



## WKYMVm-induced cross-talk between FPR2 and HGF receptor in human prostate epithelial cell line PNT1A



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### ABSTRACT

**Cross-communication between GPCRs and TKRs represents a mechanism to amplify the information exchange throughout the cell. We show that WKYMVm, an FPR2 agonist, induces the phosphorylation of Y1313/Y1349/Y1356 residues of c-Met and triggers some of the molecular responses elicited by c-Met/HGF binding, such as STAT3, PLC- $\gamma$ 1/PKC $\alpha$  and PI3K/Akt pathways. The critical role of NADPH oxidase-dependent superoxide generation in this cross-talk mechanism is supported by the finding that blockade of NADPH oxidase function prevents c-Met trans-phosphorylation and the downstream signalling cascade. These results highlight the function of FPR2 to activate a interconnected signalling network and suggest novel possibilities for therapeutic interventions.**

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### 1. Introduction

Eukaryotic cells have developed highly efficient mechanisms of receptor-mediated cell communication to coordinate and integrate extracellular signals. The formyl-peptide receptors FPR1, FPR2 and FPR3 belong to the G protein-coupled receptors (GPCR) super-family and are coupled to pertussis toxin (PTX)-sensitive  $G_i$  proteins [1]. FPR1 is efficiently activated by *N*-formyl-methionyl-leucyl-phenylalanine, whereas FPR2 shows a higher binding efficiency for WKYMVm [2]. The expression of these receptors has been demonstrated in several cell types [3] and their important biological functions are supported by the identification of high affinity host-derived agonists. [4–6].

Despite GPCRs lack intrinsic tyrosine kinase activity, tyrosine phosphorylation of a tyrosine kinase receptor (TKR) occurs in response to binding of specific agonists of several such receptors. GPCR ligands increase tyrosine phosphorylation of TKRs either by increasing the kinase activity or by inhibiting an associated protein tyrosine phosphatase [7]. These events are mediated by reactive oxygen species (ROS) whose concentration increases transiently in cells stimulated with GPCR agonists. Several evidence support the role of ROS in TKR transactivation. For instance, in human lung

cancer cells stimulation of FPR2 by WKYMVm induces ROS-dependent EGFR tyrosine phosphorylation [8] and in human carcinoma cells ROS mediate c-Met transactivation by GPCRs [9]. The deliberated and regulated generation of ROS is catalyzed by enzymes that belong to NADPH oxidase (Nox) family, constituted by membrane and cytosolic components. Phosphorylation of the regulatory cytosolic protein p47<sup>phox</sup> on several serine residues is associated with oxidase activation [10].

The c-Met tyrosine kinase receptor (c-Met) is a cell surface receptor for hepatocyte growth factor (HGF) [11]. Following HGF binding, the tyrosine kinase domain of c-Met undergoes trans-phosphorylation on the Y1234 and Y1235 residues in the activation loop [12]. Kinase activation by autophosphorylation leads to the subsequent phosphorylation of Y1349 and Y1356 residues in the C-terminal multifunctional docking site, resulting in the activation of c-Met signalling [12]. Previously, we demonstrated that PNT1A cells express a functional c-Met and cell exposure to NK1, a splice variant of HGF, induces the phosphorylation of Y1313/Y1349/Y1356 residues of c-Met which provide docking sites for the activation of intracellular signalling pathways [13].

The aims of this study were to analyze the cross-talk between FPR2 and c-Met in PNT1A cells and to identify intracellular signalling cascades triggered by the WKYMVm-mediated activation of HGF receptor. We show that stimulation of FPR2 by its agonist results in the phosphorylation of tyrosine 1313/1349/1356 residues of c-Met and promotes some of the molecular responses elicited by the binding of HGF to its receptor.

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2. Materials and methods

2.1. Antibodies and chemicals

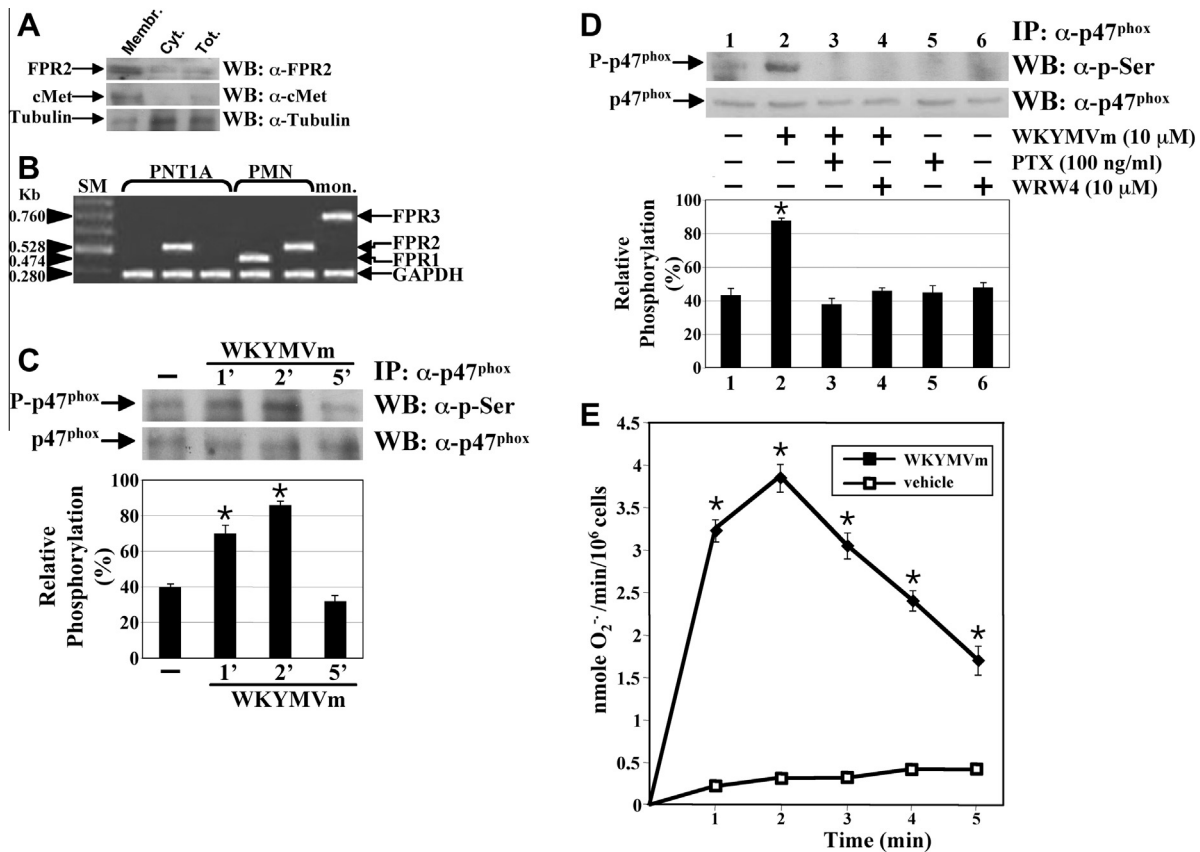
The WKYMVm and WRWWWW (WRW4) peptides were synthesized and HPLC-purified by PRIMM (Milan, Italy). SDS-PAGE reagents were from Bio-Rad (Hercules, CA, USA). Protein A/G Plus agarose, anti-active phosphorylated ERK1/2, anti-tubulin, anti-p47<sup>phox</sup>, anti-p22<sup>phox</sup>, anti-FPR2, anti-cMet, anti-STAT3, anti-cyclin A, anti-p-Y, anti-phospho-cMet(Y1313), anti-phospho-cMet(Y1349), anti-PLC- $\gamma$ 1, anti-phospho-PLC- $\gamma$ 1, anti-PKC $\alpha$ , anti-PKC $\delta$ , anti-PKC $\epsilon$  and anti-rabbit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-STAT3(-Tyr705), anti-phospho-STAT3(Ser727), anti-phospho-PI3K(p85) and anti-phospho-Akt(S473) were from Cell Signalling Technology (Danvers, MA, USA). Anti-phospho-cMet(Y1356) was purchased from Abnova (Walnut, CA, USA). Protein A-horseradish peroxidase and anti-mouse Ig-horseradish peroxidase were from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). PD098059 and SU11274 were from Calbiochem (La Jolla, CA, USA). Anti-phospho-Ser antibody, PTX, apocynin, phorbol 12-myristate 13-acetate (PMA), Wortmannin and LY294002 were from Sigma (St. Louis, MO, USA). p22<sup>phox</sup> siRNA (SI03078523) and negative control siRNA (SI03650318) were purchased from Qiagen (Hiden, Germany). c-Met siRNA (L-003156-00), FPR2 siRNA (L-005140-00) and a negative scrambled control (D-001810-10) were obtained from Dharmacon (Lafayette, CO, USA).

2.2. Cell culture

PNT1A and primary prostate epithelial cells were purchased from ATCC (Rockville, MD, USA) and were grown in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 1% L-glutamine. Cells were cultured until they reached 80% confluence, starved in serum-free RPMI for 24 h and stimulated with WKYMVm at the final concentration of 10  $\mu$ M for different times, as indicated in the figures. In other experiments, serum-deprived cells were preincubated with 50  $\mu$ M PD098059 for 90 min, or 100 ng/ml PTX for 16 h, or 2  $\mu$ M SU11274 for 16 h, or 50  $\mu$ M LY294002 for 1 h, or 0.5  $\mu$ M Wortmannin for 1 h, or 100  $\mu$ M apocynin for 2 h, or 10  $\mu$ M WRW4 for 15 min, before stimulation with 10  $\mu$ M WKYMVm for 2 min. In short interfering RNA experiments  $4 \times 10^5$  cells were incubated for 12 h with 5 nM siRNAs in RPMI containing 10% FBS in the presence of 20  $\mu$ l HiPerFect (Qiagen, Hiden, Germany). Cells were then serum-deprived for 24 h and stimulated with WKYMVm. PNT1A cells were also stimulated with 10 ng/ml PMA for different times as indicated in the figure.

2.3. Western blot and immunoprecipitation analysis

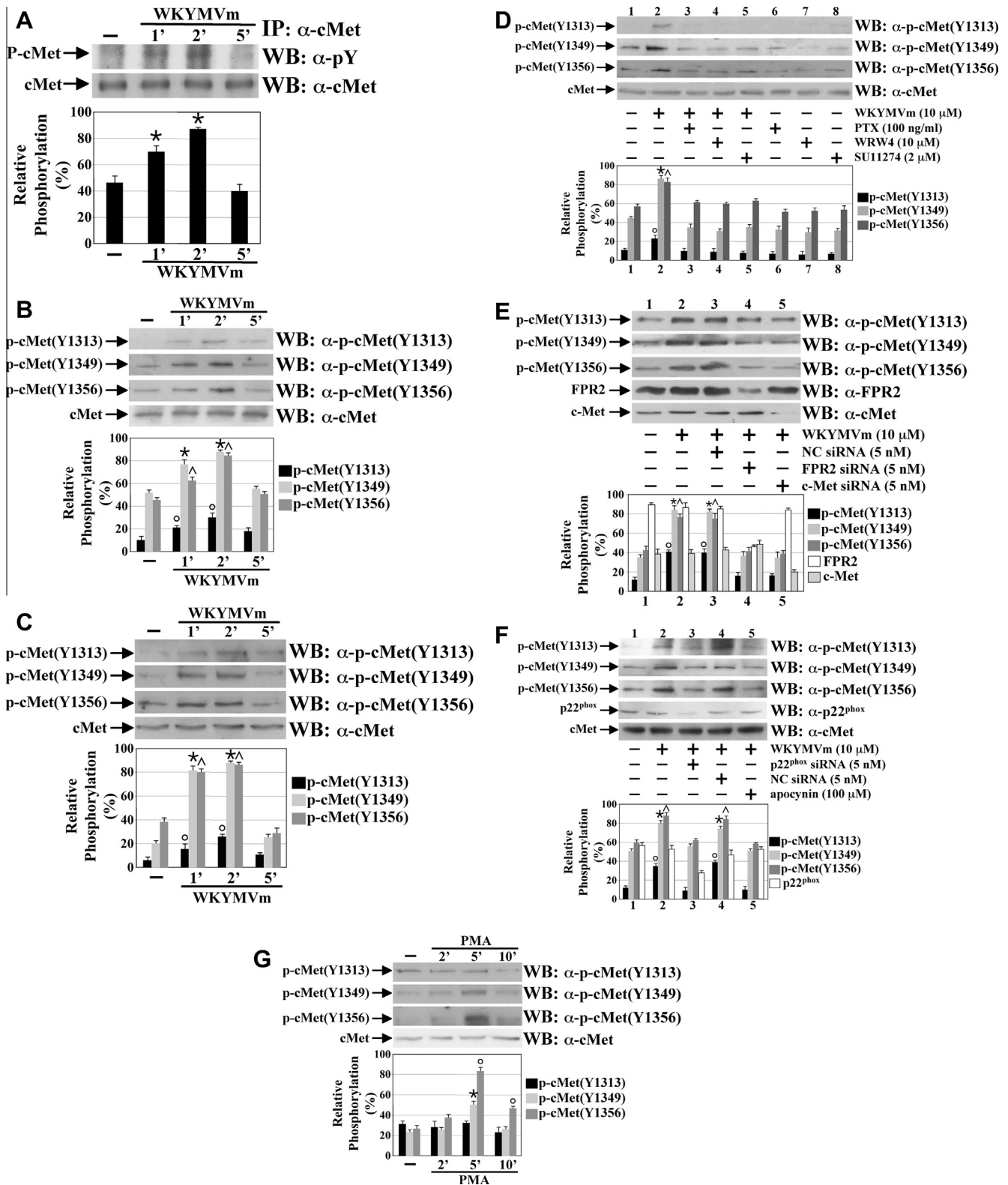
Cells were stimulated with WKYMVm in the presence or absence of the appropriate amount of specific inhibitors and rinsed with cold phosphate-buffered saline. Cell lysates were purified as previously described [8] and proteins concentration was determined by a Bio-Rad protein assay (BioRad, Hercules, CA, USA). Nu-



**Fig. 1.** FPR2 is a functional receptor in PNT1A cells. (A) Membrane (Membr.), cytoplasmic (Cyt.) and whole (Tot.) lysates (50  $\mu$ g) were analyzed by western blot with an  $\alpha$ -FPR2 antibody. (B) cDNAs from PNT1A cells, PMN and monocytes (mon.) were coamplified by using FPRs and GAPDH primers; (C) Cell were exposed to WKYMVm for the indicated times, or (D) preincubated with PTX or WRW4 before stimulation. Whole lysates (1 mg) were immunoprecipitated with an  $\alpha$ -p47<sup>phox</sup> antibody and p47<sup>phox</sup> serine phosphorylation was detected by using an  $\alpha$ -p-Ser antibody. An  $\alpha$ -p47<sup>phox</sup> antibody served as a control for protein loading. (E) Superoxide production was determined as the SOD-sensitive rate of reduction of cytochrome c. \*P < 0.05 compared with unstimulated cells.

clear proteins and membrane proteins purification were performed with a Qproteome kit (Qiagen, Hiden, Germany) and with a MemPER® Kit (Thermo Scientific, Rockford, USA), respectively, according

to the manufacturer's instructions. Western blot analysis was performed as previously described [8]. The expression of targeted proteins was detected by an ECL chemiluminescence reagent kit



**Fig. 2.** FPR2 activation results in c-Met trans-phosphorylation. (A) Whole lysates (800  $\mu$ g) from PNT1A cells were immunoprecipitated with an  $\alpha$ -cMet antibody and c-Met tyrosine phosphorylation level was detected with an  $\alpha$ -pY antibody. PNT1A (B) or primary prostate epithelial cells (C) were stimulated with WKYMVm for the indicated times. (D) PNT1A cells were preincubated with PTX or WRW4 or SU11274, or (E) with siRNAs against FPR2 (FPR2 siRNA) or c-Met (c-Met siRNA), or (F) with a siRNA against p22<sup>phox</sup> (p22<sup>phox</sup> siRNA) or with apocynin, before stimulation. A negative control siRNA (NC siRNA) was included in the experiments. (G) PNT1A cells were stimulated with PMA for the indicated times. Whole lysates (50  $\mu$ g) were subjected to immunoblotting analysis and phosphorylation of Y1313/Y1349/Y1356 residues of c-Met was detected with  $\alpha$ -p-cMet(1313),  $\alpha$ -p-cMet(1349) or  $\alpha$ -p-cMet(1356) antibodies. \* $P$  < 0.05, <sup>o</sup> $P$  < 0.05 and <sup>^</sup> $P$  < 0.05 compared with unstimulated cells.

(Amersham Pharmacia Biotech) and visualized by autoradiography. In immunoprecipitation experiments, cell lysates containing equal amounts of proteins were incubated with 3 µg of either anti-cMet or anti-p47<sup>phox</sup> antibody [8]. Proteins were resolved on a 10% SDS-PAGE and phosphorylated protein levels were quantitatively estimated by densitometry using a Discover Pharmacia scanner.

2.4. Assay of superoxide production and RT-PCR analysis

Superoxide production assay, RNA purification and RT-PCR analysis were performed as previously described [8]. The following primers were designed to amplify human FPRs coding regions: (FPR1) 5'-GACCACAGCTGGTGAACAGT-3' and 5'-GATGCAGGACGCAACACAG-3'; (FPR2) 5'-GGATTTCACCCACTGCATTT-3' and 5'-ATCCAAGTCCGACGATCAC-3'; (FPR3) 5'-GAGTTGTCCACAGGAA TCCA-3' and 5'-ATAGGCACGCTGAAGCCAAT-3'. These primers generate 474, 528 and 760 bp fragments, respectively.

2.5. Statistical analysis

All the presented data are expressed as means ± S.D. and are representative of three or more independent experiments. Statistical analyses were assessed by Student's t test for paired data. Results were considered significant at P < 0.05.

3. Results

3.1. PNT1A cells express a functional FPR2 receptor

We first analyzed the expression of FPR2 in membrane, cytoplasmic and total proteins purified by PNT1A cells. We detected

the presence of the band corresponding to FPR2 protein at the expected molecular size on membrane extracts by using an α-FPR2 antibody (Fig. 1A). By RT-PCR we detected FPR2 but not FPR1 and FPR3, providing the first evidence of FPR2 expression in these cells. (Fig. 1B). In IMR90 fibroblasts and in human lung cancer cells, stimulation of FPR2 with 10 µM WKYMVm induces p47<sup>phox</sup> phosphorylation, which is considered the key event for NADPH oxidase-dependent superoxide generation [8,14]. In immunoblot experiments we observed that p47<sup>phox</sup> results phosphorylated on serine residues within the first 2 min of stimulation (Fig. 1C) and that preincubation with PTX or with the FPR2 antagonist WRW4 peptide, significantly prevents p47<sup>phox</sup> serine phosphorylation (Fig. 1D). Furthermore, stimulation of FPR2 by WKYMVm induces NADPH oxidase-dependent superoxide generation with maximal production occurring at 2 min (Fig. 1E), indicating that FPR2 is a biologically functional receptor in PNT1A cells.

3.2. FPR2 activation promotes the phosphorylation of Y1313/Y1349/Y1356 residues of c-Met

Cross-talk between GPCRs and TKRs plays an instrumental role in orchestrating downstream signalling molecules. We analyzed the ability of FPR2 to transactivate c-Met and, in immunoblot experiments, we observed that stimulation with WKYMVm induces the time-dependent phosphorylation of the HGF receptor (Fig. 2A). In particular, the FPR2 agonist promotes the phosphorylation of Y1313/Y1349/Y1356 residues of c-Met within the first 2 min which decreases after 5 min of stimulation, both in PNT1A (Fig. 2B) and primary prostate epithelial cells (Fig. 2C). Furthermore, preincubation of PNT1A cells with the c-Met inhibitor SU11274 or PTX or WRW4 (Fig. 2D), or with siRNAs against FPR2

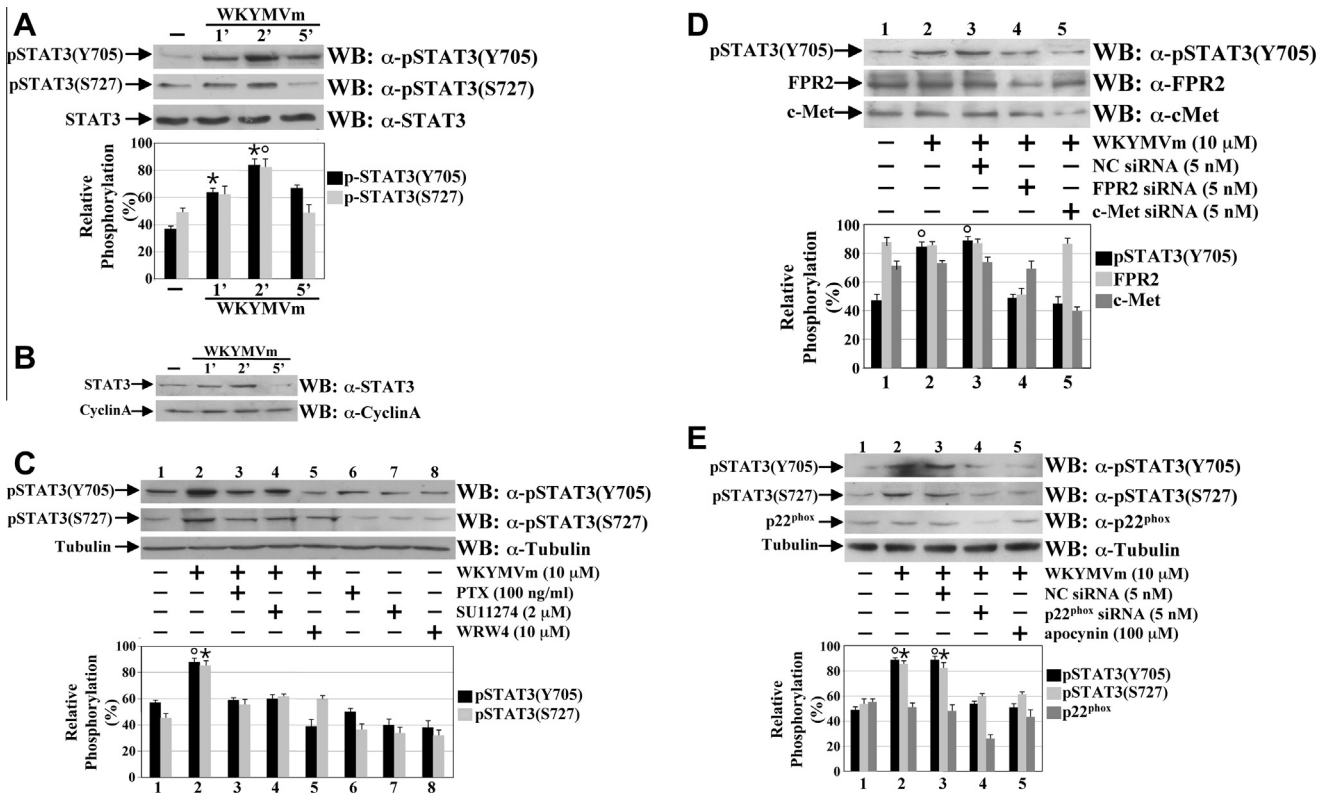


Fig. 3. FPR2 activation triggers STAT3 pathway. (A) PNT1A cells were stimulated for the indicated times with WKYMVm and specific phosphorylation of STAT3 was detected with α-pSTAT3(Y705) or α-pSTAT3(S727) antibodies. An α-STAT3 antibody served as a control for protein loading. (B) STAT3 nuclear translocation was analyzed on nuclear extracts (50 µg) with an α-STAT3 antibody. The same filter was reprobbed with an α-cyclin A antibody. (C) Cells were preincubated with PTX or SU11274 or WRW4, or (D) with FPR2 siRNA or c-Met siRNA, or (E) with a p22<sup>phox</sup> siRNA or apocynin, before WKYMVm stimulation. A NC siRNA was included in the experiments. Proteins (50 µg) were subjected to immunoblotting analysis with α-p-STAT3(Y705) or α-p-STAT3(S727) antibodies. \*P < 0.05 and \*P < 0.05 compared with unstimulated cells.

or c-Met (Fig. 2E), before WKYMVm stimulation, results in a significant reduction in the phosphorylation level of these tyrosines.

Oxidation and/or reduction of cysteine sulfhydryl groups of phosphotyrosine phosphatases (PTPases) tightly controls the activity of TKRs. Nox family is considered the main cytosolic source of ROS [10] which can contribute to TKR transactivation by inhibiting a PTPase activity [15] and, in turn, shifting the equilibrium state of TKR from non-phosphorylated to phosphorylated. We preincubated cells with the NADPH-oxidase-specific inhibitor apocynin or with a siRNA against p22<sup>phox</sup> before WKYMVm stimulation, and we observed that blockade of NADPH oxidase function prevents FPR2-induced phosphorylation of Y1313/Y1349/Y1356 residues of c-Met (Fig. 2F).

We also incubated cells with PMA, an oxidase inducer, and we observed that it promotes the phosphorylation of Y1349 and Y1356 residues, but not of Y1313 residue, only after 5 min and with a kinetic different from that induced by FPR2 stimulation (Fig. 2G). This suggests that FPR2-mediated ROS production plays a crucial role in this cross-talk mechanism.

### 3.3. FPR2/c-Met cross-talk triggers STAT3 pathway

The single multifunctional docking site located in the C-terminus of the HGF receptor contains the sequence Y<sup>1349</sup>VHVNATY<sup>1356</sup>VNV, which provides binding sites for a variety of SH2-containing signal transducers and effectors [16]. STAT3 binds to the sequence following the phosphorylated Y<sup>1356</sup> residue [17] and its association with c-Met results in the phosphorylation of a conserved tyrosine residue (Y705), which is required for promoting the dimerization of STAT3. Activated STAT3 is then translocated in the nucleus where it acts as a transcriptional factor. Full transcriptional activity and DNA binding capacity are manifested only when the serine 727 residue of STAT3 is also phosphorylated.

In time-dependent western blot experiments, we observed that WKYMVm induces the rapid phosphorylation of Y705 and S727 residues of STAT3 (Fig. 3A), as well as the nuclear translocation of activated STAT3 (Fig. 3B). We also preincubated cells with PTX or WRW4 or SU11274 (Fig. 3C), or with siRNAs against FPR2 or c-Met (Fig. 3D), and we observed that these treatments prevent the WKYMVm-induced activation of STAT3. Furthermore, we blocked NADPH oxidase function by pretreating cells with apocynin or with a siRNA against p22<sup>phox</sup> and we observed that FPR2-induced phosphorylation of Y705 and S727 residues of STAT3 was prevented (Fig. 3E).

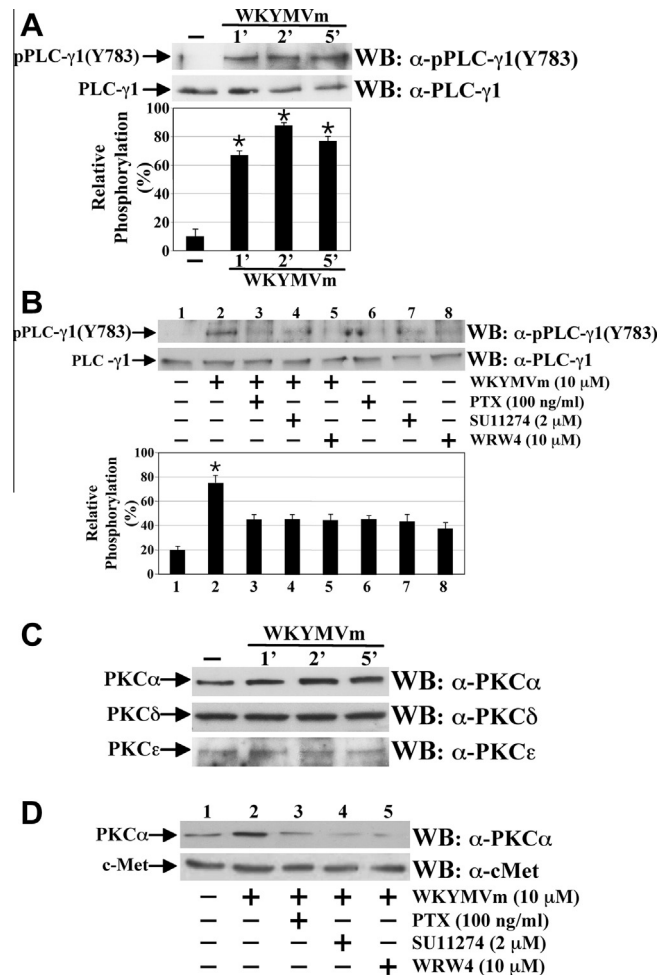
### 3.4. FPR2-induced c-Met trans-phosphorylation generates specific docking sites for PLC- $\gamma$ 1

Signal transducers that bind sequences surrounding tyrosines 1349 and 1356 residues in c-Met can interact with the receptor either directly or indirectly through the scaffolding protein Gab1, which represents the key coordinator of the cellular responses to c-Met [18]. After the interaction with the receptor, Gab1 becomes phosphorylated on several tyrosine residues that, in turn, recruit a number of signalling effectors, including PLC- $\gamma$ 1 which can weakly bind c-Met also directly [18]. We analyzed PLC- $\gamma$ 1 activation in WKYMVm-stimulated PNT1A cells and we observed in time-course experiments that the FPR2 agonist induces PLC- $\gamma$ 1 activation with maximal phosphorylation of Y783 residue occurring at 2 min (Fig. 4A). PLC- $\gamma$ 1(Y783) phosphorylation is prevented by preincubation with PTX or WRW4 or SU11274, before WKYMVm stimulation (Fig. 4B). We also analyzed the PKC isoforms activated as a consequence of the hydrolysis of phosphatidylinositol-4-5-bisphosphate by PLC- $\gamma$ 1, by analyzing the cellular partitioning of PKC isozymes in growth-arrested and WKYMVm-stimulated cells. In response to the FPR2 agonist, of the seven PKC isoenzymes that

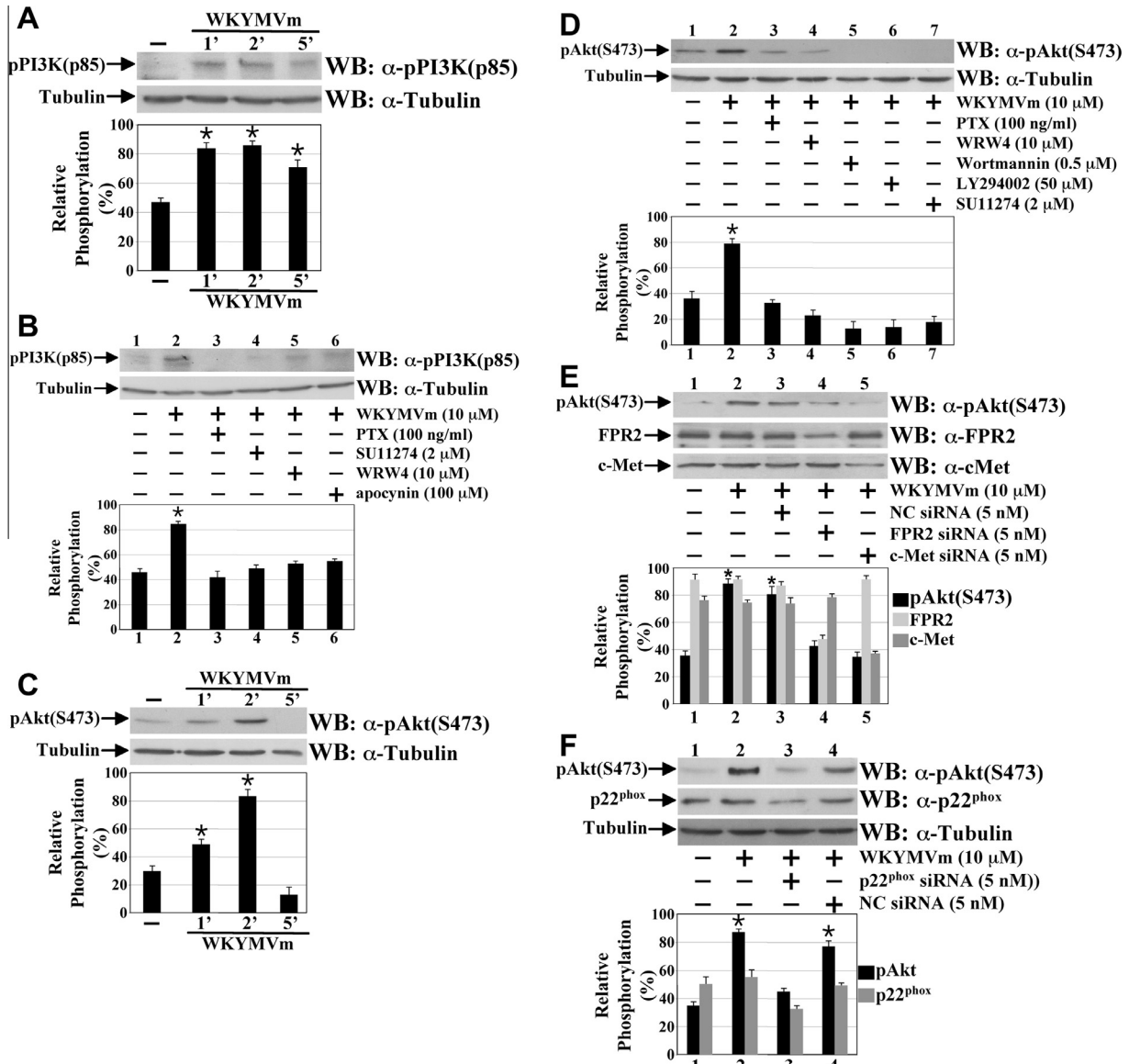
we examined (data not shown) only PKC $\alpha$  translocates to the membrane fraction and a significant increase in the amount was detected within 2 min of exposure to WKYMVm (Fig. 4C). In contrast, no translocation of PKC $\delta$  and PKC $\epsilon$  was observed (Fig. 4C). Furthermore, the G<sub>i</sub> protein specific inhibition, or a specific FPR2 antagonist, or an inhibitor of c-Met kinase activity, completely prevents PKC $\alpha$  activation (Fig. 4D), suggesting that it depends on FPR2-dependent c-Met phosphorylation.

### 3.5. FPR2-induced activation of the multifunctional docking site of c-Met triggers PI3K/Akt pathway

Nine domains of Gab1 containing a single tyrosine are phosphorylated in vitro by HGF and three of these tyrosines (Y458/Y473/Y590) bind the p85 regulatory subunit of PI3K [18]. In HGF receptor there is also another recognition motif (Y<sup>1313</sup>EVM) which can represent a potential binding site for PI3K [19]. In immunoblotting experiments we observed that WKYMVm induces PI3K(p85) phosphorylation within 2 min of stimulation (Fig. 5A). This is prevented by pretreating cells with PTX, or SU11274, or WRW4, or apocynin (Fig. 5B). PI3K is the key component for the



**Fig. 4.** FPR2-induced c-Met transactivation generates specific docking sites for PLC- $\gamma$ 1. (A) Proteins were purified from PNT1A cells exposed to WKYMVm for the indicated times, or (B) preincubated with PTX or SU11274 or WRW4, before stimulation. Fifty micrograms of lysates were analyzed with an  $\alpha$ -pPLC- $\gamma$ 1(Y783) antibody. (C) Translocation of PKC isoforms was analyzed on membrane extracts (30  $\mu$ g) with  $\alpha$ -PKC $\alpha$  or  $\alpha$ -PKC $\delta$  or  $\alpha$ -PKC $\epsilon$  antibodies. (D) Cells were preincubated with PTX, or SU11274 or WRW4 before stimulation and 30  $\mu$ g of purified membrane proteins were analyzed with an  $\alpha$ -PKC $\alpha$  antibody. The same filter was reprobbed with an  $\alpha$ -cMet antibody.



**Fig. 5.** FPR2/c-Met cross-talk triggers the activation of PI3K/Akt pathway. (A and C) Cells were exposed with WKYMVm for the indicated times or (B) preincubated with PTX or SU11274 or WRW4 or apocynin, or (D) with PTX or WRW4 or Wortmannin or LY294002 or SU11274, or (E) with FPR2 siRNA or c-Met siRNA, or (F) with a p22<sup>phox</sup> siRNA, before stimulation. A NC siRNA was included in the experiments. Proteins (50 μg) were analyzed with (A and B) an α-pPI3K(p85) antibody, or (C, D, E and F) with an α-pAkt(S473) antibody. An α-Tubulin antibody was used as a control of protein loading. \*P < 0.05 compared with unstimulated cells.

activation of Akt signalling. In the PI3K/Akt pathway, formation of 3-phosphoinositides by PI3K enables the activation of Akt by phosphoinositide-dependent protein kinases 1 and 2, which phosphorylate Akt at threonine 308 and serine 473 residues, respectively. We analyzed PI3K(p85) activity by analyzing Akt phosphorylation in response to FPR2 stimulation and the results showed that WKYMVm induces Akt(S473) phosphorylation within the same time frame as PI3K(p85) phosphorylation (Fig. 5C). The preincubation of cells with highly selective PI3K inhibitors or PTX or WRW4 or SU11274 (Fig. 5D), or with siRNAs against FPR2 or c-Met (Fig. 5E), or with a siRNA against p22<sup>phox</sup> (Fig. 5F), significantly prevents WKYMVm-induced Akt(S473) phosphorylation.

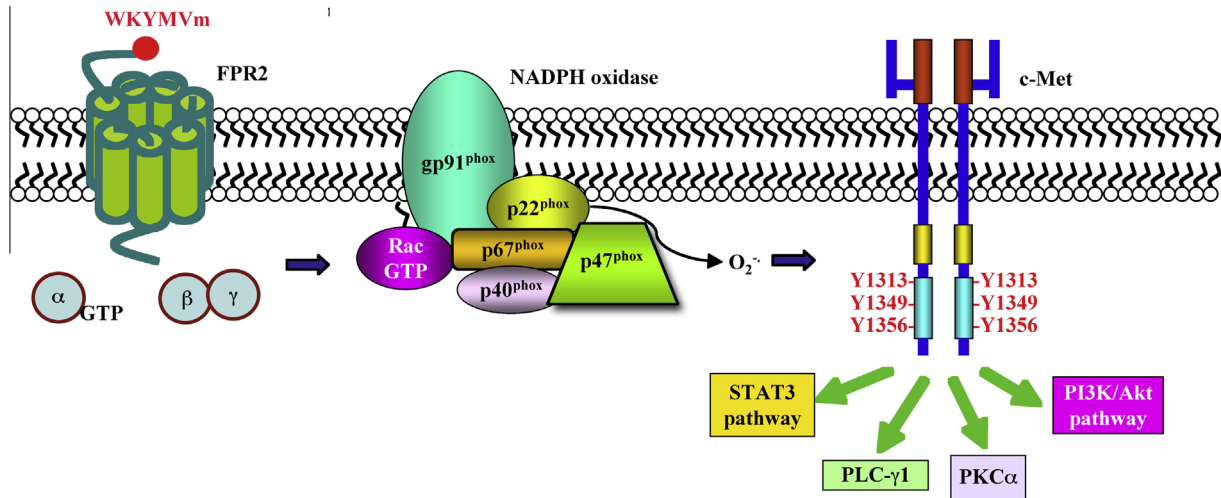
**4. Discussion**

In this study, we demonstrate that FPR2 activation results in the phosphorylation of critical tyrosines within the single multisubstrate docking site of the HGF receptor, and that superoxide gener-

ation by NADPH oxidase plays a crucial role in the transphosphorylation mechanism. We also show that these phosphotyrosines provide binding sites for a variety of SH2-containing signal transducers, promoting some of the molecular responses triggered by the binding of HGF to its receptor (Fig. 6).

In fact, we observed STAT3 activation which, in response to HGF stimulation, binds to the sequence following Y<sup>1356</sup> residue of c-Met and is required for branching morphogenesis. Our results show that STAT3 activation is prevented by blockade of FPR2, c-Met or Nox functions, suggesting that it is a part of the FPR2-dependent signalling cascade and depends on Nox activity.

Phosphotyrosines 1349 and 1356 of c-Met provide docking sites for the interaction with Shc, Src and Gab1, while Y<sup>1356</sup> residue is mainly responsible for recruitment of Grb2, PI3K and PLC-γ to the HGF receptor signalling complex [18]. Phosphorylated PLC-γ1 (Y771/Y783/Y1254 residues) is associated to HGF receptor via Gab1, and this interaction is required for HGF-mediated tubulogenesis [20]. We show that WKYMVm-induced FPR2/c-Met cross-



**Fig. 6.** Schematic representation of the WKYMVm-induced cross-talk between FPR2 and c-Met and of the critical role of superoxide generation by NADPH oxidase in the trans-phosphorylation mechanism.

talk promotes the time-dependent phosphorylation of Y783 residue of PLC- $\gamma$ 1 and, in turn, the activation of PKC $\alpha$  which is implicated in many cellular functions elicited by GPCR activation.

The activation of PI3K/Akt pathway is considered the main signalling cascade required for the induction of cell scattering and EMT [13]. Tyrosine 1313 residue of c-Met, within the Y<sup>1313</sup>EVM motif, is a potential binding site for PI3K [19] and Y<sup>1356</sup> residue is primarily responsible for recruitment of PI3K to c-Met signalling complex [18]. We show that FPR2-mediated c-Met activation induces the time-dependent phosphorylation of PI3K(p85) and Akt, and that siRNAs against FPR2, or c-Met, or p22<sup>phox</sup> completely prevents Akt activation, suggesting that the activation of PI3K/Akt pathway depends on FPR2/c-Met cross-talk and NADPH oxidase activity.

Taken together these results suggest that in PNT1A cells FPR2/c-Met cross-communication promotes the phosphorylation of critical tyrosine residues involved in many of the biological responses triggered by the HGF receptor. This results in the formation of a structural scaffold for the assembly of a signalling complex that resembles that formed in response to interaction of c-Met with its ligand. The elucidation of the intracellular signal transduction pathways that mediate the biological effects induced by FPR2 agonists is of major importance because these studies may identify potential targets and suggest novel strategies for therapeutic interventions.

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