PROGESTERONE RECEPTORS			
NEGATIVE	56	34.8 (0-156.7)	n.s
POSITIVE	45	43.1 (0-143.2)	n.s
DESMOPLASIA			
NEGATIVE	36	34.4 (0-132.3)	n.s
POSITIVE	74	36.3 (0-194.5)	n.s
PERITUMORAL INFLAMMATION			
NEGATIVE	63	35.1 (0-194.5)	n.s
POSITIVE	43	35.7 (0-156.7)	n.s
TUMOR ADVANCE			
EXPANSIVE	49	37.1 (0-194.5)	n.s
INFILTRATE	58	34.8 (0-156.7)	n.s
PERINEURAL INVASION			
NEGATIVE	100	36.7 (0-194.5)	n.s
POSITIVE	10	23.6 (0-77.9)	n.s
LYMPHOVASCULAR INVASION			
NEGATIVE	69	35.7 (0-194.5)	n.s
POSITIVE	41	33.1 (0-156.7)	n.s
TUMOR NECROSIS			
NEGATIVE	95	35.1 (0-194.5)	n.s
FOCAL	10	42.9 (17.7-132.3)	n.s
EXTENSIVE	2	56.8 (22.3-91.32)	n.s
MITOSIS			
<10	64	36.7 (0.143.2)	n.s
>10	43	35.1 (0-194.5)	n.s