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As the activation of the Notch transduction pathway induces the nuclear translocation of N1ICD and the transactivation of Notch-target genes (Miele, 2006), we initially assessed whether the ligand-activated GPER could stimulate the expression of one main Notch-regulated gene like Hes-1. Three independent lines of evidence support that indeed this is the case: (i) E2 and the selective GPER ligand G-1 increased the mRNA levels of Hes-1, (ii) E2 and G-1 triggered both the release of the Notch-1 intracellular domain (N1ICD) and its recruitments to the Hes-1 promoter sequence, (iii) E2 and G-1 transactivated the Hes-1 promoter, hence recapitulating the aforementioned findings. Notably, Hes-1 up regulation by E2 or G-1 was confirmed also in ER-positive MCF-7 cells. Interestingly, our results (see Supplementary Fig. 3) show that high doses of E2 (100 nM) were necessary to induce Hes-1 expression in MCF-7 cells, whereas Hes-1 induction was already achieved at low dosage of E2 (1 nM) in SkBr3 cells. Moreover, we found that knockdown of ERα expression had no effect on Hes-1 up-regulation in MCF-7 cells, whereas GPER silencing completely abrogated Hes-1 up-regulation by E2 or G-1. These results indicate that ER-positive and ER-negative breast cancer cells have a different dose-response to E2, presumably relying on the different binding affinity of E2 for ERα and GPER, as demonstrated in previous studies (Revankar et al., 2005; Thomas et al., 2005). Importantly, the dose response curve of E2 on Hes-1 expression in MCF-7 cells overcomes the apparent discrepancy between our data and the previous study by Rizzo and colleagues, showing that low doses of E2 reduce Delta-dependent activation of Notch signaling (Rizzo et al., 2008b).

The ability of E2 and G-1 to trigger Notch-dependent transcription was clearly demonstrated by the evidence that the up-regulation of Hes-1 was abolished by using both GSI or DN-MAML-1, a dominant-negative mutant of Master-mind like 1, which is a Notch-1 transcriptional co-activator (Wu and Griffin, 2004). Likewise, the knockdown of GPER abrogated both the induction of Hes-1 and the release of N1ICD upon E2 and G-1 treatments, hence demonstrating the involvement of GPER in the activation of Notch signaling by these ligands.

The mechanism through which GPER leads to Notch-1 activation seems to be transcriptional, as an increase of Notch-1 mRNA levels was found in response to E2 and G-1. Remarkably, this increase preceded the induction of the Hes-1 mRNA levels (see Figs. 1A, B and 3E, F), suggesting that the deregulation of Notch-1 expression mediates the effects elicited by GPER on both Notch activation and Notch-dependent transcription. Conversely, in SkBr3 cells the constitutive JAGGED-1 expression was not altered by E2 and G-1 (data not shown), indicating that the boost of Notch induced by E2 and G-1 is sufficient to activate the Notch signaling. Accordingly, it has been reported that high Notch-1 levels are associated with a poor prognosis in breast cancer (Al-Hussaini et al., 2011). Although the molecular mechanisms involved in the induction of Notch-1 by GPER remain to be fully elucidated, the cross talk between GPER and EGFR may be ruled out, as the inhibition of EGFR does not interfere with the up-regulation of Hes-1 induced by E2 or G-1 (our published data). In this regard, further studies are required to investigate whether GPER may trigger the expression of Notch-1 through other transduction mechanisms.

The aforementioned findings were recapitulated and further highlighted by evaluating the role of Notch in a relevant biological response like cell migration. Of note, the ability of E2 and G-1 to stimulate the migration of SkBr3 cells was abolished using GSI or DM-MALM-1, hence indicating that both Notch activation and Notch-dependent transcription contribute to the migratory effects elicited by E2 and G-1.

The invasive and migratory ability of cancer cells have been previously associated with EMT (Matsuno et al., 2012; Thiery, 2002), that particularly in breast tumor involved the deregulation of E-cadherin expression through Notch and its target gene Snail (Wang et al., 2010) In this regard, the up-regulation of Snail coupled to an altered expression of VE-cadherin induced by E2 and G-1 in a GSI-dependent fashion, further corroborates the contribution of the Notch-dependent transcription in cell migration and EMT triggered by these ligands.

Our results demonstrate for the first time that estrogen-activated GPER engages Notch signaling toward gene expression changes and biological responses in breast tumor cells and CAFs, hence providing a further mechanism through which estrogens may stimulate the progression of breast cancer.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2013.11.011.

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(VE)-cadherin. In this regard, it has been shown that these genes are involved in the epithelial to mesenchymal transition (EMT) which is a biological process regulated by a network of transduction pathways including Notch signaling (Grego-Bessa et al., 2004). Hence, we investigated whether E2 and G-1 induced the expression of *Snail* at both mRNA and protein levels in a Notch-dependent manner. As shown in Fig. 6A–D and in Supplementary Fig. 7A, we found that E2 and G-1, as well as JAG-1, induced the expression of *Snail* which was abrogated by co-expression of DN-MAML-1. Next, we investigated whether the up-regulation of *Snail* by E2 and G-1 is coupled to an altered expression of cell-cell adhesion molecules. As SkBr3 cells lack *E-Cadherin* expression (Hiraguri et al., 1998), we evaluated the response of *VE-Cadherin*, which is expressed in SkBr3 cells (Endo et al., 2008). As shown in Fig. 6E–H and in Supplementary Fig. 7B, E2 and G-1 reduced the expression of *VE-Cadherin* at both mRNA and protein levels, in a GSI-sensitive fashion.

3.5. The GPER and Notch pathways cooperate in cancer-associated fibroblasts (CAFs)

Given the relevant contribution of the microenvironment to cancer cell growth and invasiveness (Kalluri and Zeisberg, 2006), we examined whether the cross-talk between Notch and GPER signaling pathways occurs in CAFs which play a pivotal role in the functional interaction between stroma and cancer cells toward tumor progression. Hence, we determined that GPER mediates the mRNA and protein expression of *Hes-1* induced by E2 and G-1 in CAFs obtained from breast cancer patients, whereas the *Hes-1* response to JAG-1 was not altered transfecting cells with a shG-PER (Fig. 7A–F). Using GSI, the up-regulation of *Hes-1*, at both mRNA and protein levels, triggered by all agents was no longer observed (Fig. 7G–J). As biological counterpart, the migration of CAFs stimulated by E2, G-1 and JAG-1 was abolished using GSI, as assessed performing both wound-healing and Boyden chamber assays (Fig. 8A and B, Supplementary Figs. 8 and 9). Collectively, these data confirmed the results obtained in breast cancer cells and extended the potential of E2 and G-1 to engage the Notch signaling through GPER in main components of the tumor microenvironment like CAFs

4. Discussion

A wide number of studies have shown that the functional interactions among steroid receptors and growth factor receptors play a crucial role toward breast cancer progression (Bartella et al., 2012; Lappano et al., 2013; Vivacqua et al., 2009). In this regard, the estrogen action mediated by GPER via EGFR activation has made clear that GPER may facilitate the response to estrogens independently of the classical ERs. Accordingly, the functional cross-talk between GPER and EGFR has been documented in different types of malignancies and involved in relevant biological outcomes like the proliferation and migration of cancer cells (Maggiolini and Picard, 2010).

On the other side, deregulation of the Notch pathway by oncogenic signaling represents a further crossroad in tumor growth and
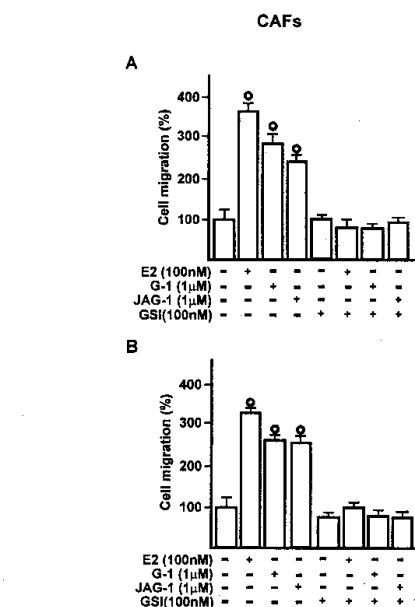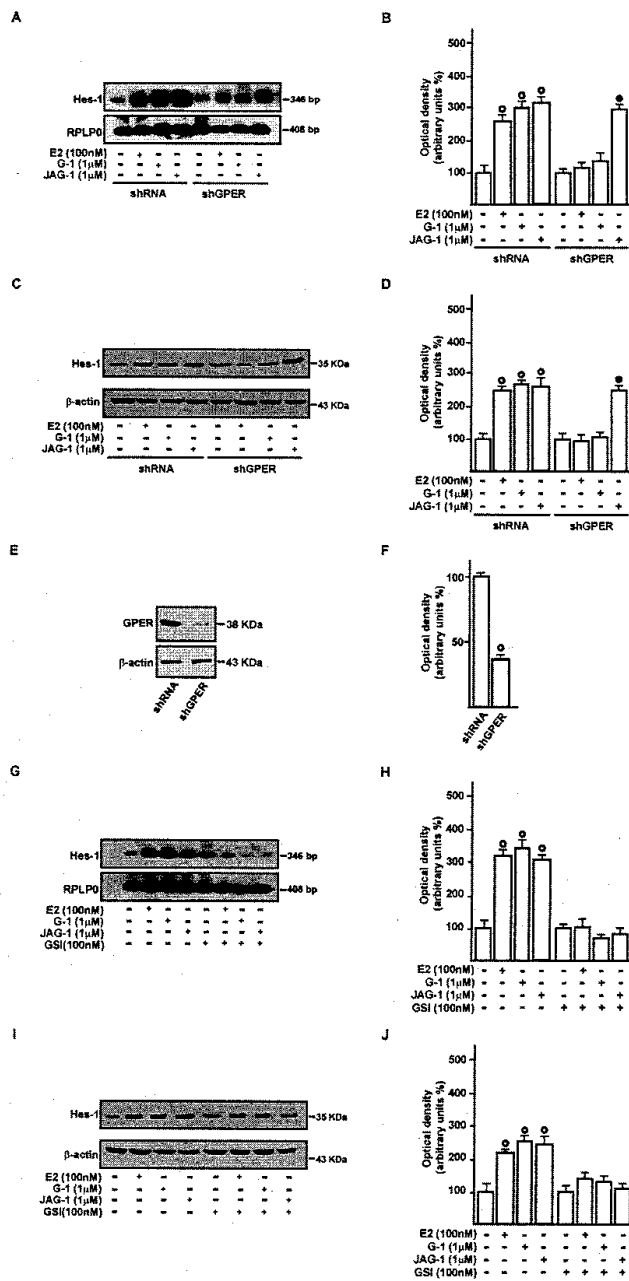
CAFs

Fig. 8. Inhibition of Notch signaling abolishes the migratory effects stimulated by E2, G-1 and JAG-1 in CAFs. (A) Wound-healing and (B) Boyden chamber assays performed in CAFs to evaluate the migration in presence of 100 nM E2, 1 μM G-1 and 1 μM JAG-1 alone or in combination with 100 nM of the γ-secretase inhibitor (GSI). Bar graphs show representative experiments with means of triplicate samples. Results were standardized to data of cells receiving vehicle which were set to 100%. (○) indicates P < 0.05 for cells receiving vehicle (−) versus treatments.

invasion (Guo et al., 2011; Rizzo et al., 2008a). In particular, the Notch interaction with ER-mediated signaling as well as growth factor receptors has been shown in breast cancer cells (Al-Hussaini et al., 2011; Guo et al., 2011; Osipo et al., 2008; Rizzo et al., 2008b). Therefore, the response of breast cancer cells to estrogens may be dependent on the repertoire of receptors expressed in different cell contexts and the downstream transduction pathways that functionally cooperate toward tumor progression.

In the present study, we have used the ER-negative SkBr3 breast cancer cells in order to evaluate in a peculiar model system the potential cross talk between the Notch pathway and the GPER signaling. Worthy, we provide novel evidence showing that ligand-activated GPER triggers Notch-mediated gene expression changes and biological responses as cell migration. These findings were confirmed in CAFs derived from breast cancer patients, suggesting that the GPER/Notch signaling may be engaged by estrogens also in the tumor microenvironment that mainly contribute to tumor development (Kalluri and Zeisberg, 2006).

Fig. 7. Ligand-activated GPER induces *Hes-1* expression in CAFs. (A) Evaluation of *Hes-1* mRNA expression in CAFs transfected for 24 h with a shRNA or a shGPER and treated for 8 h with 100 nM E2, 1 μM G-1 and 1 μM JAG-1. (B) Densitometric analysis of *Hes-1* mRNA expressions normalized to *RPLP0*. (C) Immunoblots of *Hes-1* protein expression in CAFs transfected for 24 h with a shRNA or shGPER and then treated for additional 18 h with 100 nM E2, 1 μM G-1 and 1 μM JAG-1. (D) Densitometric analysis of *Hes-1* protein expressions normalized to β-actin. (E) Immunoblots of GPER expression in CAFs transfected for 48 h with shRNA or shGPER. (F) Densitometric analysis of GPER expressions normalized to β-actin. (G) Evaluation of *Hes-1* mRNA expression in CAFs treated for 8 h with 100 nM E2, 1 μM G-1 and 1 μM JAG-1 alone or in combination with 100 nM of the γ-secretase inhibitor (GSI). (H) Densitometric analysis of *Hes-1* mRNA expressions normalized to *RPLP0*. (I) *Hes-1* protein expression in CAFs treated for 8 h with 100 nM E2, 1 μM G-1 and 1 μM JAG-1 alone or in combination with 100 nM of γ-secretase inhibitor (GSI). (J) Densitometric analysis of *Hes-1* protein expressions normalized to β-actin. Columns represent the mean ± SD of three independent experiments. (○) and (●) indicate P < 0.05 for cells receiving vehicle (−) versus treatments. In panel F, (○) indicates P < 0.05 for cells transfected with shRNA respect to cells transfected with shGPER.

CAFs

A

Hes-1 ——346 bp
RPLP0 ——408 bp

E2 (100nM)	–	+	–	–	+	–	–
G-1 (1μM)	–	–	+	–	–	+	–
JAG-1 (1μM)	–	–	–	+	–	–	+

shRNA   shGPER

B

| | E2 (100nM) | G-1 (1μM) | JAG-1 (1μM) |
shRNA   shGPER

C

Hes-1 ——35 KDa
β-actin ——43 KDa

E2 (100nM)
G-1 (1μM)
JAG-1 (1μM)

shRNA   shGPER

D

E2 (100nM)
G-1 (1μM)
JAG-1 (1μM)

shRNA   shGPER

E

GPER ——38 KDa
β-actin ——43 KDa

shRNA shGPER

F

shRNA shGPER

G

Hes-1 ——346 bp
RPLP0 ——408 bp

E2 (100nM)
G-1 (1μM)
JAG-1 (1μM)
GSI (100nM)

H

E2 (100nM)
G-1 (1μM)
JAG-1 (1μM)
GSI (100nM)

I

Hes-1 ——35 KDa
β-actin ——43 KDa

E2 (100nM)
G-1 (1μM)
JAG-1 (1μM)
GSI (100nM)
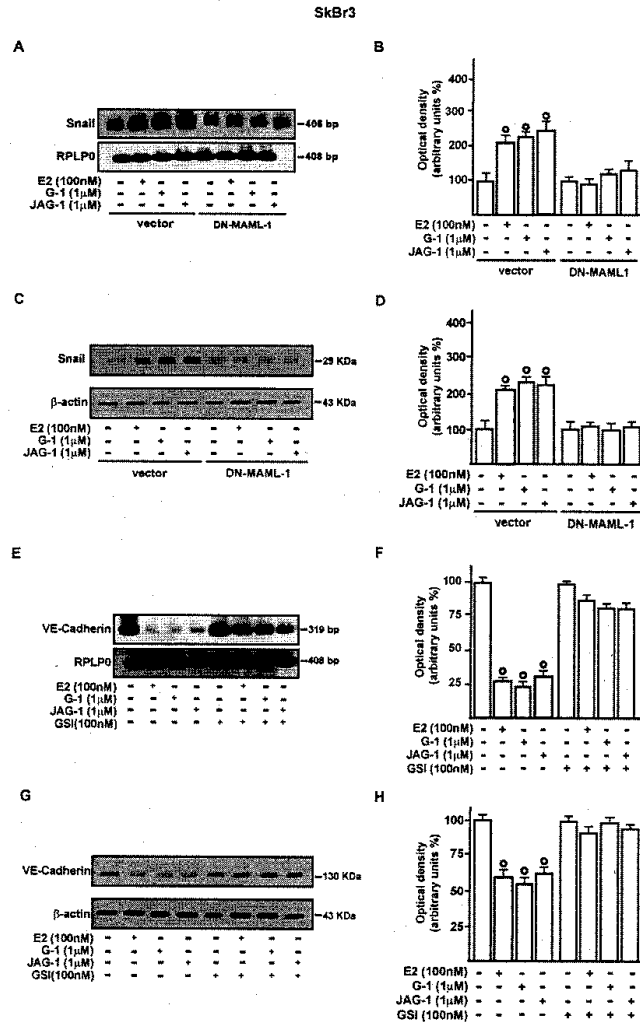
J

E2 (100nM)
G-1 (1μM)
JAG-1 (1μM)
GSI (100nM)

SkBr3

Fig. 6. Notch signaling is required for up-regulation of Snail and inhibition of VE-Cadherin expression in SkBr3 cells. (A) The up-regulation of the Snail mRNA expression induced in SkBr3 cells by a 24 h exposure to 100 nM E2, 1 μM G-1 and 1 μM JAG-1 was abolished transfecting cells for 24 h with a dominant-negative mutant of the Master-mind like 1 (DN-MAML-1) before treatments. (B) Densitometric analysis of Snail mRNA expressions normalized to RPLP0. (C) The induction of the Snail protein expression stimulated in SkBr3 cells by a 24 hours treatment with 100 nM E2, 1 μM G-1 and 1 μM JAG-1 was abrogated transfecting cells for 24 h with a dominant-negative mutant of the Master-mind like 1 (DN-MAML-1) before treatments. (D) Densitometric analysis of Snail protein expressions normalized to β-actin. (E) The down-regulation of VE-Cadherin mRNA expression in SkBr3 cells treated for 24 h with 100 nM E2, 1 μM G-1 and 1 μM JAG-1 was rescued treating cells with 100 nM of the γ-secretase inhibitor (GSI). (F) Densitometric analysis of VE-Cadherin mRNA expressions normalized to RPLP0. (G) The reduction of VE-Cadherin protein expression in SkBr3 cells treated for 24 h with 100 nM E2, 1 μM G-1 and 1 μM JAG-1 was prevented treating cells with 100 nM of the γ-secretase inhibitor (GSI). (H) Densitometric analysis of VE-Cadherin protein expressions normalized to β-actin. Columns represent the mean ± SD of three independent experiments. (○) indicates P < 0.05 for cells receiving vehicle (−) versus treatments.

signaling in the migration of SkBr3 cells. As determined by wound-healing assays, the migration of SkBr3 cells induced by E2 and G-1 as well as JAG-1 was abolished using GSI (Fig. 5A and Supplementary Fig. 4). Performing Boyden chamber assays, we confirmed the aforementioned results (Fig. 5B and Supplementary Fig. 5A) and we demonstrated that the migration induced by E2, G-1 or JAG-1 is also prevented in SkBr3 cells transfected with DN-MAML-1 (Fig. 5C and Supplementary Fig. 5B). Moreover, shGPER transfection abolished SkBr3 migration induced by E2 and G-1, whereas JAG-1-induced migration was not affected (Fig. 5D and Supplementary Fig. 6).

Taken together, these data suggest that GPER and Notch signaling pathways are involved in the migration of SkBr3 cells stimulated by E2 and G-1.

3.4. E2 and G-1 up-regulate Snail and inhibit VE-Cadherin expression through the Notch signaling

In different types of cancer cells, the Notch-target gene Snail has been shown to repress the expression of cell-cell adhesion molecules, including E-cadherins and vascular endothelial
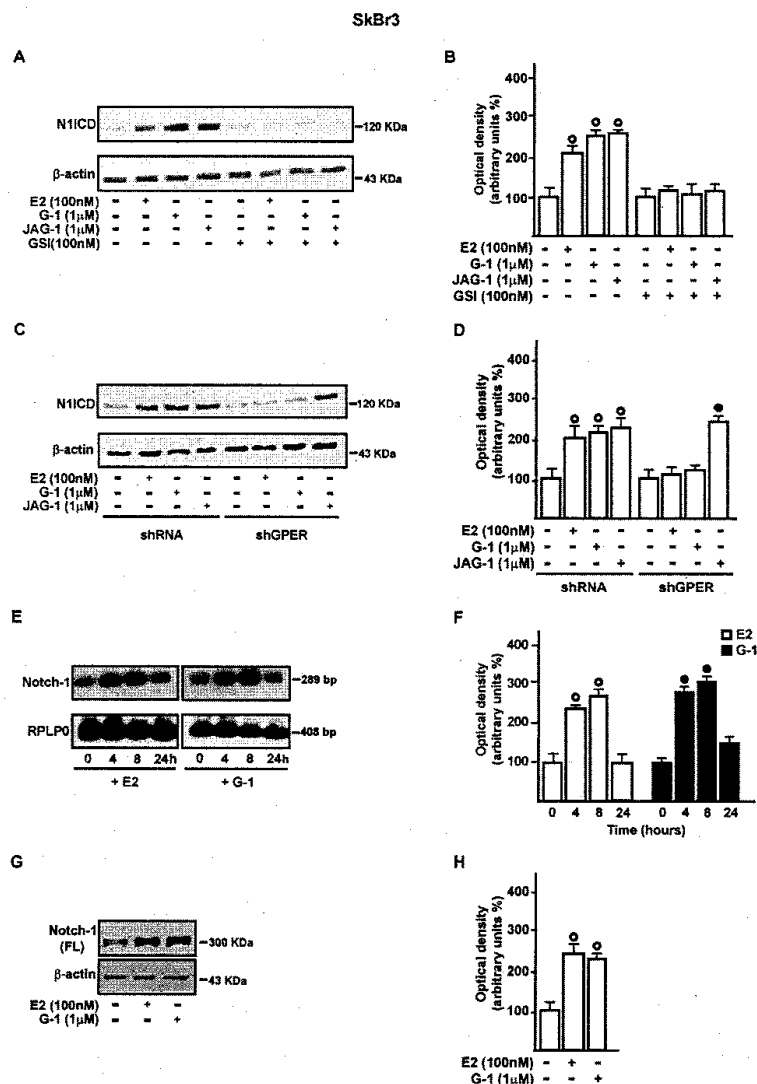
SkBr3

Fig. 3. Ligand-activated GPER up-regulates the expression of both N1ICD and Notch-1 in SkBr3 breast cancer cells. (A) N1ICD protein expression in SkBr3 cells treated for 24 h with 100 nM E2, 1 μM G-1 and 1 μM JAG-1 alone or in combination with 100 nM of γ-secretase inhibitor (GSI). (B) Densitometric analysis of N1ICD protein expressions normalized to β-actin. (C) N1ICD protein expression in SkBr3 cells transfected for 24 h with a shRNA or shGPER and then treated for additional 24 hours with 100 nM E2, 1 μM G-1 and 1 μM JAG-1. (D) Densitometric analysis of N1ICD protein expressions normalized to β-actin. (E) mRNA expression of Notch-1 in SkBr3 cells treated with 100 nM E2 and 1 μM G-1, as indicated. (F) Densitometric analysis of Notch-1 mRNA expressions normalized to RPLP0. (G) Notch-1 full length (Notch-1 FL) protein expression in SkBr3 cells treated for 8 h with 100 nM E2 and 1 μM G-1. (H) Densitometric analysis of Notch-1 FL protein expressions normalized to β-actin. Columns represent the mean ± SD of three independent experiments. (○) and (●) indicate P < 0.05 for cells receiving vehicle (−) versus treatments.

membrane-release of N1ICD in a GSI-sensitive manner. In addition, the abrogation of GPER expression by shGPER abolished only the membrane-release of N1ICD induced by E2 and G-1 (Fig. 3C and D), suggesting that GPER ligands trigger Notch activation.

As SkBr3 cells express constitutive levels of JAG-1 (data not shown), we asked whether the effects of E2 and G-1 on Notch activation could rely on the up-regulation of Notch-1 expression. As shown in Fig. 3E–H, we found that E2 and G-1 induced both

mRNA and protein levels of Notch-1, further supporting the afore-mentioned data.

Performing ChIP experiments in SkBr3 cells, we next determined that E2 and G-1 together with JAG-1 induce in a GSI dependent fashion the recruitment of N1ICD to the RBP-J/CSL binding site located within the Hes-1 promoter sequence (Fig. 4A). In accordance with these results, the transactivation of a Hes-1 promoter reporter gene induced by E2, G-1 and JAG-1 was no longer evident in
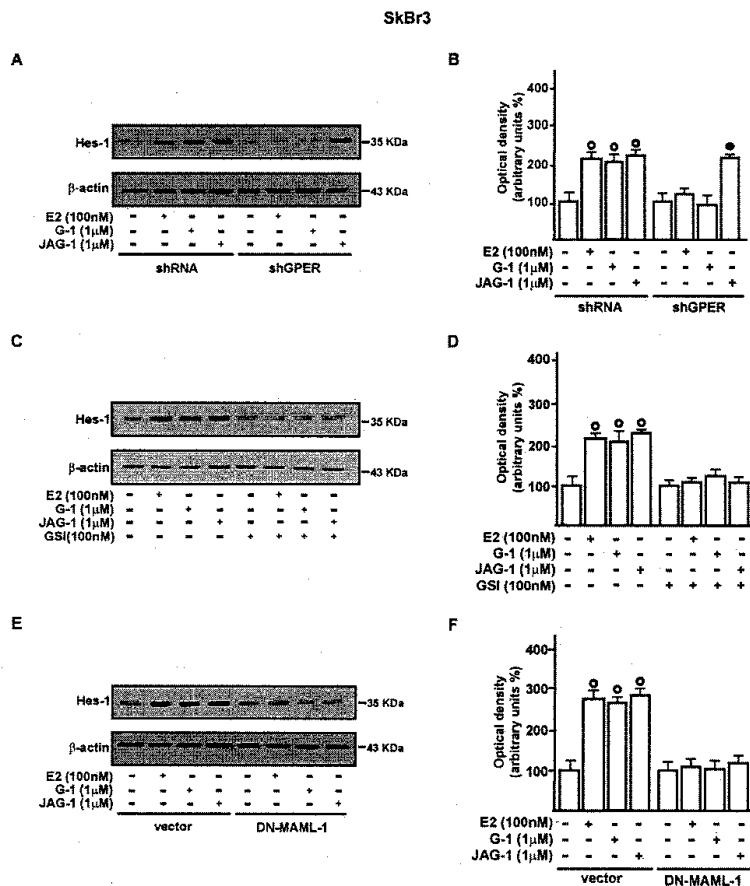
SkBr3

Fig. 2. Ligand-activated GPER induced the Notch-dependent expression of *Hes-1* at protein level in SkBr3 breast cancer cells. (A) Immunoblots of *Hes-1* protein expression in SkBr3 cells transfected for 24 h with a shRNA or shGPER and then treated for additional 24 h with 100 nM E2, 1 μM G-1 and 1 μM JAG-1. (B) Densitometric analysis of *Hes-1* protein expressions normalized to β-actin. (C) *Hes-1* protein expression in SkBr3 cells treated for 24 hours with 100 nM E2, 1 μM G-1 and 1 μM JAG-1 alone or in combination with 100 nM of γ-secretase inhibitor (GSI). (D) Densitometric analysis of *Hes-1* protein expressions normalized to β-actin. (E) *Hes-1* protein expression in SkBr3 cells transfected for 24 h with an empty vector or a dominant-negative mutant of the Master-mind like 1 (DN-MAML-1) and then treated for additional 24 h with 100 nM E2, 1 μM G-1 and 1 μM JAG-1. (F) Densitometric analysis of *Hes-1* protein expressions normalized to β-actin. Columns represent the mean ± SD of three independent experiments. (○) and (●) indicate $P < 0.05$ for cells receiving vehicle (−) versus treatments.

Supplementary Fig. 1C). In order to corroborate these results, we transfected SkBr3 cells with a dominant-negative mutant of the Master-mind like 1 (DN-MAML-1), which is a transcriptional co-activator of Notch-1 (Guo et al., 2011). As shown in Fig. 1I and J and in Supplementary Fig. 1D, the induction of *Hes1* mRNA by E2, G-1 and JAG-1 was no longer observed in presence of DN-MAML-1. To confirm these findings, we also evaluated *Hes-1* protein levels by western blot analysis. As shown in Fig. 2A–F, we found in all treatments described above, variations of *Hes-1* protein which reflected those of mRNAs.

Next, to corroborate the role elicited by GPER in mediating the Notch dependent transcription of *Hes-1*, upon E2 and G-1 treatments, we evaluated the expression of *Hes-1* mRNA also using the ER-positive MCF-7 breast cancer cells (Supplementary Fig. 2A–H). As previous reports have shown that in ER-positive breast cancer cells low doses of E2 may inhibit Notch activity (Rizzo et al., 2008a), we evaluated the expression of *Hes-1* mRNA by increasing

concentrations of E2 in both MCF-7 (ER-positive) and SkBr3 (ER-negative) breast cancer cells. As shown in Supplementary Fig. 3A–C, in SkBr3 cells, E2 induces the expression of *Hes-1* mRNA already at concentration of 1 nM. In contrast, in MCF-7 cells the expression of *Hes-1* mRNA was up-regulated only at higher concentrations of E2 (Supplementary Fig. 3D–F). Moreover, ERα silencing by shERα had no effect on the up-regulation of *Hes-1* mRNA by E2 (Supplementary Fig. 3G–K).

3.2. E2 and G-1 induce Notch activation and the recruitment of N1ICD to the Hes-1 promoter sequence

Having established that E2 and G-1 trigger *Hes-1* expression in a GSI dependent fashion, we then verified whether these ligands induce the γ-secretase-mediated release of Notch intracellular domain (N1ICD), which is a hallmark of Notch activation. As shown in Fig. 3A and B, both E2 and G-1 along with JAG-1 stimulated the
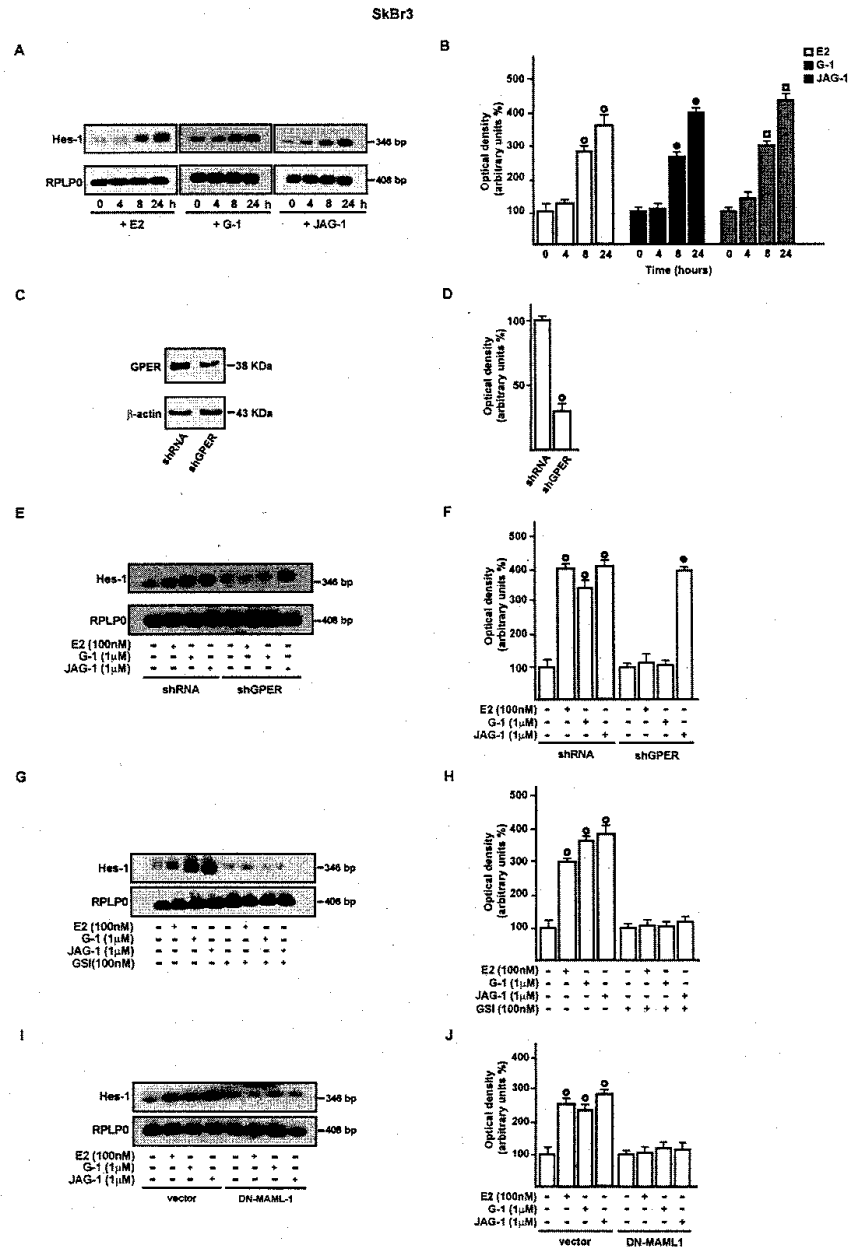
SkBr3

Fig. 1. Ligand-activated GPER induces the Notch-dependent expression of *Hes-1*mRNA in SkBr3 breast cancer cells. (A) mRNA expression of *Hes-1* in SkBr3 cells treated with 100 nM E2, 1 μM G-1 and 1 μM JAG-1, as indicated. (B) Densitometric analysis of *Hes-1* mRNA expressions normalized to *RPLP0*. (C) Immunoblots of GPER expression in SkBr3 cells transfected for 48 h with shRNA or shGPER. (D) Densitometric analysis of GPER expressions normalized to β-actin. (E) Expression of *Hes-1* mRNA in SkBr3 cells transfected with shRNA or shGPER for 24 h and then treated with 100 nM E2, 1 μM G-1 and 1 μM JAG-1 for additional 24 h. (F) Densitometric analysis of *Hes-1* mRNA expressions normalized to *RPLP0*. (G) mRNA expression of *Hes-1* in SkBr3 cells treated for 24 h with 100 nM E2, 1 μM G-1 and 1 μM JAG-1 alone or in combination with 100 nM of γ-secretase inhibitor (GSI). (H) Densitometric analysis of *Hes-1* mRNA expressions normalized to *RPLP0*. (I) mRNA expression of *Hes-1* in SkBr3 cells transfected for 24 h with an empty vector or a dominant-negative mutant of the Master-mind like 1 (DN-MAML-1) and then treated for additional 24 h with 100 nM E2, 1 μM G-1 and 1 μM JAG-1. (J) Densitometric analysis of *Hes-1* mRNA expressions normalized to *RPLP0*. Columns represent the mean ± SD of three independent experiments. (○), (●) and (□) indicate $P < 0.05$ for cells receiving vehicle (−) versus treatments. In panel D, (○) indicates $P < 0.05$ for cells transfected with shRNA respect to cells transfected with shGPER

obtained using ImageJ software, are presented as optical density (OD; expressed in arbitrary units) relative to the control (RPLP0).

2.5. Real-time PCR

Gene expression was evaluated also by real-time PCR as we previously described (Lappano et al., 2011). For Hes-1, Snail, VE-Cadherin, and the ribosomal protein 18S, which was used as a control gene to obtain normalized values, the primers were: 5'-TCAACACGACACCGGATAAA-3' (Hes-1 forward) and 5'-CCGCGAGCTATCTTTCTTCA-3' (Hes-1 reverse); 5'-CTTCCAGCAGC-CCTACGAC-3' (Snail forward) and 5'-CGGTGGGGTTGAGGATCT-3' (Snail reverse); 5'-TTTCCAGCAGCCTTTCTACCA-3' (VE-Cadherin forward) and 5'-GGAAGAACTGGCCCTTGTCA-3' (VE-Cadherin reverse) and 5'-GGCGTCCCCCAACTTCTTA-3' (18S forward) and 5'-GGGCATCACAGACCTGTTATT-3' (18S reverse), respectively.

2.6. Chromatin Immunoprecipitation (ChIP) assay

Cells were grown in 10-cm plates and then exposed to ligands for 8 h. Thereafter, cells were cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleared with sonicated salmon DNA/protein A agarose (Upstate Biotechnology, Inc., Lake Placid, NY) and immunoprecipitated with the anti-Notch-1 (C-20) antibody or non specific IgG (Santa Cruz Biotechnology, DBA, Milan, Italy). Pellets were washed, eluted with a buffer consisting of 1% SDS and 0.1 mol/L NaHCO₃, and digested with proteinase K. DNA was obtained by phenol/chloroform extraction and precipitated with ethanol. A 4 µl volume of each sample was used as template to amplify by real-time PCR a RBP-J binding site corresponding to −167 to +6 located in the 5'-flanking region of Hes-1 gene. The primers used were Fwd: 5'-CAGACCTTGTGCCTGGCG-3' and Rev: 5'-TGTGATCCCTAGGCCCTG-3'. Data were normalized with respect to unprocessed lysates (input DNA). Inputs DNA quantification was performed by using 4 µl of the template DNA. The relative antibody-bound fractions were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as percent differences with respect to the relative inputs.

2.7. Plasmids

The luciferase reporter plasmid Hes1-Luc (−467 to +46 of the Hes1 promoter with the luciferase gene) was a kind gift from Dr. Hee-Sae Park (Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University, Yongbong-dong, Buk-ku, Gwangju, Republic of Korea). The plasmid encoding dominant-negative MAML-1(DN-MAML-1) was a gift from Dr. M. Bocchetta (Cardinal Bernardin Cancer Center, Loyola University Chicago, Illinois, USA). The Sure Silencing (sh) ERα and the respective control plasmid (shRNA), generated in pGeneClip Puromycin Vector, were purchased from SA Bioscience Corp. (Frederick, MD, USA) and used according to the manufacturer's recommendations. Short hairpin RNA construct against human GPER (shGPR30/shGPER) and the unrelated shRNA control construct were previously described (Pandey et al., 2009).

2.8. Transfection, luciferase assays and gene silencing experiments

For luciferase assays, cells were plated into 24-well plates with 500 µl of regular growth medium/well the day before transfection. Cell medium was replaced with medium supplemented with 1% charcoal-stripped (CS) FBS lacking phenol red and serum on the day of transfection, which was performed using the X-treme Gene 9 reagent as recommended by the manufacturer (Roche Diagnostics, Milan, Italy) with a mixture containing 0.5 µg of reporter plasmid and 2 ng of pRL-TK. After 5–6 h, ligands were added and cells were incubated for 24 h. Luciferase activity was then measured using the Dual Luciferase Kit (Promega, Milan, Italy) according to the manufacturer's recommendations. Firefly luciferase activity was normalized to the internal transfection control provided by the Renilla luciferase activity. The normalized relative light unit values obtained from cells treated with vehicle were set as one-fold induction upon which the activity induced by treatments was calculated. For the gene silencing experiments, cells were plated into 10-cm dishes, transfected in serum-free medium for 24 h before treatments using X-treme Gene 9 (Roche Diagnostics, Milan, Italy), according to the manufacturer's recommendations with shRNA, shGPER, shERα, DN MAML-1 and the unrelated empty vector.

2.9. Wound-healing assay

SkBr3 cells and CAFs were seeded into 12-well plates in regular growth medium. When at 70–80% confluence, the cells were washed once and medium was replaced with 2.5% charcoal-stripped FBS. Cells were then pre-treated with GSI and treated with E2, G-1 and JAG-1. Then, a p200 pipette tip was used to scratch the cell monolayer. We evaluated cell migration in three independent experiments after 24 h of treatment; data are expressed as a percentage of cells in the wound area upon treatment compared with cells receiving vehicle.

2.10. Transwell cell migration assay

Migration assays were performed with SkBr3 cells and CAFs in triplicate using Boyden chambers (Costar Transwell, 8 mm polycarbonate membrane). For knockdown experiments, cells were transfected with the plasmid DN-MAML1 or with an empty vector construct using X-treme Gene 9 reagent in medium without serum. After 24 h, cells were seeded in the upper chambers. Treatments were added to the medium without serum in the bottom wells. After 24 h, cells on the bottom side of the membrane were fixed, stained with GIEMSA (Sigma–Aldrich Milan, Italy), photographed and counted.

2.11. Statistical analysis

Statistical analysis was performed using analysis of variance followed by Newman–Keuls testing to determine differences in means. p-Values < 0.05 are considered statistically significant.

3. 3 Results

3.1. E2 and G-1 induce Notch-dependent expression of Hes-1

In order to investigate a possible functional interaction between GPER and Notch signaling, we first investigated whether ligand-activated GPER triggers Notch-dependent transcription. Hence, we evaluated the Hes-1 mRNA expression in SkBr3 cells treated with E2, G-1 or the soluble Notch ligand JAG-1. As shown in Fig. 1A and B and in Supplementary Fig. 1A, all three ligands induced the levels of Hes-1 particularly upon 24 h treatments. To assess the direct involvement of GPER in the up-regulation of Hes-1, we transfected SkBr3 cells with a shGPER which abrogated the expression of Hes-1 induced by E2 and G-1 (Fig. 1C–F and Supplementary Fig. 1B). In contrast, in SkBr3 cells transfected with the shGPER and exposed to JAG-1, the induction of Hes-1 was still evident (Fig. 1E and F and Supplementary Fig. 1B). To confirm the Notch-dependent up-regulation of Hes-1 by E2 and G-1, we used the γ-secretase inhibitor named GSI which impaired the Hes-1 response to these treatments along that promoted by JAG-1 as expected (Fig. 1G and H and

the EGFR transduction pathway, the activation of the MAPK cascade, PI3K kinase and phospholipase C signaling (Maggiolini and Picard, 2010). Moreover, in ER-negative cancer cells like SkBr3 cells or cancer-associated fibroblasts (CAFs), GPER contributed to the stimulation of migration as its silencing drastically reduced the pro-migratory effects of 17β-estradiol (E2) and G-1, which involved EGFR-dependent activation of one important GPER target gene named connective tissue growth factor (*CTGF*) (Madeo and Maggiolini, 2010; Pandey et al., 2009).

The Notch signaling and its crosstalk with several transduction pathways plays an important role in different aspects of breast cancer biology, including cell growth, EMT transition and cell migration (Guo et al., 2011). Notch consists of a family of single-pass transmembrane receptors (Notch1-4), which can be activated by the interaction with membrane-tethered ligands, including Dll (Delta-like 1-4) and Jagged (Jagged 1-2) (Miele, 2006). Up-regulated expression of Notch receptors and/or their ligands have been found in several human malignancies, including breast cancer (Al-Hussaini et al., 2011; Kopan and Ilagan, 2009). In addition, the expression of the Notch ligand Jagged-1 has been correlated with more aggressive malignant features (Reedijk et al., 2005). Upon ligand activation, Notch is cleaved by an ADAM metalloproteinase and γ-secretase and thereafter the membrane-released Notch intracellular domain (NICD) translocates to the nucleus (Guo et al., 2011). In the nucleus, NICD releases the expression of Notch target genes by recruiting transcriptional regulators, hence converting the RBP-J/CSL transcriptional repressor complex in a transcriptional activator (Yin et al., 2010). The most characterized transcriptional targets of Notch signaling are bHLH transcriptional repressors of the *Hes* (*Hes-1 to 7*) and *Hey* (*Hey-1 and 2, L*) subfamilies (Iso et al., 2003). Notch-induced activation of *Hes* and *Hey* genes play an important role in cell fate determination during organ development (Guo et al., 2011) Furthermore, Notch-dependent expression of *Hes* and *Hey* genes has been described in different types of cancer cells and correlates with Notch tumorigenic activities (Miele, 2006). An additional Notch-target gene is the zinc-finger transcriptional repressor *Snail*, which has been shown to trigger EMT by directly repressing E-cadherin expression (Wang et al., 2010). Notch-dependent up-regulation of *Snail* and the consequent E-cadherin repression represent the main pathway mediating the Notch-dependent migration in diverse tumor cells (Chen et al., 2010; Guo et al., 2011; Matsuno et al., 2012).

In the present study, we provide novel evidence showing that ligand-activated GPER triggers Notch activation and expression of Notch target genes. Moreover, we have assessed that Notch signaling contributes to GPER-mediated migration in ER-negative breast cancer cells and cancer-associated fibroblasts.

2. Materials and methods

2.1. Reagents

17β-Estradiol (E2) was purchased from Sigma–Aldrich (Milan, Italy); γ-Secretase inhibitor cbz-Leu-Leu-Nle-CHO (GSI) and 1-[4-(-6-bromobenzol[1,3]diodo-5-yl)-3a,4,5,9b-tetrahidro3H-cyclopenta[c]quinolin-8yl]-ethanone (G-1) were purchased from Calbiochem (Merck KGaA, Frankfurt, Germany); Notch ligand Jagged-1 (DSL Peptide 188-204) (JAG-1) was obtained from AnaSpec (DBA Milan, Italy). E2 was dissolved in ethanol while G-1, GSI and JAG-1 were solubilized in DMSO.

2.2. Cell cultures

The SkBr3 breast cancer cells were maintained in RPMI-1640 (Invitrogen, Gibco, Milan, Italy) without phenol red, supplemented with 10% fetal bovine serum (FBS). MCF7 breast cancer cells were maintained in DMEM with phenol red supplemented with 10% FBS. Cells were switched to medium without serum the day before experiments for immunoblots, reverse transcription polymerase chain reaction (RT-PCR) and chromatin immunoprecipitation (ChIP). Both cell lines were grown in a 37 °C incubator with 5% CO_2. CAFs were extracted as previously described (Madeo and Maggiolini, 2010; Pupo et al., 2012, 2013) maintained in a mixture of MEDIUM 199 and HAM'S F-12 (1:1) supplemented with 10% FBS. Primary cells cultures of breast fibroblasts were characterized by immunofluorescence. Briefly cells were incubated with human anti-vimentin (V9) and human anti-cytokeratin 14 (LL001), all antibodies were from Santa Cruz Biotechnology, DBA (Milan, Italy). In order to assess fibroblasts activation, we used anti-fibroblast activated protein α (FAPα) antibody (H-56) purchased from Santa Cruz Biotechnology, DBA (Milan, Italy) (data not shown). We used CAFs passaged for up to five population doublings for the experiments performed using these cells. CAFs were also switched to medium without serum the day before experiments for immunoblots and reverse transcription polymerase chain reaction (RT-PCR)

2.3. Western blotting

Western blotting experiments were performed as previously described (Madeo et al., 2010). Briefly, cells were exposed to ligands, and then lysed in a buffer containing 1% SDS and a mixture of protease inhibitors. Equal amounts of whole protein extract were resolved on SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Amersham Biosciences, Milan, Italy), probed overnight at 4 °C with antibodies against *Hes-1* (H-140), *Notch-1* (C-20), Snai 1 (G-7), VE-Cadherin (N-14), ERα (F-10), GPER (N-15) and β-actin (C-2) purchased from Santa Cruz Biotechnology (DBA, Milan, Italy), and then revealed using the ECL® Western Blotting Analysis System (GE Healthcare, Milan, Italy). Results of densitometric analyses of Western blots, obtained using ImageJ software, are presented as optical density (OD; expressed in arbitrary units) relative to the control (β-actin).

2.4. Reverse transcription and quantitave RT-PCR

Total RNA was extracted using Trizol commercial kit (Invitrogen, Milan, Italy) according to the manufacturer's protocol. RNA was quantified spectrophotometrically and its quality was checked by electrophoresis through agarose gel stained with ethidium bromide. Only samples that were not degraded and showed clear 18S and 28S bands under ultraviolet light were used for real-time PCR. Total cDNA was synthesized from RNA by reverse transcription using the murine leukemia virus reverse transcriptase (Invitrogen, Milan, Italy) following the protocol provided by the manufacturer. The expression of selected genes was quantified by quantitative RT-PCR carried out as previously described (Maggiolini et al., 1999). *Hes-1*, *Notch-1*, *Snail*, *VE-cadherin* and the internal control *RPLP0* (also known as *36B4*) cDNAs yielded bands of 346, 289, 406, 319 and 408 bp with 21, 25, 22, 23 and 10 PCR cycles, respectively. The primers pairs used to amplify the fragments were: 5'-CCCAGCCAGTGTCAACACGAC-3' (forward) and 5'-ATTAACGCCCTCGCACGTGG-3' (reverse) for *Hes-1*; 5'-GCATAG-TCCAAAAAGCTCCTG-3' (forward) and 5'-GTGCACTCTTGGCATACA-CAC-3' (reverse) for *Notch-1*; 5'-CTCACCGGCTCCTTCGTCCT-3' (forward) and 5'-ACACGCCTGGCACTGGTACT-3' (reverse) for *Snail*; 5'-TCTCCGCAATAGACAAGGAC-3' (forward) and 5'-AGTAAGATGG-CTACCACTGC-3' (reverse) for *VE-Cadherin*; 5'-CTCAACATCT-CCCCCTTCTC-3' (forward) and 5'-CAAATCCCATATCCTCGTCC-3' (reverse) for *RPLP0*. Results of densitometric analyses of blots,

GPER activates Notch signaling in breast cancer cells and cancer-associated fibroblasts (CAFs)

Marco Pupo[a], Assunta Pisano[a], Sergio Abonante[b],
Marcello Maggiolini[a,*], Anna Maria Musti[a,c,**]

[a] Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Rende, Italy
[b] Regional Hospital, Cosenza, Italy
[c] Institute for Clinical Neurobiology, University of Würzburg, Würzburg, Germany

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ABSTRACT

The G protein-coupled receptor GPR30/GPER has been shown to mediate rapid effects of 17β-estradiol (E2) in diverse types of cancer cells. Here, we provide evidence for a novel crosstalk between GPER and the Notch signaling pathway in breast cancer cells and cancer-associated fibroblasts (CAFs). We show that E2 and the GPER selective ligand G-1 induce both the γ-secretase-dependent activation of Notch-1 and the expression of the Notch target gene Hes-1. These inductions are prevented by knocking down GPER or by using a dominant-negative mutant of the Notch transcriptional co-activator Master-mind like-1 (DN-MAML-1), hence suggesting the involvement of GPER in the Notch-dependent transcription. By performing chromatin-immunoprecipitation experiments and luciferase assays, we also demonstrate that E2 and G-1 induce the recruitment of the intracellular domain of Notch-1 (N1ICD) to the Hes-1 promoter and the transactivation of a Hes-1-reporter gene, respectively. Functionally, the E2 and G-1-induced migration of breast cancer cells and CAFs is abolished in presence of the γ-secretase inhibitor GSI or DN-MAML-1, which both inhibit the Notch signaling pathway. In addition, we demonstrate that E2 and G-1 prevent the expression of VE-Cadherin, while both compounds induce the expression of Snail, a Notch target gene acting as a repressor of cadherins expression. Notably, both GSI and DN-MAML-1 abolish the up-regulation of Snail-1 by E2 and G-1, whereas the use of GSI rescues VE-Cadherin expression. Taken together, our results prove the involvement of the Notch signaling pathway in mediating the effects of estrogenic GPER signaling in breast cancer cells and CAFs.

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1. Introduction

Estrogens regulate critical signaling pathways involved in the control of cell proliferation and differentiation in reproductive and non-reproductive tissues (Liang and Shang, 2013). These steroids influence also the pathological processes of hormone-dependent tumors, like breast cancer, activating the estrogen receptor (ER)α and ERβ which act as transcription factors binding to cognate the responsive elements located in the promoter regions of target genes (Ascenzi et al., 2006; Panno et al., 1996).

Although estrogens act mostly by this classic genomic mechanism, they are also able to rapidly activate transduction pathways in an ER-independent manner. In the last few years the membrane-associated G protein-coupled receptor GPR30/GPER has been widely shown to mediate signals triggered by estrogens, antiestrogens and phyto-xenoestrogens, including the quick MAPK activation, the induction of early gene expression, the proliferation and migration in different types of cancer cells (Albanito et al., 2007, 2008a,b; Chimento et al., 2012; De Marco et al., 2013; Filice et al., 2009; Lappano et al., 2010; Maggiolini et al., 2004; Pupo et al., 2012; Recchia et al., 2011; Santolla et al., 2012; Thomas et al., 2005; Vivacqua et al., 2006a,b, 2012). Moreover, the identification of synthetic molecules acting either as agonistic or antagonist ligands of GPER has extended our knowledge regarding the estrogenic GPER signaling (Bologa et al., 2006; Dennis et al., 2011; Lappano et al., 2012a,b; Rosano et al., 2012). Among these molecules, the GPER-agonist ligand G-1 has been shown to induce both gene expression changes and proliferation in diverse tumor cells. In this regard, several studies have shown that these effects mediated by ligand-activated GPER require a functional interaction with

* Corresponding author at: Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, 87036 Rende, Italy. Tel.: +39 0984 493076; fax: +39 0984 493458.
** Corresponding author at: Institute for Clinical Neurobiology, University of Würzburg, D-97078 Würzburg, Germany. Tel.: +49 0931 201 44030; fax: +49 0931 201 44009.
E-mail addresses: marcellomaggiolini@yahoo.it (M. Maggiolini),
E.Musti_A@klinik.uni-wuerzburg.de (A.M. Musti).

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Table V. Contingency table: ER—/PR+ versus ER—/PR—

	ER—/PR+	ER—/PR—
CVD+	8	41
CVD—	65	176
Total	73	217

Odds ratio, 0.53; $P < 0.16$.

DISCUSSION

It is well-known that blood vessel walls, and other target organs such as the breast, have a large number of ERs and PRs for which distribution is variable from person to person, thereby rendering a reasonable number of persons who may be more sensitive to the hormone effects.[9]

Estrogen has been shown to increase venous capacitance, altering blood vessel walls.[10,11] In fact, estrogen has effects on vascular smooth muscle contraction evoked by activation of both receptor- and potential-operated calcium channels.[12] Moreover, ERs have been localized in the nucleus and cytoplasm of human arterial and venous endothelial cells and human vascular smooth muscle cells.[13]

Mashiah et al.[14] conducted a study in which ERs and PRs were investigated in the walls of normal and varicose veins. ER levels were lower in nonvaricose segments of varicose veins compared with normal veins. In varicose segments, ERs were more abundant than in the nonvaricose parts of the same vein, especially in women; contrasting results were found for PRs.

Raffetto et al.[15,16] showed that sex differences in venous function reflect reduced constriction and enhanced venous dilation in women because of direct venous relaxation effects of estrogen on specific receptors.

ERs and PRs have been studied in clinical BC for more than 20 years. The percentage of ER+ cells is quite low (10%—20%) in normal mammary gland, and increases in proliferative disease, whereas ER+ cells are found in 50% to 80% of breast tumors, suggesting that an elevated receptivity to estrogens in these tissues is involved in their higher risk of tumorigenesis. ER/PR status is essential in making decisions about endocrine therapy, and positive receptor status correlates with favorable prognostic features, including a lower rate of cell proliferation and histologic evidence of tumor differentiation. Tumors that express both ER and PR have the greatest benefit from hormonal therapy, but those containing only ER or PR still have significant responses.[3]

Moreover, endocrine therapy with selective estrogen receptor modulators (SERMs) has been associated with an increased risk of venous thromboembolic events. SERMs bind with high affinity to ERs and promote either estrogen agonist or estrogen antagonist effects, depending on the target tissue. SERMs exert estrogen antagonist effects on the breast but agonist and harmful effects on venous endothelium.[13,17] The hypothesis that the distribution of venous ERs and PRs may reflect the hormonal status of BC tissue could explain adverse events involving the venous system as being manifestations of CVD and, in the case of treatment with SERMs, the occurrence of acute venous thromboembolic events.

In some clinical cases reported in the literature, the diagnosis of BC has been predicted even by some acute thromboembolic phenomena.[18,19]

In the present series, 4 cases of superficial vein thrombosis of upper limbs occurred within 1 year from diagnosis of BC.

This study shows a strong association between BC and the concomitant presence of clinical manifestations related to CVD, and this association is strongly influenced by the receptor status of BC. The presence of ER+ status is associated with more severe clinical manifestations of venous disease; 99.17% of patients who have ER+ status and CVD had a clinical stage between grades C3 and C6 of the CEAP classification.

Moreover, a worsening of clinical conditions according to CEAP classification have been observed within 1 year from diagnosis of BC in patients with ER+ status and CVD compared with those with ER— status.

In contrast, the absence of ER+ status did not seem to be associated to the occurrence of venous disease, and the presence of PR+ status seems to be even protective against the onset of venous disease.

BC and CVD seem to be closely linked by sex-hormone related mechanisms that remain unknown, which must be elucidated by further studies.

Moreover, these findings suggest that future clinical evidence is needed to clarify the benefit of systematic compression-stocking use and prophylactic administration of anticoagulants, such as low-molecular-weight heparin, to prevent clinical worsening of CVD and thromboembolic events, respectively, in patients with BC.

Table II. Receptor status versus clinical classification of CVD

Receptor status	Classification	% (N)
ER+/PR+	C1s (telangiectasies or reticular veins + symptoms)	7.63% (41)
834 patients	C2 (varicose veins)	51.58% (277)
537 (64.38%) with CVD	C3 (edema)	28.11% (151)
	C4 (pigmentation, eczema, lipodermatosclerosis)	5.40% (29)
	C5 (healed venous ulcer)	2.04% (11)
	C6 (active venous ulcer)	5.27% (28)
ER+/PR−	C1s (telangiectasies or reticular veins + symptoms)	47.61% (20)
67 patients	C2 (varicose veins)	4.76% (2)
42 (62.68%) with CVD	C3 (edema)	9.52% (4)
	C4 (pigmentation, eczema, lipodermatosclerosis)	23.80% (10)
	C5 (healed venous ulcer)	11.90% (5)
	C6 (active venous ulcer)	2.38% (1)
ER−/PR+	C1s (telangiectasies or reticular veins + symptoms)	75.00% (6)
73 patients	C2 (varicose veins)	12.5% (1)
8 (10.95%) with CVD	C3 (edema)	12.5% (1)
	C4 (pigmentation, eczema, lipodermatosclerosis)	0% (0)
	C5 (healed venous ulcer)	0% (0)
	C6 (active venous ulcer)	0% (0)
ER−/PR−	C1s (telangiectasies or reticular veins + symptoms)	90.24% (37)
217 patients	C2 (varicose veins)	7.31% (3)
41 (18.89%) with CVD	C3 (edema)	2.43% (1)
	C4 (pigmentation, eczema, lipodermatosclerosis)	0% (0)
	C5 (healed venous ulcer)	0% (0)
	C6 (active venous ulcer)	0% (0)

Table III. Contingency table: ER+/PR+ versus ER−/PR−

	ER+/PR+	ER−/PR−
CVD+	537	41
CVD−	297	176
Total	834	217

Odds ratio, 7.76; $P < 0.00001$.

Table IV. Contingency table: ER+/PR− versus ER−/PR−

	ER+/PR−	ER−/PR−
CVD+	42	41
CVD−	25	176
Total	67	217

Odds ratio, 7.21; $P < 0.00001$.

(ER−/PR+) and patients with negative status for both kind of receptors (ER−/PR−) were affected by CVD (Table II). Moreover, from a clinical viewpoint, 99.17% (239 of 241) of patients with severe manifestations of CVD (C3−C6) were included in ER+ groups (ER+/PR+; ER+/PR−) (Tables III and IV).

The presence of BC was associated with concomitant CVD clinical manifestations in both ER+ groups (ER+/PR+ group: odds ratio [OR], 7.76; $P < 0.00001$; ER+/PR− group: OR, 7.21; $P < 0.00001$), whereas no association was seen in the ER−/PR+ group (OR, 0.53; $P < 0.016$), and the presence of PR+ in this latter group seems to even be protective against CVD (Table V).

In addition, based on the available data from medical records, in the ER+ groups with CVD

($N = 579$), 151 patients (26.08%), despite appropriate treatment, advanced a clinical class of CEAP classification within a year from BC diagnosis, compared with 2 (4.08%) of the 49 patients in the ER− groups with CVD.

In the 151 patients in the ER+ groups, clinical worsening went from C2 to C3 in 66 patients (43,70%) and from C3 to C4 in 38 patients (25.16%), and from C4 to C6 in 47 patients (31.12%).

In the 2 patients in the ER− groups, clinical worsening went from C2 to C3.

Moreover, 4 events of superficial vein thrombosis of the upper limb occurred within a year from the time of diagnosis of BC in the ER+ groups with CVD (0.69%), whereas no thromboembolic events occurred in the ER− groups nor in patients with BC without CVD.

Table I. Patient characteristics

Characteristic	Classification	% (N)
Age (median, 54 yr; range, 25—83 yr)	0—29 yr	5.36% (61)
	30—49 yr	25.21% (287)
	50—59 yr	27.94% (316)
	60—69 yr	19.94% (227)
	70—79 yr	13.97% (159)
	≥ 80 yr	7.73% (88)
Body mass index	Underweight: < 18.5 kg/m^2	14.49% (165)
	Normal: 18.5—24.9 kg/m^2	46.22% (526)
	Overweight: 25.0—29.9 kg/m^2	31.01% (353)
	Obese: 30.0—39.9 kg/m^2	7.73% (88)
	Extremely obese: ≥ 40 kg/m^2	0.52% (6)
Diabetes		7.46% (85)
Smoking		26.97% (307)
Hormone replacement therapy		2.54% (29)
BC stage	Stage I	25.74% (293)
	Stage II	36.55% (416)
	Stage III	27.68% (315)
	Stage IV	10.01% (114)
ER/PR status	ER+/PR+	73.28% (834)
	ER+/PR—	5.88% (67)
	ER—/PR+	6.41% (73)
	ER—/PR—	19.68% (217)
Venous disease (n = 628)	C1s (telangiectasies or reticular veins + symptoms)	9.13% (104)
	C2 (varicose veins)	24.86% (283)
	C3 (edema)	13.79% (157)
	C4 (pigmentation, eczema, lipodermatosclerosis)	3.42% (39)
	C5 (healed venous ulcer)	1.40% (16)
	C6 (active venous ulcer)	2.54% (29)

ER+, estrogen receptor—positive status; ER—, estrogen receptor—negative status; PR+, progesterone receptor—positive status; PR—, progesterone receptor—negative status.

the medical records from 2 clinical centers (University Magna Graecia of Catanzaro and Annunziata Hospital of Cosenza) of 1138 female patients with BC for whom information about ER and PR status was available, to characterize the association between BC and CVD.

The author referred to the Clinical-Etiology-Anatomy-Pathophysiology (CEAP) classification for the clinical evaluation of signs and symptoms related to venous disease.[8]

Only for C1 class (telangiectasia or reticular veins), because telangiectasies are highly prevalent in the healthy adult population, the authors considered venous disease to be present in patients who also complained of having symptoms such as aching, pain, tightness, heaviness, and muscle cramps attributable to venous dysfunction.

For classes C2 through C6, the diagnostic evaluation of CVD, contained in medical records, was obtained through an office visit, including a history and clinical examination, that involved the use of a hand-held Doppler scanner or duplex color scanning.

Patient characteristics are shown in Table I.

Patient characteristics and the presence of clinical manifestations indicative of CVD were studied in relation to the receptor status of BC to determine any correlation within these variables.

Statistical analysis was performed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Of 1138 patients, 628 (55.18%) had CVD; 358 patients (31.45%) had clinical manifestations of mild severity (C1—C2), whereas 239 (21%) had severe clinical manifestations (C3—C6).

If patients are subdivided by receptor status, one can assume that the 64.38% of patients with positive status for ERs and PRs (ER+/PR+) and 62.68% of patients with positive status for ERs and negative status for PRs (ER+/PR—) had clinical manifestations related to CVD, whereas only 10.95% of patients with negative status for estrogen receptors and positive status for progesterone receptors

Clinical Research

Breast Cancer and Venous Disease: A Retrospective Cohort Study

Raffaele Serra,[1] Gianluca Buffone,[1] Anna Maria Miglietta,[2] Sergio Abonante,[2] Vincent Giordano,[3] Maria Renne,[4] Marina Lugarà,[5] and Stefano de Franciscis,[1] Catanzaro and Cosenza, Italy

Background: Breast cancer (BC) and chronic venous disease (CVD) are in some way related to hormonal effects, and often the clinical manifestations of CVD intersect with the clinical course of BC. This article describes the correlations between these clinical conditions.
Methods: A total of 1138 female patients with BC were retrospectively reviewed in a 5-year period to obtain clinical information about the frequency and characteristics of contemporary CVD and the relative correlations with estrogen and progesterone receptor status.
Results: The presence of BC was associated with concomitant CVD clinical manifestations in patients with positive estrogen receptor status, whereas no association was found in patients with negative estrogen receptor status. The presence of negative estrogen receptor status associated with positive progesterone receptor status seemed to be even protective against CVD. Patients with more severe manifestations of CVD had positive estrogen receptor status.
Conclusions: BC and CVD seem to be strongly associated. Positive estrogen receptor status may predispose to a more severe clinical course of venous disease when it occurs in patients with BC.

INTRODUCTION

Breast cancer (BC) is one of the most common forms of cancer and chronic venous disease (CVD) is one of the most common diseases in women.

[1]Chair of Surgery, University Magna Graecia of Catanzaro, Catanzaro, Italy.

[2]Breast Unit, Annunziata Hospital, Cosenza, Italy.

[3]Plastic Surgery Unit, Annunziata Hospital, Cosenza, Italy.

[4]Surgical Oncology Unit, Campanella Foundation for Cancer Research and Care, University Magna Graecia of Catanzaro, Catanzaro, Italy.

[5]Chair of Internal Medicine, University Magna Graecia of Catanzaro, Catanzaro, Italy.

Correspondence to: Raffaele Serra, MD, PhD, Department of Medical and Surgical Science, University Magna Graecia of Catanzaro, Catanzaro, Italy, Viale Europa, Germaneto 88100 Catanzaro; E-mail: rserra@unicz.it

The expression of estrogen receptor (ER) and progesterone receptor (PR) has been shown to have prognostic significance for patients with BC and can be a predictive marker for endocrine therapy in their clinical management.[1–3]

Clinical and epidemiologic observations regarding varicose veins, such as their predominance in woman,[4,5] and the occurrence of venous stasis during sex hormone therapy, the luteal phase, and pregnancy suggest a sex hormone dependency of venous disease.[6,7]

Assuming that the distribution of ERs and PRs may overlap, both in the breast tissue and in the venous system, the clinical manifestation of CVD could be expected to be highly prevalent in patients with BC with positive ER and/or PR status.

MATERIAL AND METHODS

Over a 5-year period (January 2007–December 2011), the authors gathered anamnestic and clinicopathologic data through a retrospective review of

VIA SAN MARTINO SNC
87100 COSENZA (CS)
Telefono   0984.681060 Fax 0984681064
Partita Iva 01987250782

Centri di Costo
Periodo Dal [01/10/2013]
Al [31/12/2013]
Pagina N°.   I

Centro Costo 41	S.S.D. Senologia chirurgica				
Mastro	Conto	Sotto Conto	Descrizione		Tot.Costo
501	1	101	501.01.0101 Medicinali con A.I.C.		2.333,20
501	1	401	501.01.0401 Prodotti dietetici		42,90
501	1	501	501.01.0501 Materiale per la profilassi (Vaccini)		93,68
501	1	601	501.01.0803 Materiali Diagnostici		17,69
501	1	801	501.01.0801 Dispositivi Medici		45.774,66
501	1	901	501.01.0802 Materiale Protesici		6.136,00
Totali Centro di Costo		**41**	**S.S.D. Senologia chirurgica**		**54.398,12**
Totali Generali					**54.398,12**

COSENZA lì 24/01/2014

VIA SAN MARTINO SNC
87100 COSENZA (CS)
Telefono   0984.681060 Fax 0984681064
Partita Iva 01987250782

Centri di Costo
Periodo Dal [01/01/2013]
Al [31/12/2013]
Pagina N°.   5

Tipo FA	FARMACO	Centro 41	S.S.D. Senologia chirurgica		Sub 1	4.6.1 REPARTO			
Codice	ATC-CND	Descrizione Articolo				Scarico	Reso	Differenza	Tot.Costo
DR0100903	R05CB01	Acetilcisteina im.ev. 300 mg.				70,00		70,00	17,71
P00001896	R06AE07	CETIRIZINA RATIOPHARM 20CPR RIV. 10MG				20,00		20,00	0,53
F00007044	V03AF04	LEDERFOLIN 10 CPR 7,5 MG				10,00		10,00	0,98
Totali Categoria Prodotti		**FA FARMACO**							**8.442,39**

Tipo MT	MATERIA PRIMA	Centro 41	S.S.D. Senologia chirurgica		Sub 1	4.6.1 REPARTO			
Codice	ATC-CND	Descrizione Articolo				Scarico	Reso	Differenza	Tot.Costo
P00000341		Olio di vaselina				1,00		1,00	7,32
Totali Categoria Prodotti		**MT MATERIA PRIMA**							**7,32**

Tipo PC	Prodotto chimico puro	Centro 41	S.S.D. Senologia chirurgica		Sub 1	4.6.1 REPARTO			
Codice	ATC-CND	Descrizione Articolo				Scarico	Reso	Differenza	Tot.Costo
P00004902	01	Perossido di idrogeno 10 volumi in soluzione acquosa stabilizzata F.U.				6,00		6,00	2,18
Totali Categoria Prodotti		**PC Prodotto chimico puro**							**2,18**

Tipo PF	PARAFARMACO	Centro 41	S.S.D. Senologia chirurgica		Sub 1	4.6.1 REPARTO			
Codice	ATC-CND	Descrizione Articolo				Scarico	Reso	Differenza	Tot.Costo
F00030763	4	RESOURCE ENERGY 200ML VARI GUSTI				39,00		39,00	42,90
Totali Categoria Prodotti		**PF PARAFARMACO**							**42,90**

Tipo PMC	Presidio Medico Chirurgico	Centro 41	S.S.D. Senologia chirurgica		Sub 1	4.6.1 REPARTO			
Codice	ATC-CND	Descrizione Articolo				Scarico	Reso	Differenza	Tot.Costo
P00004903	01	Clorexidina gluconato o di gluconato al 4% in soluzione acquosa con				2,00		2,00	4,03
P00005531	01	Soluzione o gel a base idroalcolica (concentrazione alcoolica 60-80%) -				4,00		4,00	4,75
Totali Categoria Prodotti		**PMC Presidio Medico Chirurgico**							**8,78**
Totali Sub Centro Costo		**1 4.6.1 REPARTO**							**8.503,57**
Totali Centro di Costo		**41 S.S.D. Senologia chirurgica**							**183.391,81**

COSENZA li 24/01/2014

RIEPILOGHI GENERALI

Riepilogo Generale per METODICA

Codice	Descrizione	Interni Q.ta	Importo	Interni Urgenti Q.ta	Importo	Esterni Q.ta	Importo	Esterni Urgenti Q.ta	Importo	Pronto Soccorso Q.ta	Importo	Totale Q.ta	Importo
2	Ecografia	3	132,21	0	0,00	0	0,00	0	0,00	0	0,00	3	132,21
1	Radiologia	408	6.565,54	7	127,80	0	0,00	0	0,00	0	0,00	415	6.693,34
4	RM	36	8.978,40	0	0,00	0	0,00	0	0,00	0	0,00	36	8.978,40
3	TC	52	7.272,10	3	419,90	0	0,00	0	0,00	0	0,00	55	7.692,00
	Totale Generale	*499*	*22.948,25*	*10*	*547,70*	*0*	*0,00*	*0*	*0,00*	*0*	*0,00*	*599*	*23.495,95*

Maione dirigente S.C. dell'ASP di Catanzaro – Distretto di Lamezia Terme, in sostituzione della dott.ssa Alberta Talarico;

— **di confermare**, quali componenti di cui ai decreti dirigenziali n. 426 del 23 gennaio 2009 e n. 15638 del 12.11.2010:
   - il Dirigente del Servizio "Politica del Farmaco" del Dipartimento Tutela della Salute
   - il dott. De Sarro Giovanbattista ed il dott. Rotiroti Domenico Antonio – Farmacologi delle Università della Regione Calabria
   - la dott.ssa De Francesco Adele (Azienda Ospedaliera Universitaria Mater Domini Catanzaro) – Farmacista Ospedaliera
   - il dott. Anastasio Luigi (Azienda Sanitaria Provinciale di Vibo Valentia) – Medico ospedaliero di area medica
   - il dott. De Nardo Gennaro – Medico di Assistenza Primaria
   - il dott. Grotteria Salvatore – Medico Pediatra di libera scelta

— **di stabilire** che, nelle more della nomina del Dirigente del Servizio Politica del Farmaco in corso di espletamento, è individuato, quale componente regionale del Gruppo di Lavoro, il Dirigente del Settore "Area LEA" del Dipartimento;

Svolgerà le funzioni di Segretario un Funzionario dipendente del Dipartimento Tutela della Salute;

— **di dare atto** che ai componenti non spetta alcun compenso se non il rimborso delle spese di viaggio e di soggiorno derivanti dalla partecipazione ai lavori, debitamente documentate, ad esclusione del personale dipendente dal S.S.R. le cui spettanze saranno erogate dalle Aziende di appartenenza;

— **di pubblicare** il presente decreto sul Bollettino Ufficiale della Regione Calabria, ai sensi della L.R. 19/01, nel suo testo integrale.

Il Dirigente del Settore
Dr. Luigi Rubens Curia

Il Dirigente Generale
Dr. Antonino Orlando

IL DIRIGENTE GENERALE

PREMESSO CHE il Servizio competente per l'istruttoria ha accertato:

- che con delibera di Giunta Regionale n.235 del 13 aprile 2004 è stato istituito il Prontuario Terapeutico Ospedaliero Regionale (PTOR),ai sensi della Legge Regionale n.18/90;

- che con Decreto Dirigenziale n.426 del 23 gennaio 2009 è stato costituito un Gruppo di Lavoro con compiti di revisione e aggiornamento periodico del suddetto PTOR(oggi PTR) secondo criteri di rigore scientifico, di rilevanza clinica e di economicità;

- che con D.D.R. n. 15638 del 12.11.2010 è stato integrato il Gruppo di Lavoro PTR;

TENUTO CONTO

- che il Dott. Bruno Nardo, componente del suindicato Gruppo di Lavoro, non più in servizio presso l'Azienda Ospedaliera di Cosenza, con nota n. 0010212/UOR del 09.04.2013 del Direttore Generale dell'Azienda Ospedaliera di Cosenza è stato sostituito dal dott. Sergio Abonante, dirigente della SSD Senologia Chirurgia dell'Azienda Ospedaliera di Cosenza;

- che la dott.ssa Alberta Talarico, già farmacista dirigente dell'ASP di Catanzaro e posta in quiescenza dal 01.01.2013, anch'essa componente del citato Gruppo di Lavoro, è stata sostituita, in seno allo stesso, dalla dott.ssa Maria Rosaria Maione, farmacista dirigente S.C. dell'ASP di Catanzaro – Distretto di Lamezia Terme;

ATTESA la necessità di modificare il sopracitato Gruppo di Lavoro, procedendo alla sostituzione del dott. Bruno Nardo in quanto non più in servizio presso l'Azienda Ospedaliera Annunziata di Cosenza e della dott.ssa Alberta Talarico posta in quiescenza dal 01.01.2013;

VISTO il D.D.G. n. 7793 del 14 maggio 2010 che individua lo stesso gruppo di Lavoro per la stesura delle linee guida regionali per la prescrizione di categorie di farmaci che maggiormente incidono sulla spesa;

VISTA la L.R. 13.05.1996 n.7 e successive modifiche e integrazioni;
VISTO il D.P.G.R. n.354 del 24.06.1999 e successive modifiche e integrazioni;
VISTA la Legge Regionale n.34 del 2002 e s.m.i. e ritenuta la propria competenza;
VISTI la delibera di G.R. n.634 del28.09.2010 ed il D.P.G.R. n.269 del 15.10.2010;

VISTO il documento istruttorio redatto dal responsabile del procedimento ai sensi e per gli effetti della Legge Regionale n.19/2001;

SU proposta del Dirigente del Settore che, vista l'istruttoria e per le motivazioni espresse nell'atto, attesta la legittimità e la regolarità tecnica del presente provvedimento;

DECRETA

Per quanto espresso in premessa che si intende integralmente riportato:

- **di individuare** quali nuovi componenti del Gruppo di Lavoro per la revisione ed aggiornamento periodico del PTR, già costituito con decreto dirigenziale n.426 del 23 gennaio 2009, il dott. Sergio Abonante, dirigente della SSD Senologia Chirurgia Azienda Ospedaliera Annunziata di Cosenza, in sostituzione del dott. Bruno Nardo e la dott.ssa Maria Rosaria

REGIONE CALABRIA
GIUNTA REGIONALE
Dipartimento Tutela della Salute e Politiche Sanitarie

Dipartimento	13
Settore	3
Servizio	8

DECRETO DIRIGENTE DEL

(ASSUNTO IL) 03 MAG. 2013 *PROT. N.* OO26 8)

CODICE N. _____

" Registro dei decreti dei Dirigenti della Regione Calabria"

N. 6842 DEL _____

07 MAG. 2013

OGGETTO: Decreto Dirigenziale n. 426 del 23 gennaio 2009 - Gruppo di Lavoro Prontuario Terapeutico Regionale – Sostituzione componenti.

Settore Ragioneria
Ai sensi dell'Art.44 della L.R. 4.2.02 n°.8
si esprime parere favorevole in ordine alla
regolarità contabile e, nel contempo, si
attesta che per l'impegno assunto esiste
copertura finanziaria.
Il Dirigente di Settore
Dr.ssa Rosaria Guzzo

Pubblicato sul BURC n.° ..... del ....

Parte _____