Identification of a GαGβγ, AKT and PKCα signalome associated with invasive growth in two genetic models of human breast cancer cell epithelial-to-mesenchymal transition

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Abstract. The epithelial-to-mesenchymal transition (EMT) confers an aggressive subtype associated with chemotherapy resistance in epithelial cancers. However, the mechanisms underlying the EMT and its associated signaling dysfunctions are still poorly understood. In two genetic models of MCF-7 breast cancer cells induced to EMT by *WISP-2* silencing and Snail transformation, we investigated the status of several signaling elements downstream of G-protein receptors (GPR) and their functional roles in the invasive growth potential. We report that the E-cadherin repressors Slug, Zeb1/2 and Twist are overexpressed in these EMT cells characterized by a triple negative phenotype (loss of estrogen ERα and progesterone PRA/PRB receptors, no *HER2* amplification), combined with loss of the alternative GPR30 estrogen receptor and induction of the invasive growth in collagen type I gels. Ectopic Snail expression suppressed *WISP-2* transcripts and down-regulated *WISP-2* gene promoter expression in transfected cells. Accordingly, *WISP-2* transcripts and Wisp-2 protein were depleted in these two convergent models of BC cell EMT. The EMT caused dominance of several proinvasive pathways downstream of GPR, including GαGβγ subunits, PKCα, AKT and c-Jun induction, constitutive activation of the actin-remodeling GTPase Rac1, coupled with growth responses (more cells at S and G2/M phases of the cell cycle), in line with inhibition of the $p27^{kip1}$/cyclin-dependent kinase CDK3 cascade. RNA interference or selective inhibitors targeting GαGβγ subunits (BIM-46187, gallein), PKCα (Gö6976, MT477, sh-RNAs) and PI3K-AKT (wortmannin) alleviated the invasive phenotype. In contrast, MCF-7 cells in EMT showed signaling independence to inhibitors of HER family tyrosine kinases and the mitogen- and stress-activated protein kinases. Our study suggests that the signaling protagonists GαGβγ, PKCα and PI3K-AKT are promising candidates as predictive molecular biomarkers and therapeutic targets in the management of clinical BC in EMT.

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Abbreviations: BC, breast cancer; CDK3, cyclin-dependent kinase-3; EMT, epithelial-to-mesenchymal transition; ERα, estrogen receptor-α; ERK1/2, extracellular signal-regulated kinases 1/2, p38 kinases; GPR, G-protein receptors; GαGβγ, G-proteins α, β and γ; HB-EGF, heparin-binding EGF-like growth factor; HER, epidermal receptor family; JNK, c-Jun NH2 terminal kinases; MAPK/SAPK, mitogen- and stress-activated protein kinases; MMP, matrix metalloproteinase; PI3K, phosphoinositide 3-kinase; PKCα, protein kinase C-α; PLC, phospholipase C; PKA, protein kinase A; PRA/PRB, progesterone receptors A and B; PTx, pertussis toxin; qRT-PCR, quantitative real-time polymerase chain reaction; sh-RNAs, short-herpin RNAs; TGFβ, transforming growth factor-β; TNBC, triple-negative breast cancer

Key words: WISP-2, Snail, GPR30, Rac1 GTPase, protein kinase C-α, short-herpin RNAs, p27, HER family, prognosis, targeted-therapy

Introduction

The metastatic cascade integrates the invasive growth of cancer cells in primary tumors and their subsequent dissemination to vital organs. Cancer cell invasion and metastasis share many parallels with the epithelial-to-mesenchymal transition (EMT) phenotype, initially described during normal embryonic development (1,2). The signals that initiate and regulate EMT derive

from intrinsic genetic alterations in cancer cells as well as from signaling crosstalk with the tumor stroma microenvironment and autocrine or paracrine effectors such as TGFβ, Wnt, and EGF (1-5). The full EMT is mainly characterized by disruption of epithelial cell polarity and adherens junctions, loss of the cell-cell adhesion molecule E-cadherin, nuclear localization of β-catenin, and gain of the mesenchymal markers N-cadherin, vimentin and fibronectin. Several EMT transcription factors such as Snail, Slug, Zeb1, Zeb2 and Twist can act in concert as claudin (*CLDN*) and E-cadherin (*CDH1*) gene repressors and display multiple connections with oncogenes and tumor suppressor genes implicated in the progression and dissemination of localized epithelial cancers to organ-specific metastasis (1,2). For instance, the zinc-finger protein Snail can act as a repressor of the ERα estrogen receptor during EMT in breast cancer (BC) (6,7). In addition, we have previously shown that *WISP-2/CCN5* silencing promoted EMT via activation of the TGFβ signaling pathways in BC (8). WISP-2/CCN5 is a 29-kDa protein, which belongs to the cysteine-rich 61/connective tissue growth factor/nephroblastoma overexpressed (CCN) family and underexpressed in several human epithelial cancers (8).

We, and others, have identified G-protein receptors (GPR) and their immediate downstream signaling transducers, the Gα/Gβγ subunits, as critical protagonists driving the neoplasia and drug resistance in epithelial cancers of the colon, prostate and breast (9-12). These main GPR signaling cascades include the transforming elements phospholipases C (PLC), protein kinases C (PKC), cAMP-activated protein kinase, phosphoinositide 3-kinases (PI3K), the serine/threonine kinases AKT, the mitogen-activated and stress-activated protein kinase (MAPK/SAPK) family, i.e., ERK1/2 (extracellular signal-regulated kinases), p38 kinases and JNK (c-Jun NH2 terminal kinases). GPR ligands such as lysophosphatidic acid, endothelin, and thrombin transactivate and crosstalk with HER1 through PKC and protease-dependent proHB-EGF shedding (13,14). The best example to map such crosstalk in BC is the GPR30/HER1 transactivation cascade using GPR30 as an alternative non-nuclear estradiol receptor. Of note, GPR30 is now considered as a marker of the metastatic potential in BC (15,16). Consistently, GPR signaling pathways are now considered in targeted therapies against cancer, as recently exemplified by the discovery of small molecule inhibitors targeting Gα or Gβγ subunits (17-21).

Herein, we characterized the dominant signaling transduction cascades regulating the invasive growth in two genetic BC models following EMT induction by Snail overexpression or by *WISP-2* silencing (3,6). Indeed, the physiological function and interplay between Snail, WISP-2/CCN5 and other EMT transcription factors is not fully understood in BC. Therefore, we further characterized these transformed cells, in regard to the expression of several EMT transcription factors and markers with special reference to the BC triple-negative phenotype (22) and their dependence to HER family tyrosine kinases. In the present study, we established for the first time a common molecular signature illustrated by the constitutive activation of the GαGβγ, AKT and PKCα signaling pathways in two genetic models of breast cancer cell EMT. It is anticipated that our data will contribute to improve the stratification of BC in EMT and identification of novel prognostic markers in order to provide new clues in targeted therapies and personalized medicine.

Materials and methods

Cell culture, transfections and gene promoter-reporter assay. MCF-7 breast cancer cell lines expressing SNAIL-WT and SNAIL-6SA, control (scrambled sh-RNA, sh-CON) and *WISP-2* silenced (sh-WISP-2) MCF-7 cells were grown as described previously (3,6). HEK293 and HeLa cells were transiently transfected for 36 h by the *WISP-2*-Luc promoter (23) and pGL3 plasmids (Lipofectamine, Invitrogen). Luciferase activity (Promega, Charbonnières, France) was measured after 48 h and normalized for transfection efficiency by a β-galactosidase-expressing vector and the Galacto-Star system (Applied Biosystems, Life Technologies).

Cancer cell growth and invasion. Cells were cultured in the presence of BIM-46187 or gallein for 48 h and pertussis toxin (PTx) for 24 h and counted in Beckmann Coulter counter (Roissy, France). Cell cycle analysis and invasion assays were performed as described (24,25). The invasion index was calculated as the ratio of the number of invading cells divided by the total number of cells in ten randomly selected microscopic fields (DMI 3000B, Leica, Wetzlar, Germany). Where indicated, samples were stained with H&E or anti-vimentin, anti-cytokeratin and anti-E-cadherin antibodies.

Immunoblotting. Whole cell extracts were prepared in RIPA buffer containing a cocktail of proteases (Complete®, Roche, Meylan, France) and phosphatase inhibitors (PhosSTOP®, Roche). Immunoblot analysis was performed as described (24). The primary monoclonal (mAb) antibodies: anti-Snail, -Slug, -p-ERK1/2 (Thr202/Tyr204), -HER2, -p-HER2 (Tyr1221/1222), -HER3, polyclonal (pAb) antibodies anti-$p27^{kip1}$, -ERK1/2, -p-PKCα (Thr638), -p-AKT (Ser473), -AKT, -HER1, -p38 (Cell Signaling, Ozyme, Saint-Quantin-en-Yvelines, France). mAb anti-Twist, -E-cadherin, -CDK3, -p-JNK (Thr183/Tyr185), -p-c-Jun (Ser63), pAb anti-Zeb1, -Zeb2, -p-p38 (Thr180/Tyr182), -c-Jun, -JNK, -PKCβII, -PKCδ (Santa Cruz, Ozyme). mAb anti-vimentin (Sigma-Aldrich), anti-ERα, -Wisp-2 (Abcam, Paris, France), anti-PRA/PRB (Menarini Diagnostics, Novocastra, Newcastle, UK), α-tubulin (Amersham, GE Healthcare) PKCα (BD Bioscience, Le-Pont-de-Claix, France), pAb anti-GPR30 GeneTex (Interchim, Montluçon, France). Secondary antibodies against mouse and rabbit primary antibodies (Jackson ImmunoResearch, Interchim).

Quantitative RT-PCR analysis. Total RNA was extracted and purified from cellular pellets using the RNeasy Plus® Mini Kit (Qiagen, Les Ulis, France). cDNAs were synthesized according to the protocol for First Strand cDNA Synthesis® (Fermentas, Villebon-sur-Yvette, France) using the RevertAid™ H Minus M-MuLV reverse transcriptase. The real-time PCR using Maxima SYBR Green/ROX qPCR Master Mix® (Fermentas) was performed as described previously (3). The primers for SNAIL, SLUG, ZEB1, ZEB2, TWIST, CDH1, JUN, PKCα, Gα (i1, 2, 3), Gα (o, 12, 13, q, 11 and s), HER2, HER3 and GAPDH were supplied from Qiagen; WISP-2 and HER1 from Sigma-Aldrich (PCR primer sequences are available upon request).

Rho and Rac activation assays. MCF-7 cells were lysed in lysis buffer according to the manufacturer's protocol. Equal

amounts of protein were incubated in equal volumes of either GST-tagged fusion-proteins corresponding to the p21 binding domain of p21-activated kinase-1 (PAK-1) or Rhotekin Rho binding domain bound to agarose beads (Millipore, Billerica, MA). The total amount of GTPases in cell lysates and their corresponding GTP-bound fractions were detected by immunoblot using monoclonal antibodies against RhoA (Santa Cruz Ozyme) and Rac1 (Transduction Laboratories, Lexington, KY). The relative intensities were determined by Image J software.

Short-hairpin RNAs and PRKCA silencing. Human PKCα sh-RNA expression vectors (pGFP-V-RS, OriGene, Rockville, MD) were used for stable silencing of *PRKCA* (*PKCα*) in MCF-7-SNAIL-6SA by Lipofectamine. These vectors encode a mixture of four PKCα sh-RNAs, puromycin resistance and the green fluorescent protein (GFP). The corresponding control cells were transfected with the scrambled (Scr) sh-RNA cassette. Transfection efficiencies were monitored by GFP. Stable clones were isolated by puromycin selection.

Chemical inhibitors. PD98059, SB203580, SP600125, U73122, KT5720 and Gö6976 were from Calbiochem (San Diego, CA); BIM-46187 (IPSEN Laboratories, Les Ulis, France); marimastat, PTx, and gallein (Tocris Bioscience, Biozol Diagnostica, Germany); MT477 (Medisyn Technologies Inc., Minnetonka, MN); Iressa (AstraZeneca, Macclesfield, UK). Pertuzumab/Omnitarg/2C4 and Trastuzumab/Herceptin (Genentech Inc., San Francisco, CA), Lapatinib/GW572016 (GlaxoSmithKline, Brentford, UK), Cetuximab/C225/Erbitux (ImClone Systems Inc., New York, NY) and Panitumumab/Vectibix (Amgen, Thousand Oaks, CA).

Statistical analyses. Statistical analyses were performed using Microsoft Excel. For type I collagen invasion assay SPSS version 12.0 software (IBM) was used for mean, SEM and Student's t-test. In all tests, the level of statistical significance was set at $P<0.05$.

Results

Induction of EMT transcription factors in sh-WISP-2 and SNAIL-6SA-transformed MCF-7 cells. The two models of MCF-7 breast cancer cells in EMT have been described previously. The first model, SNAIL-6SA, was established by constitutive activation of the Snail transcription factor, with replacement of six Ser to Ala in the GSK3β-phosphorylation consensus motif (6). The second model, sh-WISP-2, has silenced *WISP-2*/*CCN5* gene by short-hairpin RNA targeting *WISP-2*/*CCN5* transcripts (3). MCF-7 cells are normally organized as tightly adhesive cubical epithelial clusters when spread on the culture substratum, showing intercellular junctions (Fig. 1A). Stable expression of the EMT transcription factor Snail-WT had no apparent impact on the epithelial morphology. In contrast, *WISP-2* silencing and Snail-6SA transformation induced extensive scattering and marked changes in the morphology of cells displaying elongated mesenchymal-like structure with filopodia protrusions in culture, reduced cellular adherence, spread and loss of cell-cell contacts, all criteria consistent with the induction of a more migratory phenotype. These features confirm the morphological transitions previously reported for these two genetic MCF-7 models in EMT (3,6). We further examined the relative expression of a series of EMT-related transcription factors by western blot analysis and quantitative real-time polymerase chain reaction (qRT-PCR) (Fig. 1B and C). Immunoblot analysis revealed that the Snail, Slug, Zeb1/2 and Twist EMT transcription factors were barely detectable in parental MCF-7 epithelial cells. Interestingly, constitutive expression of Snail-WT was not sufficient to induce the expression of Slug, Zeb1/2 and Twist in MCF-7 cells as well as E-cadherin repression or vimentin induction. These data indicate that Snail-WT transformed cells are not engaged in a partial or full EMT (4). In contrast, constitutive expression of the proteasome-resistant, stable mutant Snail-6SA was associated with induction of Slug, Zeb1/2 and Twist proteins (Fig. 1B) as well as E-cadherin repression and vimentin induction, two canonical EMT signatures. The same four EMT transcription factors are induced by EMT in sh-WISP-2 cells which however, remain Snail-negative. Thus, the EMT of sh-WISP-2 cells is Snail-independent in clear contrast to the critical impact of Snail-6SA that drives the EMT phenotypes in MCF-7-SNAIL-6SA cells. In MCF-7-SNAIL-WT immunoblots, we detected an additional slow-migrating 33 kDa band, which most likely corresponds to phosphorylated Snail via a casein kinase CK1 and GSK3β-dependent post-transcriptional modification (6). In addition, we detected several bands corresponding to Snail fragments that were absent in the MCF-7-SNAIL-6SA variant (Fig. 1B). Consistently, once phosphorylated, Snail is exported from the nucleus to the cytoplasm for subsequently βTrCP-mediated ubiquitination and fragmentation/degradation.

As shown in Fig. 1C, qRT-PCR analysis of the transcripts encoding these EMT transcription factors and markers mirror our western blot analysis. Most interestingly, WISP-2 transcripts were down-regulated in SNAIL-6SA cells, but not in SNAIL-WT cells, suggesting that *WISP-2* depletion may contribute or play a significant role in the EMT induced by Snail-6SA in MCF-7 cells. In support of this notion, the promoter luciferase reporter assay Fig. 1D shows that Snail functions as a transcriptional repressor at the *WISP-2* promoter. In contrast, Zeb1 was inefficient in mimicking such *WISP-2* promoter repression in 293T cells but reduced this activity by 30% in HeLa cells. Western blot analysis confirmed that WISP-2 was expressed in both parental and SNAIL-WT cells but was fully down-regulated in SNAIL-6SA and sh-WISP-2 MCF-7 cells (data not shown).

Induction of functional EMT responses and triple-negative phenotypes in sh-WISP-2 and SNAIL-6SA-transformed MCF-7 cells. The invasive growth of MCF-7 cells following EMT is illustrated by the capacity to invade native collagen type I gels and marked stimulation of cell proliferation (2.5- to 3.4-fold) in SNAIL-6SA and sh-WISP-2 cells, respectively (Fig. 2A). In comparison, parental MCF-7 cells were non-invasive in collagen type I gels. Deregulated growth of EMT cells is supported by a striking decrease in the fraction of cells at the G0/G1 transition of the cell proliferation cycle associated with high proportion of cells at S or G2/M phases (2- and 3-fold increase, respectively) (Fig. 2B). This growth response is not sustained by survival responses and corresponding changes in the sub-G1 fractions. In agreement, EMT cells were characterized by a reduction of $p27^{kip1}$ levels (Fig. 2C), a dual tumor suppressor involved in the regulation of the cell cycle, contact growth inhibition, differentiation,

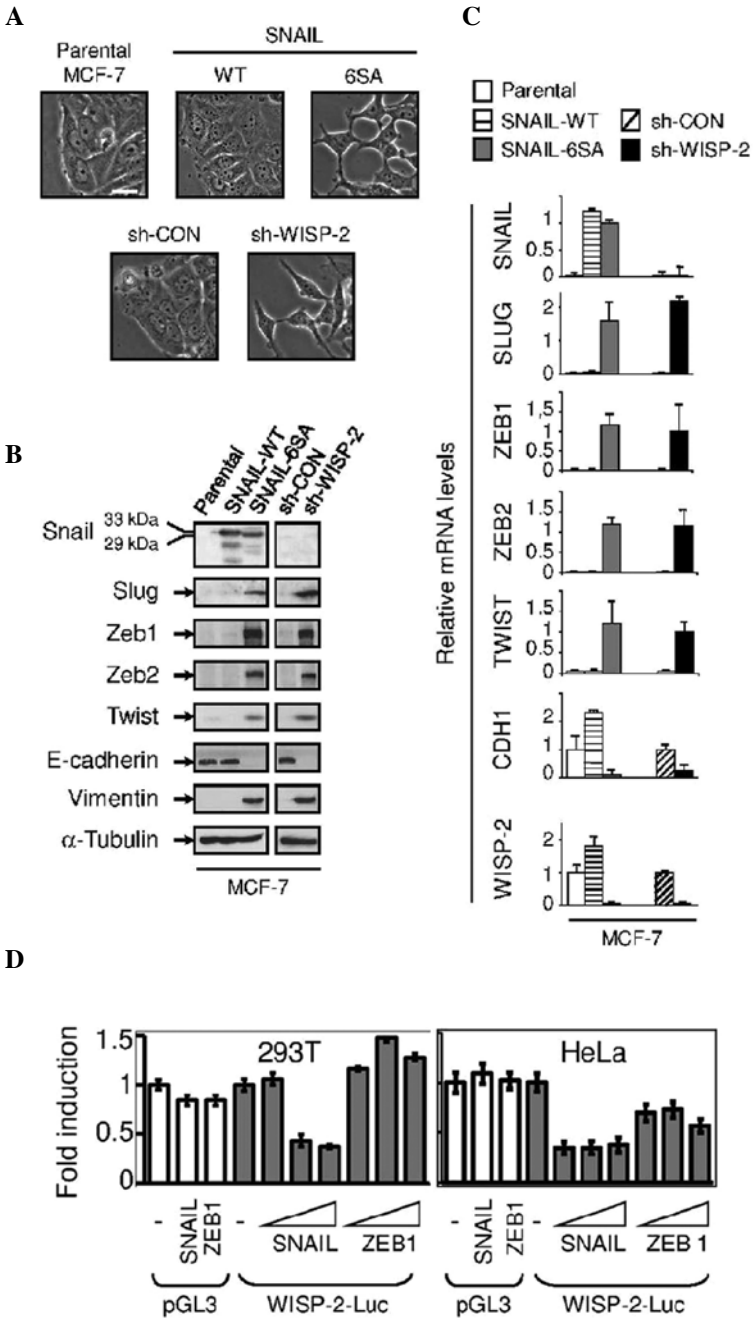

Figure 1. Implication of EMT transcription factors on morphotype, epithelial and mesenchymal markers in MCF-7 cell EMT. (A), Phase-contrast microscopy of stably transfected MCF-7 cells showing the epithelial morphology of parental MCF-7 cells and their derivatives, control MCF-7 cells (sh-CON, SNAIL-WT) in comparison with transitions to mesenchymal phenotypes observed in SNAIL-6SA and sh-WISP-2 cells. The images are recorded by System Time-lapse imaging microscopy (Biostation® IM-Q, Nikon), at magnification of x20. Scale bar, 60 μm. (B), Immunoblot analysis and relative expression of EMT transcription factors Snail, Slug, Zeb1 and 2, and Twist in SNAIL-6SA and sh-WISP-2-transfected cells, versus parental and control MCF-7 cells (SNAIL-WT and sh-CON cells). The EMT markers E-cadherin (epithelial criteria) and vimentin (mesenchymal criteria) indicate a full mesenchymal conversion observed in SNAIL-6SA and sh-WISP-2 cells. Total protein loading was 40 μg and α-tubulin was used as a loading control. (C), Comparative qRT-PCR analysis of the transcripts encoding these EMT transcription factors and markers using GAPDH for normalization. Data are expressed as relative transcript levels (arbitrary units). (D), Transcriptional impact of Snail and Zeb1 on the expression of the *WISP-2* promoter gene in HEK293 and HeLa cells. Data are means ± SEM of 3 experiments performed in triplicate (qRT-PCR, gene reporter assays) or are representative of three other experiments (immunoblots).

cell motility and metastasis susceptibility to carcinogen-induced tumors (26-29). Several mechanisms are involved in the functional expression, stability and localization of p27, including the loss of the E-cadherin tumor suppressor observed in EMT cells (29). Conversely, the downstream p27 effector cyclin-dependent kinase-3 (CDK3) is consistently induced at high levels in EMT cells, in accordance with the negative role played by p27 on CDK3 expression (30). Convergent with the EMT and its consequent impact on cell proliferation, differentiation and its opposing effects on p27/CDK3, both sh-WISP-2 and SNAIL-6SA cells exhibit a triple-negative phenotype TN (31) illustrated by a drastic repression of the nuclear estrogen receptors ERα, progesterone receptors PRA/PRB, and absence of *HER2* amplification (Fig. 2D). It should be noted that this EMT and TN phenotype was further characterized by the loss of the alternative estrogen receptor GPR30.

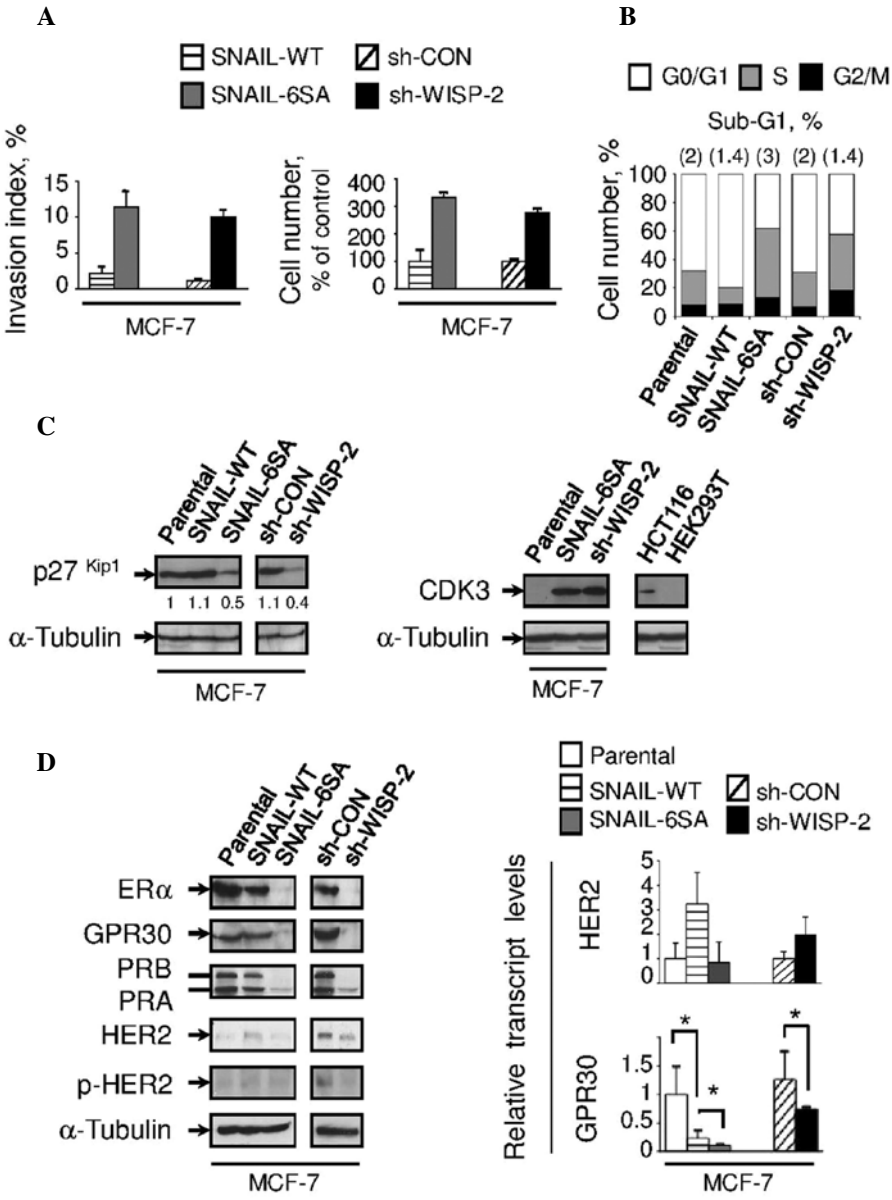

Figure 2. Induction of proliferation, invasion and loss of mammary epithelial markers during EMT in MCF-7 cells. (A), Control and EMT cells were tested for their ability to invade native type I collagen in a 24-h invasion assay (left). For the proliferation assays, cells were seeded in 12-well plates at the density of 20,000 cells/well and cultured for 72 h before direct cell counting. Columns are mean of 3-5 independent experiments. (B), Cell cycle analysis was determined by flow cytometry in parental and control MCF-7 cells (SNAIL-WT, sh-CON) and their EMT counterparts (SNAIL-6SA and sh-WISP-2). The percentages of cells in G0/G1, S and G2/M phases of the cell cycle are indicated and the sub-G1 fractions are quoted in parenthesis on the top of each corresponding bar. (C), $p27^{kip1}$ down-regulation and CDK3 overexpression in MCF-7 cells induced to the EMT phenotype. These two cell cycle regulators were quantified by autoradiography as arbitrary units versus α-tubulin signals in immunoblots prepared from parental, control and EMT cells. Human colon cancer cells HCT116 and HEK293 cells were respectively used as positive and negative controls for CDK3 expression. (D), Left: Immunoblots prepared from parental, control and EMT cells were analyzed for the expression of ERα GPR30, PRA and PRB, total and phosphorylated forms of HER2 (p-HER2 phosphorylation at the Tyr1221/1222 motifs). Right: Comparative qRT-PCR analysis of the transcripts encoding HER2 and GPR30. Data are means ± SEM of 3 experiments performed in duplicate (qRT-PCR, invasion assay, cell number determination) or are representative of three other experiments (immunoblots). SEM. *P<0.05 or better versus control cells.

Proinvasive signaling pathways involved in sh-WISP-2 and SNAIL-6SA-transformed MCF-7 cells. We analyzed the possible contribution of several proinvasive pathways in the EMT cells, using selective inhibitors targeting critical protagonists of invasive growth in epithelial cancer cells. First, we explored the requirement of matrix metalloproteinases (MMPs) in the invasive phenotype induced by the EMT in MCF-7 cells. Marimastat, a pan inhibitor of MMP-1, -2, -9, -14 (MT1-MMP) and MMP-7 (matrilysin), decreased by 50-60% collagen type I invasion in MCF-7 cells under EMT (Fig. 3A). EMT-induced invasiveness was strongly reversed by chemical inhibitors of GPR signaling, including BIM-46187 (Gα subunits), PTx (Gαi/o subunits), gallein (Gβγ subunits), U73122 (PLC), Gö6976 (Ca^{2+}-dependent PKCα and βI), the new PKCα inhibitor MT477 (32), and wortmannin (PI3K). In contrast, collagen invasion was insensitive to the PKA inhibitor KT5720. Consistent with our data, the inhibitory effects of the two PKC inhibitors coincided with accumulation and phosphorylation of the PKCα protein in EMT cells, as indicated by immunoblot analysis of this PKC isoform in SNAIL-6SA and sh-WISP-2 cells (Fig. 3B). Increased levels of PKCα were therefore directly connected with global activation of the enzyme and Thr638 phosphorylation. The

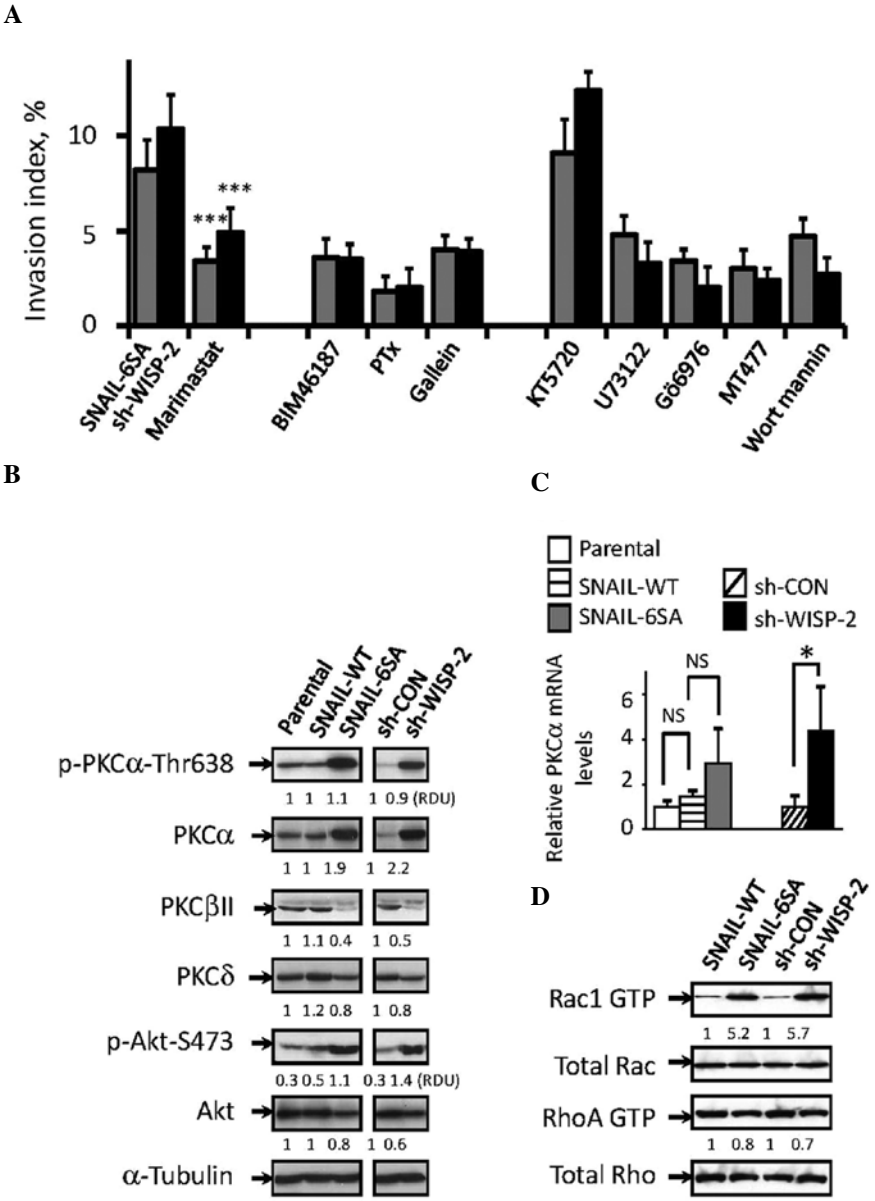

Figure 3. Invasive phenotype induced by MCF-7 cell EMT and oncogenic pathways downstream of GαGβγ subunits. (A), EMT cells were tested for their ability to invade collagen type I gels for 24 h in the presence or absence of chemical inhibitors of GPR signaling (BIM-46187: 1 μM, PTx: 0.1 μg/ml, gallein: 5 μM), PKA (KT5720, 1 μM), PLC (U73122, 1 μM), PKCα/βI (Gö6976, 40 nM and MT477, 1 μM) and PI3K (wortmannin, 1 nM). The pan inhibitor of MMPs 1-2-7 and -14 marimastat was tested at 10 μM. Data are means ± SEM of 3 experiments. ***P<0.005 or better. (B), Immunoblot analysis of total and phosphorylated forms of PKCs (isoforms α, βII, δ) and AKT in parental, control and EMT cells. Total protein loading was 40 μg and α-tubulin was used as a loading control. The PKCα and AKT phosphorylation levels were assessed using relative densitometric units (RDU) according to the densitometry of the phosphorylated form signals (RDU) versus the corresponding total PKCα and AKT signals (control = 1). (C), Quantitative RT-PCR analysis of the transcripts encoding PKCα. (D), Relative density of the Rac-GTP and Rho-GTP immunoreactive bands was quantified, according to total Rac and Rho levels detected by direct immunoblotting (arbitrary units). Data are means ± SEM of 3 experiments performed in duplicate (qRT-PCR and invasion assays) or are representative of two other experiments (immunoblots).

observed PKCα overexpression in EMT cells was directly associated with accumulation of PKCα transcripts in SNAIL-6SA cells (3-fold increase) and sh-WISP-2 cells, 4-fold (Fig. 3C). In contrast, we observed a down-regulation of PKCβII protein and no change in PKCδ expression levels (Fig. 3B). In parallel with sensitivity of the invasive SNAIL-6SA and sh-WISP-2 cells to the PI3K inhibitor wortmannin (Fig. 3A), we demonstrated in Fig. 3B that EMT is correlated with a 3- to 4-fold increase in the intrinsic activity of AKT in the Ser473 phosphorylated form, with no change in total AKT expression.

Remodeling of the actin cytoskeleton during cancer cell scattering, motility and invasion is tightly controlled by the differential activation and spatiotemporal interplay of the small GTPases Rho and Rac during cellular signaling, invasion and EMT (4,10,33). Accordingly, a strong activation of the GTP-bound forms of Rac1 was induced in SNAIL-6SA and sh-WISP-2 cells, without inducing RhoA-GTP levels that remained down-regulated by 20-30% in EMT cells (Fig. 3D). In contrast, the intrinsic activity of the mitogen- and stress-activated protein kinases MAPK-SAPK ERK1/2, p38 and JNK in the phosphorylated forms was found strongly down-regulated, according to their stable expression levels in both SNAIL-6SA and sh-WISP-2 cells (data not shown). In agreement with these findings, signaling inhibitors targeting these Ser/Thr kinases,

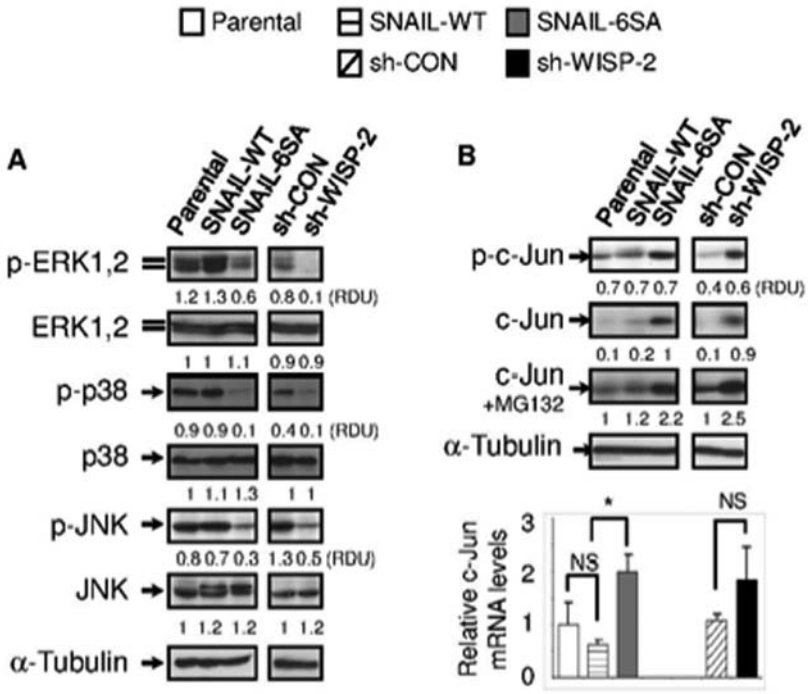

Figure 4. Expression and phosphorylation levels of MAPK/SAPK and Jun during MCF-7 cell EMT. (A) Total and phosphorylated forms of ERK1/2 (Thr 202/Tyr 204), p38 (Thr 180 / Tyr 182), JNK (Thr 183 / Tyr 185) and Jun (Ser 63) were assayed and quantified in parental, control and EMT cells using the corresponding antibodies. (B) Quantitative RT-PCR analysis of the transcripts encoding c-Jun. Data are means ± SEM of 3 experiments performed in duplicate (qRT-PCR assays) or are representative of two other experiments (immunoblots). *Significantly different at P<0.05 from the control levels.

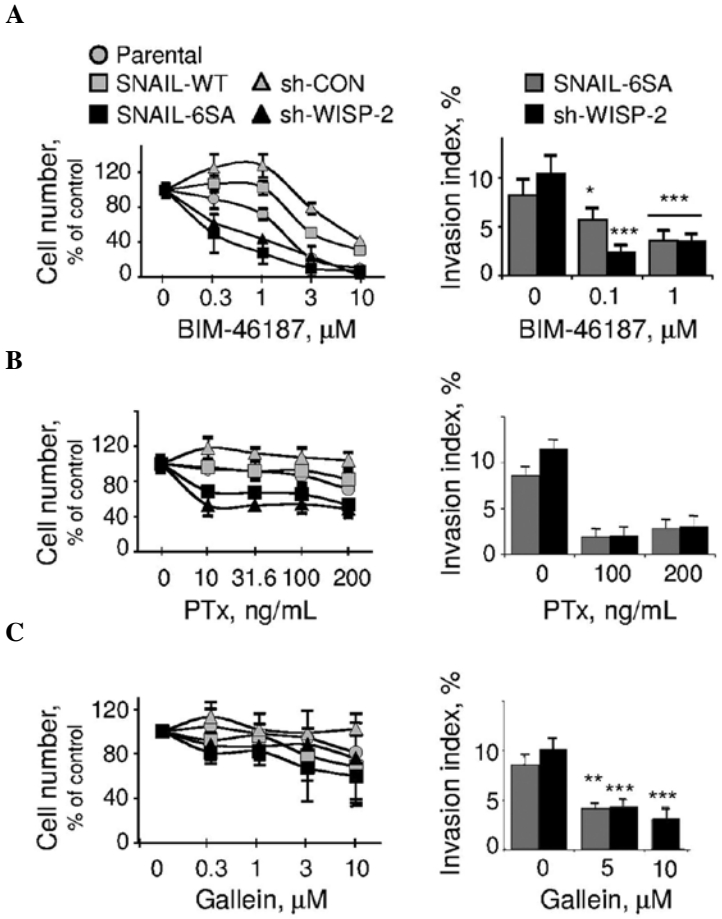

Figure 5. Critical role of GαGβγ subunits in invasive growth responses induced by MCF-7 cell EMT. GPR signaling transduced by heterotrimeric G-protein subunits Gα and Gβγ complex was alleviated using increasing concentrations of pharmacologic inhibitors targeting: (A), Gα subunits (BIM-46187 pan-inhibitor); (B and C), selectively Gαi and Gαo subunits (PTx) or Gβγ subunits (gallein). Cellular proliferation (left panels) and invasion (right panels) were tested as described in the Legends to Figs. 1 and 2. Significant inhibition at *P<0.05, **P<0.01 and ***P<0.005.

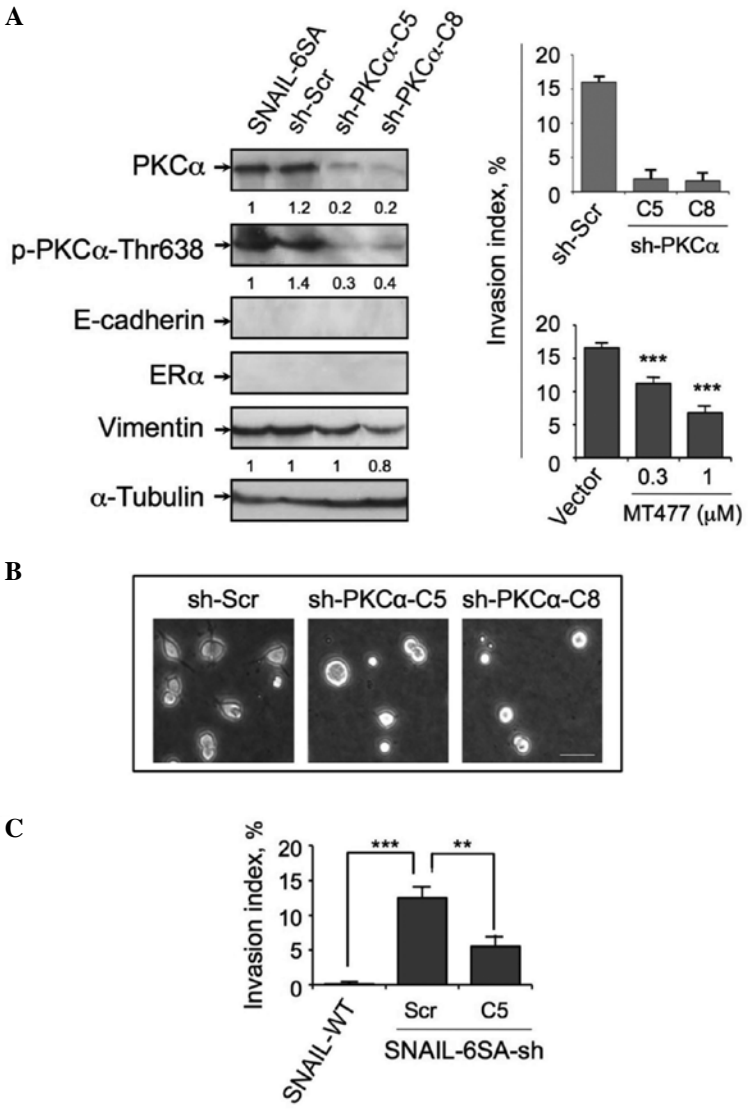

Figure 6. Functional consequences of *PRKCA* silencing on the invasive phenotype induced by MCF-7 cell EMT. (A), Left: Expression and phosphorylation levels of PKCα in Snail-6SA transformed cells and their corresponding *PRKCA* (*PKCα*) silenced counterparts. These clonal derivative cells were compared to parental SNAIL-6SA cells by immunoblot analysis to confirm their reduced levels of total and phosphorylated PKCα forms. Right: Negative impact of *PKCα* silencing on the invasive phenotype of SNAIL-6SA transformed MCF-7 cells in EMT (top). Dose-effect of the PKCα inhibitor MT477 on the invasive potential of SNAIL-6SA cells transfected by the control empty vector pGFP-V-RS (bottom). Control vector and sh-Scr cells, *PKCα* silenced cells, and SNAIL-6SA cells were tested for their ability to invade collagen type I gels for 24 h. Data are means ± SEM of 3 experiments (invasion assays) or are representative of one other experiment (immunoblots). (B), Phase-contrast microscopy of control sh-Scr and *PKCα* silenced cells (clones C5 and C8) cultured for 24 h in the collagen type I invasion assay. Scale bar, 100 μm. Significantly different at ***P<0.005. (C), MCF-7 SNAIL-WT cells were compared to SNAIL-6SA cells (sh-Scr and silenced sh-PKCα MCF-7 cells, Clone 5) for their ability to invade collagen type I gels in the 14-day assays. Data collected from each experimental condition are recorded from 10 microscopic fields. Significantly different at **P<0.01 or better.

respectively PD98059 (ERK1/2), SB203580 (p38) and SP600125 (JNK) were ineffective to counteract the invasive potential of MCF-7 cells in EMT (data not shown). It is anticipated that these EMT and TN phenotypes are likely to show resistance to small molecules and antibodies targeting MAPK/SAPK and the HER family. Most interestingly, and in accordance with elevation in c-Jun transcript levels, we observed a strong accumulation of c-Jun protein (5-9 times) in parallel with a global increase of the c-Jun Ser-phosphorylated form (Fig. 4). These findings are coherent with a predominant role of the CDK3-c-Jun axis compared with the relative deficiency of the two EMT models in JNK activity. We also found that c-Jun protein levels in MCF-7 cells are strongly elevated by the proteasome inhibitor MG132, suggesting that both transcriptional and post-transcriptional mechanisms are underlying the up-regulation of c-Jun, a molecular component of the AP1 oncogene involved in cancer cell transformation (34).

We next addressed the impact of the HER family members in determination of the invasive phenotype in EMT cells. HER1 and HER3 were, respectively, up- and down-regulated at the protein and transcript levels in SNAIL-6SA and sh-WISP-2 cells (data not shown). Most interestingly, tyrosine kinase inhibitors and neutralizing antibodies targeting HER family members were inefficient to inactivate the constitutive invasive potential of MCF-7 cells in EMT (data not shown).

Functional implication of Gα and Gβγ signaling in the invasive growth of MCF-7 cancer cells in EMT. According to our data

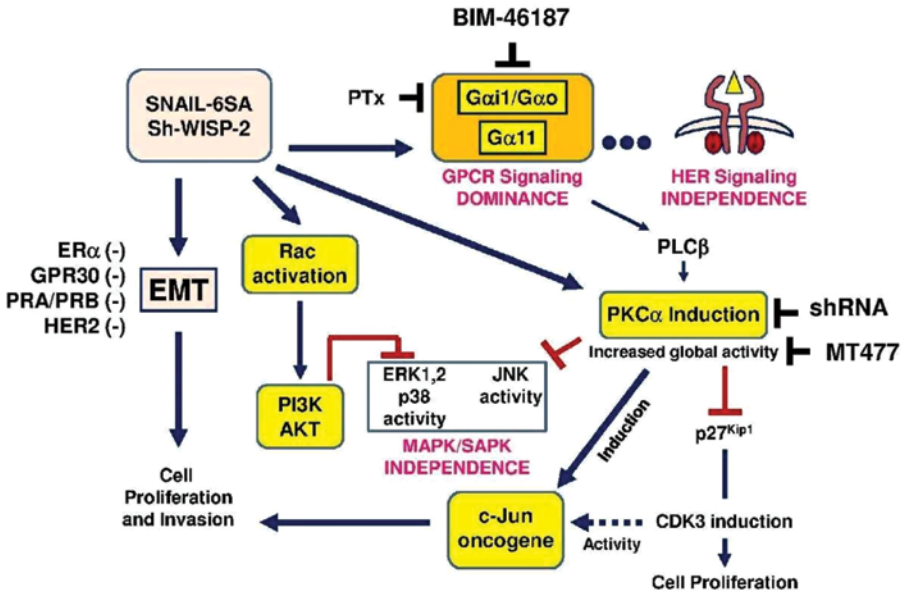

Figure 7. Molecular signature of the EMT induced by Snail activation and *WISP-2* silencing in MCF-7 cells. This tentative signalome take into account the consequences of the EMT on: a) cancer cell proliferation and invasion; b) implication of signaling networks using heterotrimeric G-proteins Gα/Gβγ, c) PKCα-$p27^{kip1}$-CDK3 cascade targeting the c-Jun/AP1 oncogenic pathway; d) the PI3K/Rac/AKT cascade; e) the independence of the EMT-associated invasive phenotype versus MAPK/SAPK, HER family pathways and other critical operators including the loss of ERα, PRA/PRB, HER and GPR30 signaling addition as molecular criteria of triple-negative breast cancers. This signalome is fully valid in two separate genetic models of MCF-7 induced to EMT and TN phenotypes. Such a similarity and convergence is well explained by the common implication of several genetic protagonists, including the expression of Slug, Twist and Zeb1/2 and WISP-2 depletion in the two models. This coincidence led us to anticipate the functional and clinical significance of these signaling elements in breast cancer patients.

presented in Fig. 3A, we undertook a comparative study on the potency of GαGβγ inhibitors to inactivate the invasive growth of MCF-7 cells in EMT. Interestingly, we discovered that the Gα subunit inhibitor BIM-46187 (12,19,20) potently abolished the growth of MCF-7 cells in EMT (IC_{50} = 0.3-0.7 μM), versus their parental and control counterparts (Fig. 5A, left). Indeed, the IC_{50} of BIM-46187 producing half-maximal inhibition on cell proliferation was 10-fold lower in SNAIL-6SA cells (IC_{50} = 0.3 μM) than for SNAIL-WT (IC_{50} = 3 μM). Similarly, the IC_{50} of BIM-46187 was almost 30-fold lower (IC_{50} = 0.3 μM) in sh-WISP-2 cells compared to sh-CON cells (IC_{50} = 10 μM). Both PTx and gallein (18) were much less efficient (partial inhibition, Fig. 5B and C). Indeed, gallein (0.3-1 μM for 48 h) had no effect on the proliferation of MCF-7 cells (Fig. 5C). Both SNAIL-6SA and sh-WISP-2 cells appeared to be more sensitive to PTx than controls.

At low concentrations (0.1 μM), BIM-46187 was also very efficient in reversing the invasive behavior of EMT cells (Fig. 5A, right). The invasion index was reduced by 27 and 80% in SNAIL-6SA and sh-WISP-2 cells, respectively. Both PTx and gallein reduced the invasive phenotype of the EMT cells by 60-70%, with similar potencies in SNAIL-6SA and sh-WISP-2 cells. These data encouraged us to establish the rationale of the high sensitivity of EMT cells to the Gα subunit inhibitor BIM-46187. Based on the partial inhibitory effects of PTx and gallein on the invasive growth of SNAIL-6SA and sh-WISP-2 cells, we hypothesized that at least two classes of PTx-sensitive and insensitive Gα-protein subunits might be implicated in these two EMT genotypes. To verify this possibility, we investigated by qRT-PCR the expression levels of several Gα subunits transcripts encoding *bona fide* potential oncogenic signaling protagonists (data not shown). In both EMT cells, we observed a remarkable induction of the PTx-sensitive Gα subunit members Gαi1 (7-fold) and Gαo (10- to 20-fold). Transcript levels encoding the PTx-insensitive member Gα11 subunit were also up-regulated 2- to 3-fold. No significant changes in transcript levels were noted for the other Gα subunit members considered. Increased Gαi2 transcripts were detected in SNAIL-6SA cells only.

Impact of PRKCA silencing on the invasive potential and EMT markers in SNAIL-6SA-transformed MCF-7 cells. To further assess the role of PKCα in the EMT and invasive phenotypes, we silenced the *PRKCA* gene by specific sh-RNAs. As shown in Fig. 6A (left), both SNAIL-6SA and control cells transfected by the sh-RNA scrambled sequence (sh-Scr) expressed significant amounts of PKCα protein and the corresponding phosphorylated Thr638 variant. As expected, both total and phosphorylated PKCα protein levels were strongly down-regulated in the silenced clonal derivatives (sh-PKCα-C5 and -C8). Silencing efficiency reached to 80-84% cellular depletion of total and phosphorylated PKCα. The functional impact of *PRKCA* silencing was therefore examined on cancer cell invasion and EMT markers. Both sh-C5 and sh-C8-silenced clones were fully invalided for the ability to invade collagen type I gels as shown in 24-h invasion assays and phase-contrast microscopy (Fig. 6A and B, respectively). In the 24-h invasion assay, the PKCα inhibitor MT477 also reversed the invasive potential of control empty vector (pGFP-V-RS)-transfected SNAIL-6SA cells (Fig. 6A, right). Coherently, PKCα sh-RNAs reduced the invasive potential of SNAIL-6SA cells (sh-Scr sequence) by 60% in the 14-day invasion assay (Fig. 6C). Interestingly, *PRKCA* silencing reverted the mesenchymal morphotype of the EMT, in MCF-7 cells as judged by the conversion of the spindle-like cell

morphology into cohesive epithelial-like cell layers observed in both sh-PKCα-C5 and -C8 silenced cells (data not shown). However, *PRKCA* silencing had no impact on the persistence of the EMT markers characterized by vimentin expression and of deficiencies in ERα and E-cadherin expression as determined by Western blotting (Fig. 6A) and *in situ* immunocytochemistry of invasive MCF-7 cells in native collagen type I gels (data not shown).

Discussion

Our data reveal that the two genetic models of EMT in BC (3,6) are characterized by comparable expression of transcription factors whose expression (Slug, Zeb1/2 and Twist) or repression (Wisp-2) have been causally linked to invasive growth, a critical EMT signature. Importantly, the Snail-induced EMT phenotype of MCF-7 cells was characterized by Wisp-2 down-regulation via the *WISP-2* promoter. These findings indicate a potential role for TGFβ signaling in the Snail-induced EMT model, since Wisp-2 was recently identified as a repressor of TGFβ type II receptor-mediated transcription and EMT in WISP-2-silenced MCF-7 cells (8). Together with our previous findings, the current results support a new paradigm for Wisp-2, initially described as a secreted factor, and playing important roles in EMT prevention as a transcription factor.

These two genetic models of EMT are further characterized by the convergent expression of the triple negative phenotype. Triple negative breast cancers (TNBC) are usually associated with poor prognosis, short progression-free survival, higher risk of distant recurrence and a basal-like phenotype as identified by gene expression profiling. Targeted therapy and predictive markers are currently unavailable for this heterogeneous subgroup of breast cancer patients representing about 15% of all breast cancers (22,28). Interestingly, our two EMT models are also expressing a common signaling signature illustrated by the dominance of GαGβγ subunits, PKCα, c-Jun and PI3K/AKT pathways (Fig. 7). Of note, PKCα is currently considered as a clinical marker of aggressiveness in breast cancer and has been reported to play multiple roles in cell cycle checkpoint, cancer cell differentiation and transformation (35,36). PKCα levels correlate with migratory and multidrug resistance phenotypes, consistent with the EMT promoter activity of PKC acknowledged in epithelial cancers and tumor metastasis (1,37). For example, c-Jun can be activated by PKCα through p27 inhibition and CDK3 up-regulation (30,37,38). In agreement, we find that silencing and inhibition of PKCα activity by sh-RNAs or the kinase inhibitor MT477 alleviated the invasive growth of MCF-7 cells in EMT. This effect was not associated with a full reversion of the EMT phenotype via the mesenchymal-to-epithelial transition (1,2), as shown here by the persistence of the EMT markers in *PRKCA*-silenced SNAIL-6SA cells. One possible explanation is the multiplicity of dominant and convergent EMT signaling pathways identified in our models. Both WISP-2 silencing and Snail transformation generated a constitutive activation of and dependence to the PI3K-AKT axis through a hierarchical Gαo/i and Gβγ/PI3K/Rac1 signaling cascade leading to cytoskeletal rearrangements and invasive growth during cancer cell progression. Consistent with this, the Rac exchange factor P-Rex1 has been described as an essential mediator of Rac1 activation and pleiotropic responses in breast cancer cells (39). This guanine exchange factor is synergistically activated by Gβγ subunits and PI3K inducers and integrates signals from GPRs, several tyrosine kinase receptors, including those of the HER and type I insulin-like growth factors families. Interestingly, P-Rex has been described to function as a signaling connector between the PI3K/AKT/mTOR axis and Rac activation (39-41). Recent data support the notion that both mTORC1 and C2 complex are playing critical roles in the regulation of EMT and metastasis (41). Therefore, it will be interesting to establish the relative contribution of P-Rex and other Rac GEFs as signaling protagonists of the PI3K/AKT/mTOR signaling cascade in future investigations of EMT.

We demonstrated here that the constitutive activation of these pathways is subjected to direct and indirect activation via intrinsic mechanisms (Rac and AKT activity) and global expression (Gα subunits, PKCα, CDK3 and c-Jun), respectively. Additional transcriptional and post-transcriptional controls mediated by multiple EMT transcription factors and interplay support this scenario (Fig. 1). For example, CDK3 was shown to activate c-Jun and to increase AP1 activity, resulting in enhanced anchorage-independent cell transformation (38). AP1 oncogene is required for the transcriptional induction of matrilysin MMP-7 and determination of the invasive phenotype by src in human colon cancer cells (34). Overall, the participation of putative autocrine effectors in the orchestration of these two EMT programs can not be excluded. In particular, it will be important to explore the interplay between Wnt and TGFβ signaling in conjunction to autocrine agents targeting GPR, PI3K/AKT and PKCα signals for the EMT phenotype, as indicated by the induction of TGFβRII in the *WISP-2* silenced cells (1,4,12,34,42,43). TGFβ signals are connected with Smad and co-Smad family members and non-Smad pathways including MAPK/SAPK, PI3K-AKT-mTOR and the small GTPases Rho, Rac and Cdc42. Our results revealed an unsuspected down-regulation of the MAPK/SAPK cascade in the highly proliferative and invasive EMT MCF-7 cells, in full agreement with their lack of response to classical mitogen inhibitors. Our data are also consistent with the fact that the PI3K-AKT axis and PKCα are both described as efficient repressors of ERK1/2, p38 and JNK (44-46). In addition, the invasive growth of EMT cells was independent of the HER family tyrosine kinases and representatives of the TN phenotype, including the alternative estrogen receptor GPR30.

In conclusion, we characterized two genetic models of human breast cancer cells presenting EMT and TN phenotypes associated with a complex dominant signalome comprising GαGβγ subunits, PKCα, PI3K/AKT axis and the p27/CDK3/c-Jun cascade. These protagonists may thus constitute critical determinants for combined therapies using interfering agents, dual kinase and multikinase inhibitors. To validate this assumption, further studies are therefore expected in order to investigate at the preclinical level the therapeutic potential of these inhibitors and interference on the growth and metastatic dissemination of breast tumor xenografts induced by *WISP-2*-silenced and Snail-transformed MCF-7 cells in immunodeficient mice. In addition, we have not assessed the stem cell state of these two EMT models in cell culture conditions, during their primary invasive growth and progression to high-grade malignancy. In the long term, the clinical

value of these signaling elements as predictive markers and therapeutic targets should be assessed in TN breast cancer tumors showing EMT phenotypes. Our findings also provide a rational basis to improve the sub-classification of ERα-negative tumors and TNBC to provide new clues for the diagnosis, clinical outcomes and treatment of breast cancer patients.

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References

1. Sabbah M, Emami S, Redeuilh G, Julien S, Prévost G, Zimber A, Ouelaa R, Bracke M, De Wever O and Gespach C: Molecular signature and therapeutic perspective of the epithelial-to-mesenchymal transitions in epithelial cancers. Drug Resist Updat 11: 123-151, 2008.
2. Thiery JP, Acloque H, Huang RY and Nieto MA: Epithelial-mesenchymal transitions in development and disease. Cell 139: 871-890, 2009.
3. Fritah A, Saucier C, De Wever O, Bracke M, Bièche I, Lidereau R, Gespach C, Drouot S, Redeuilh G and Sabbah M: Role of WISP-2/CCN5 in the maintenance of a differentiated and noninvasive phenotype in human breast cancer cells. Mol Cell Biol 28: 1114-1123, 2008.
4. Boissan M, De Wever O, Lizarraga F, Wendum D, Poincloux R, Chignard N, Desbois-Mouthon C, Dufour S, Nawrocki-Raby B, Birembaut P, Bracke M, Chavrier P, Gespach C and Lacombe ML: Implication of metastasis suppressor NM23-H1 in maintaining adherens junctions and limiting the invasive potential of human cancer cells. Cancer Res 70: 7710-7722, 2010.
5. Sin S, Bonin F, Petit V, Meseure D, Lallemand F, Bièche I, Bellahcène A, Castronovo V, De Wever O, Gespach C, Lidereau R and Driouch K: Role of the focal adhesion protein kindlin-1 in breast cancer growth and lung metastasis. J Natl Cancer Inst 103: 1323-1337, 2011.
6. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M and Hung MC: Dual regulation of Snail by GSK-3β-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat Cell Biol 6: 931-945, 2004.
7. Dhasarathy A, Kajita M and Wade PA: The transcription factor snail mediates epithelial to mesenchymal transitions by repression of estrogen receptor-alpha. Mol Endocrinol 21: 2907-2918, 2007.
8. Sabbah M, Prunier C, Ferrand N, Megalophonos V, Lambein K, De Wever O, Nazaret N, Lachuer J, Dumont S and Redeuilh G: CCN5, a novel transcriptional repressor of the transforming growth factor β signaling pathway. Mol Cell Biol 31: 1459-1469, 2011.
9. Régnauld K, Nguyen QD, Vakaet L, Bruyneel E, Launay JM, Endo T, Mareel M, Gespach C and Emami S: G-protein alpha(olf) subunit promotes cellular invasion, survival, and neuroendocrine differentiation in digestive and urogenital epithelial cells. Oncogene 21: 4020-4031, 2002.
10. Nguyen QD, De Wever O, Bruyneel E, Hendrix A, Xie WZ, Lombet A, Leibl M, Mareel M, Gieseler F, Bracke M and Gespach C: Commutators of PAR-1 signaling in cancer cell invasion reveal an essential role of the Rho-Rho kinase axis and tumor microenvironment. Oncogene 24: 8240-8251, 2005.
11. Le Floch N, Rivat C, De Wever O, Bruyneel E, Mareel M, Dale T and Gespach C: The proinvasive activity of Wnt-2 is mediated through a noncanonical Wnt pathway coupled to GSK-3beta and c-Jun/AP-1 signaling. FASEB J 19: 144-146, 2005.
12. Dessein AF, Stechly L, Jonckheere N, Dumont P, Monté D, Leteurtre E, Truant S, Pruvot FR, Figeac M, Hebbar M, Lecellier CH, Lesuffleur T, Dessein R, Grard G, Dejonghe MJ, De Launoit Y, Furuichi Y, Prévost G, Porchet N, Gespach C and Huet G: Autocrine induction of invasive and metastatic phenotypes by the MIF-CXCR4 axis in drug-resistant human colon cancer cells. Cancer Res 70: 4644-4654, 2010.
13. Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C and Ullrich A: EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 402: 884-888, 1999.
14. Kveiborg M, Instrell R, Rowlands C, Howell M and Parker PJ: PKCα and PKCδ regulate ADAM17-mediated ectodomain shedding of heparin binding-EGF through separate pathways. PLoS One 6: e17168, 2011.
15. Maggiolini M and Picard D: The unfolding stories of GPR30, a new membrane-bound estrogen receptor. J Endocrinol 204: 105-114, 2010.
16. Filardo EJ, Quinn JA, Bland KI and Frackelton AR: Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via transactivation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol 14: 1649-1660, 2000.
17. Lappano R and Maggiolini M: G protein-coupled receptors: novel targets for drug discovery in cancer. Nat Rev Drug Discov 10: 47-60, 2011.
18. Bonacci TM, Mathews JL, Yuan C, Lehmann DM, Malik S, Wu D, Font JL, Bidlack JM and Smrcka AV: Differential targeting of Gβγ-subunit signaling with small molecules. Science 312: 443-446, 2006.
19. Prévost GP, Lonchampt MO, Holbeck S, Attoub S, Zaharevitz D, Alley M, Wright J, Brezak MC, Coulomb H, Savola A, Huchet M, Chaumeron S, Nguyen QD, Forgez P, Bruyneel E, Bracke M, Ferrandis E, Roubert P, Demarquay D, Gespach C and Kasprzyk PG: Anticancer activity of BIM-46174, a new inhibitor of the heterotrimeric Gα/Gβγ protein complex. Cancer Res 66: 9227-9234, 2006.
20. Ayoub MA, Damian M, Gespach C, Ferrandis E, Lavergne O, De Wever O, Banères JL, Pin JP and Prévost GP: Inhibition of heterotrimeric G protein signaling by a small molecule acting on Gα subunit. J Biol Chem 284: 29136-29145, 2009.
21. Kimple AJ, Bosch DE, Giguère PM and Siderovski DP: Regulators of G-protein signaling and their Gα substrates: promises and challenges in their use as drug discovery targets. Pharmacol Rev 63: 728-749, 2011.
22. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM, Lønning PE, Brown PO, Børresen-Dale AL and Botstein D: Repeated observation of breast tumor sub-types in independent gene expression data set. Proc Natl Acad Sci USA 100: 8418-8423, 2003.
23. Fritah A, Redeuilh G and Sabbah M: Molecular cloning and characterization of the human WISP-2/CCN5 gene promoter reveal its upregulation by oestrogens. J Endocrinol 191: 613-624, 2006.
24. De Wever O, Sobczak-Thépot J, Vercoutter-Edouart AS, Michalski JC, Ouelaa-Benslama R, Stupack DG, Bracke M, Wang JYJ, Gespach C and Emami S: Priming and potentiation of DNA damage response by fibronectin in human colon cancer cells and tumor-derived myofibroblasts. Int J Oncol 39: 393-400, 2011.
25. De Wever O, Hendrix A, De Boeck A, Westbroek W, Braems G, Emami S, Sabbah M, Gespach C and Bracke M: Modeling and quantification of cancer cell invasion through collagen type I matrices. Int J Dev Biol 54: 887-896, 2010.

26. Wander SA, Zhao D and Slingerland JM: p27: a barometer of signaling deregulation and potential predictor of response to targeted therapies. Clin Cancer Res 17: 12-18, 2011.
27. Larrea MD, Wander SA and Slingerland JM: p27 as Jekyll and Hyde: regulation of cell cycle and cell motility. Cell Cycle 8: 3455-3461, 2009.
28. Denicourt C, Saenz CC, Datnow B, Cui XS and Dowdy SF: Relocalized p27Kip1 tumor suppressor functions as a cytoplasmic metastatic oncogene in melanoma. Cancer Res 67: 9238-9243, 2007.
29. St Croix B, Sheehan C, Rak JW, Flørenes VA, Slingerland JM and Kerbel RS: E-cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27(KIP1). J Cell Biol 142: 557-571, 1998.
30. Braun K, Hölzl G, Soucek T, Geisen C, Möröy T and Hengstschläger M: Investigation of the cell cycle regulation of cdk3-associated kinase activity and the role of cdk3 in proliferation and transformation. Oncogene 17: 2259-2269, 1998.
31. Chacón RD and Costanzo MV: Triple-negative breast cancer. Breast Cancer Res 12 (Suppl 2): 3, 2010.
32. Jasinski P, Welsh B, Galvez J, Land D, Zwolak P, Ghandi L, Terai K and Dudek AZ: A novel quinoline, MT477: suppresses cell signaling through Ras molecular pathway, inhibits PKC activity, and demonstrates in vivo anti-tumor activity against human carcinoma cell lines. Invest New Drugs 26: 223-232, 2008.
33. Raftopoulou M and Hall A: Cell migration: Rho GTPases lead the way. Dev Biol 265: 23-32, 2004.
34. Rivat C, Le Floch N, Sabbah M, Teyrol I, Redeuilh G, Bruyneel E, Mareel M, Matrisian LM, Crawford HC, Gespach C and Attoub S: Synergistic cooperation between the AP-1 and LEF-1 transcription factors in activation of the matrilysin promoter by the src oncogene: implications in cellular invasion. FASEB J 17: 1721-1723, 2003.
35. Lønne GK, Cornmark L, Zahirovic IO, Landberg G, Jirström K and Larsson C: PKCalpha expression is a marker for breast cancer aggressiveness. Mol Cancer 9: 76-90, 2010.
36. Delage S, Chastre E, Empereur S, Wicek D, Veissiére D, Capeau J, Gespach C and Cherqui G: Increased protein kinase C alpha expression in human colonic Caco-2 cells after insertion of human Ha-ras or polyoma virus middle T oncogenes. Cancer Res 53: 2762-2770, 1993.
37. Gao Q, Tan J, Ma P, Ge J, Liu Y, Sun X and Zhou L: PKC alpha affects cell cycle progression and proliferation in human RPE cells through the downregulation of p27kip1. Mol Vis 15: 2683-2695, 2009.
38. Cho YY, Tang F, Yao K, Lu C, Zhu F, Zheng D, Pugliese A, Bode AM and Dong Z: Cyclin-dependent kinase-3-mediated c-Jun phosphorylation at Ser63 and Ser73 enhances cell transformation. Cancer Res 69: 272-281, 2009.
39. Hernández-Negrete I, Carretero-Ortega J, Rosenfeldt H, Hernández-García R, Calderón-Salinas JV, Reyes-Cruz G, Gutkind JS and Vázquez-Prado J: P-Rex1 links mammalian target of rapamycin signaling to Rac activation and cell migration. J Biol Chem 282: 23708-23715, 2007.
40. Wertheimer E, Gutierrez-Uzquiza A, Rosemblit C and Lopez-Haber C, Soledad Sosa M, Kazanietz MG: Rac signaling in breast cancer: a tale of GEFs and GAPs. Cell Signal 24: 353-362, 2012.
41. Gulhati P, Bowen KA, Liu J, Stevens PD, Rychahou PG, Chen M, Lee EY, Weiss HL, O'Connor KL, Gao T and Evers BM: mTORC1 and mTORC2 regulate EMT, motility, and metastasis of colorectal cancer via RhoA and Rac1 signaling pathways. Cancer Res 71: 3246-3256, 2011.
42. Hendrix A, Maynard D, Pauwels P, Braems G, Denys H, van den Broecke R, Lambert J, van Belle S, Cocquyt V, Gespach C, Bracke M, Seabra MC, Gahl WA, De Wever O and Westbroek W: Effect of the secretory small GTPase Rab27B on breast cancer growth, invasion, and metastasis. J Natl Cancer Inst 102: 866-880, 2010.
43. Dupouy S, Viardot-Foucault V, Alifano M, Souazé F, Plu-Bureau G, Chaouat M, Lavaur A, Hugol D, Gespach C, Gompel A and Forgez P: The neurotensin receptor-1 pathway contributes to human ductal breast cancer progression. PLoS One 4: e4223, 2009.
44. Rommel C, Clarke BA, Zimmermann S, Nuñez L, Rossman R, Reid K, Moelling K, Yancopoulos GD and Glass DJ: Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. Science 286: 1738-1741, 1999.
45. Gratton JP, Morales-Ruiz M, Kureishi Y, Fulton D, Walsh K and Sessa WC: Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. J Biol Chem 276: 30359-30365, 2001.
46. Wang N, Li Z, Tian F, Feng Y, Huang J, Li C and Xie F: PKCα inhibited apoptosis by decreasing the activity of JNK in MCF-7/ADR cells. Exp Toxicol Pathol doi: 10.1016/j.etp, 2010.