Sphingosine 1-phosphate signaling axis mediates neuropeptide S-induced invasive phenotype of endometriotic cells

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Endometriosis is a chronic gynecological syndrome characterized by endometrial cell invasion of the extra-uterine milieu, pelvic pain and infertility. Treatment relies on either symptomatic drugs or hormonal therapies, even though the mechanism involved in the onset of endometriosis is yet to be elucidated. The signaling of sphingolipid sphingosine 1-phosphate (S1P) is profoundly dysregulated in endometriosis. Indeed, sphingosine kinase (SK) 1, one of the two isoenzymes responsible for S1P biosynthesis, and $S1P_1$, $S1P_3$ and $S1P_5$, three of its five specific receptors, are more highly expressed in endometriotic lesions compared to healthy endometrium. Recently, missense coding variants of the gene encoding the receptor 1 for neuropeptide S (NPS) have been robustly associated with endometriosis in humans. This study aimed to characterize the biological effect of NPS in endometriotic epithelial cells and the possible involvement of the S1P signaling axis in its action. NPS was found to potently induce cell invasion and actin cytoskeletal remodeling. Of note, the NPS-induced invasive phenotype was dependent on SK1 and SK2 as well as on $S1P_1$ and $S1P_3$, given that the biological action of the neuropeptide was fully prevented when one of the two biosynthetic enzymes or one of the two selective receptors was inhibited or silenced. Furthermore, the RhoA/Rho kinase pathway, downstream to S1P receptor signaling, was found to be critically implicated in invasion and cytoskeletal remodeling elicited by NPS. These findings provide new information to the understanding of the molecular mechanisms implicated in endometriosis pathogenesis, establishing the rationale for non-hormonal therapeutic targets for its treatment.

Introduction

Endometriosis is a benign gynecological inflammatory syndrome with chronic and debilitating symptoms. From 6% to 10% of women of reproductive age are estimated to be affected and the prevalence peaks up to 50% among infertile women. Endometriosis is defined by the presence of endometrial-like tissue outside the uterus due to endometrial cell invasion of the extra-uterine tissues [1]. The most accepted theory

Abbreviations
BSA, bovine serum albumin; CIB1, calcium and integrin binding protein 1; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; F12, Ham's F-12; FBS, fetal bovine serum; NPS, neuropeptide S; NPSR1, neuropeptide S receptor 1; RhoA, Ras homolog family member A; ROCK, Rho kinase; ROS, reactive oxygen species; RT-PCR, real-time polymerase chain reaction; S1P, sphingosine 1-phosphate; S1PRs, sphingosine 1-phosphate receptors; SK, sphingosine kinase; SPL, sphingosine 1-phosphate lyase; Spns2, spinster homolog 2; SPP, sphingosine 1-phosphate phosphatase.

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regarding endometriosis pathogenesis describes the shedding of endometrial tissue through fallopian tubes according to the retrograde menstruation theory of John Sampson [2]. Endometriosis shares numerous features with metastatic cancer, especially the invasiveness of endometrial cells once they reach the extra-uterine environment. Furthermore, actin cytoskeletal remodeling of endometriotic cells is fundamental for the adhesion and the invasion of the mesothelial barrier [3] and to colonize peritoneual surfaces [4]. Current endometriosis treatments mostly rely on surgery and hormonal therapies with oral contraceptive pills (estro-progestin combination), progestins, gonadotropin-releasing hormone agonists or antagonists and aromatase inhibitors [5,6]. Endometriosis, in fact, is characterized by an impairment in sex steroid hormones balance, with increased estrogen sensitivity, progesterone resistance, along with inflammation. Moreover, painkillers drugs (e.g., non-steroidal anti-inflammatory drugs) are needed to control symptoms [7]. Since therapy relies exclusively on either hormonal or symptomatic drugs and has prominent side effects, the elucidation of the complex molecular mechanisms involved in the pathogenesis of endometriosis is an unmet need in order to identify innovative non-hormonal pharmacological targets. Recently, sphingolipids have emerged as new leading actors in endometriosis, in particular sphingosine 1-phosphate (S1P) is clearly involved in the onset of the disease [8–14]. S1P is a powerful bioactive molecule that regulates many cellular and tissue responses such as inflammation, neurogenesis, cell survival, migration, and tumorigenesis [15]. S1P is generated by sphingosine kinases (SK1 and SK2) that catalyze sphingosine phosphorylation, while its degradation can occur via two distinct pathways: the irreversible breakdown catalyzed by S1P lyase (SPL) and the reversible dephosphorylation brought about by S1P phosphatases (SPP1 and SPP2) [16]. Although S1P metabolism takes place inside the cells, the pleiotropic effects of the sphingolipid in many instances rely on the so-called "inside-out" mechanism of action, that implies ligation to a family of five specific G-protein coupled receptors ($S1P_{1-5}$) subsequently to the export into the extracellular microenvironment mediated by the specific transporter spinster homolog 2 (Spns2) or the unselective ABC transporters [17].

Recently, a robust link between gene variants encoding neuropeptide S receptor 1 (NPSR1), a G-protein coupled receptor, and endometriosis has been reported in humans [18]. In particular, missense coding variants of the NPSR1 gene, found to be expressed in the glandular epithelium of eutopic and ectopic endometrium, are significantly associated with stage III and IV endometriosis. Interestingly, blockade of NPSR1 with the selective antagonist SHA68 reduced monocyte migration as well as pain and inflammation in endometriotic mouse models [18], underlining a new possible non-hormonal therapeutic target for endometriosis. However, the biological action of NPS in endometriotic cells and the molecular mechanism implicated are presently unknown.

Here, the possible modulation of invasion and cytoskeletal remodeling by NPS in endometriotic epithelial 12Z cells has been investigated. Moreover, the involvement of S1P signaling axis in the biological action exerted by the neuropeptide was examined.

Results

NPS stimulates cell invasion and actin cytoskeletal remodeling of epithelial endometriotic cells

In order to characterize the biological effect of NPS in endometriotic cells, Boyden chamber experiments were performed to evaluate the pro-invasive action of the neuropeptide in human endometriotic epithelial 12Z cells. Results illustrated in Fig. 1A showed that the treatment with 100 nM NPS for 6 h potently stimulated cell invasion (approx. 3-fold). In addition, the possible modulation of cytoskeletal architecture induced by NPS was evaluated by staining F-actin filaments employing TRITC-phalloidin. Confocal microscopy images showed that cell treatment with 100 nM NPS for 45 min induced a strong increase of fluorescence associated to F-actin as well as profound changes in cytoskeletal structure, due to increased thickening, bundling, and overall organization of microfilaments (Fig. 1B).

NPS activates SK1 and SK2 in endometriotic epithelial cells

Given that the metabolism and signaling of the bioactive lipid S1P are profoundly dysregulated in endometriosis [10,12,19] and NPSR1 has been proposed as innovative non-hormonal target for the disease [18], we investigated the involvement of S1P signaling axis in NPS-induced biological effects in endometriotic cells. We first examined whether human endometriotic epithelial 12Z cells express the enzymes involved in S1P metabolism and the S1PRs. RT-PCR analysis revealed both biosynthetic enzyme isoforms SK1 and SK2 along with the catabolic enzymes SPL, SPP1 and SPP2, at mRNA level (Fig. 2A). In addition, endometriotic cells expressed the SK1-activating protein CIB1 (calcium and integrin binding protein 1),

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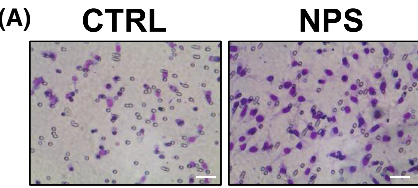

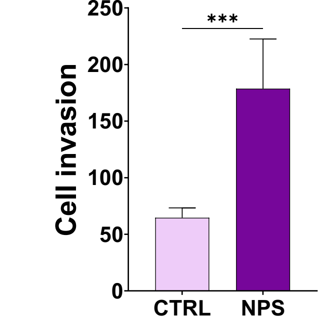

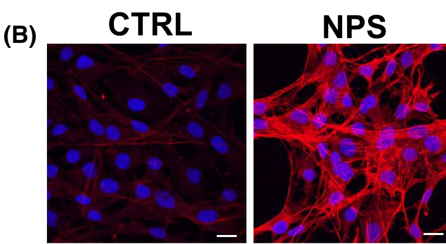

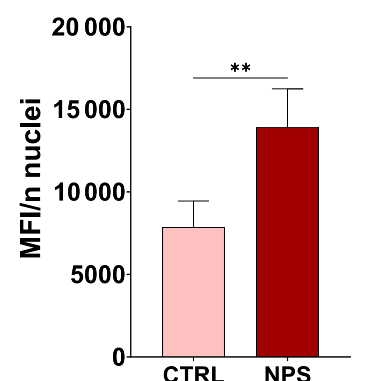

Fig. 1. NPS stimulates endometriotic epithelial cell invasion. (A) Cell invasion across Geltrex-coated porous membranes of Boyden chambers was determined in endometriotic epithelial cells challenged with 100 nM NPS for 6 h. Scale bar: 25 μm. Data are mean ± SD of three independent experiments performed in triplicate. NPS induced cell invasion of endometriotic epithelial cells in a statistically significant manner (Student's *t*-test, $***P < 0.001$). (B) F-Actin polymerization was determined in endometriotic epithelial cells seeded on matrix-coated glass coverslips and treated with 100 nM NPS for 45 min before being fixed and stained with TRITC-labeled phalloidin and DAPI. Scale bar: 10 μm. The histogram represents the quantification of the intensity of TRITC-labeled phalloidin fluorescence normalized to the number of nuclei. Data are mean ± SD of eight fields quantified in three independent experiments. NPS increases phalloidin fluorescence in a statistically significant manner, Student's *t*-test ($**P < 0.01$). MFI, mean fluorescence intensity.

all the specific receptor isoforms, $S1P_{1–5}$, as well as the selective transporter Spns2 (Fig. 2A). Next, we investigated whether the neuropeptide was capable to activate SKs in endometriotic cells. Since SK1 and SK2 activation and translocation to the plasma membrane is dependent on their phosphorylation [20,21], western blot analysis using specific anti-phospho-SK1 or anti-phospho-SK2 antibodies was performed as readout of their activation. Data reported in Fig. 2B showed that the treatment with 100 nM NPS rapidly and transiently augmented the phosphorylation of SK1 and SK2 starting from 5 and 15 min of treatment, respectively. In agreement, Western blot analysis performed in membrane fractions showed that the treatment with 100 nM NPS for 15 min significantly enhanced the amount of SK1 and SK2 associated to the membrane, thus increasing the extent of the enzymes with a favorable access to the hydrophobic substrate sphingosine, exclusively available at membrane compartment (Fig. 2C).

S1P signaling axis mediates NPS-induced biological effects in endometriotic epithelial cells

Next, the potential role of SK1 or SK2 activation in the biological effects induced by the neuropeptide was examined. For this purpose, cells were pretreated with the specific SK1 pharmacological inhibitor PF-543 (1 μM) or the SK2 inhibitor ABC294640 (1 μM) before being challenged with 100 nM NPS and cell invasion (Fig. 3A) and cytoskeletal remodeling (Fig. 3B) were then investigated. Results illustrated in Fig. 3A highlighted that the inhibition of SK1 or SK2 totally prevented the NPS-induced cell invasion. Moreover, the blockade of SK1 or SK2 activity abolished F-actin polymerization induced by NPS (Fig. 3B). To further confirm the involvement of both SK isoforms in the biological response evoked by NPS in endometriotic cells, SK1 or SK2 were knocked-down by RNA interference. When SK1 or SK2 were efficiently silenced by specific siRNA (Fig. 4A,B), the increase of cell invasion (Fig. 4C) and the remodeling of cytoskeleton (Fig. 4D) induced by NPS were blocked. Taken together, these data demonstrate an essential role of SK1 and SK2 in the NPS-induced invasive phenotype of endometriotic cells.

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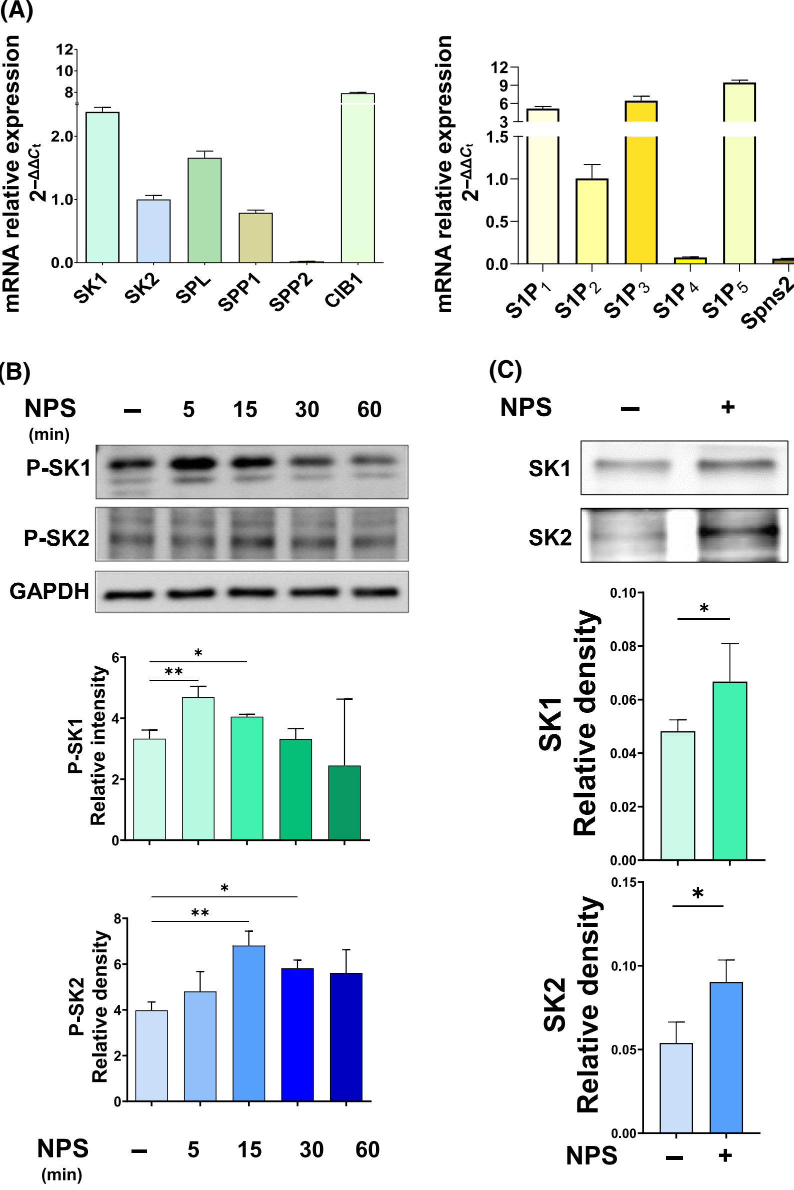

Fig. 2. NPS activates SK1 and SK2 in endometriotic epithelial cells. (A) RT-PCR analysis was performed in subconfluent endometriotic epithelial cells using TaqMan Gene Expression Assay probes specific for human SK1, SK2, SPL, SPP1, SPP2 and CIB1 (left panel), and $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, $S1P_5$ and Spsn2 (right panel). Results, analyzed with the $2^{-\Delta\Delta C_t}$ method, were normalized to β-actin housekeeping gene and expressed as fold change over a reference gene, SK2 (left panel) or $S1P_2$ (right panel). Data are mean ± SD of three independent experiments performed in triplicate. (B) Endometriotic epithelial cells were serum-starved for 18 h and then treated for different time intervals (5–60 min) with 100 nM NPS. Western blot analysis was performed using specific anti-phospho(Ser225)-SK1 and anti-phospho(Thr578)-SK2 antibodies. A blot representative of three independent experiments with analogous results is shown. The histograms represent the densitometric analysis of three independent experiments. Data are the mean ± SD and are reported as protein expression normalized to GAPDH. The increase in phospho-SK1 and phospho-SK2 content induced by NPS was found to be statistically significant by one-way ANOVA followed by Bonferroni's *post hoc* test (*$P < 0.05$; **$P < 0.01$). (C) Serum-starved endometriotic epithelial cells were incubated with 100 nM NPS for 15 min. Western blot analysis of SK1 and SK2 was performed in membrane fractions obtained as described in the method section. A blot representative of three independent experiments is shown. The histograms represent the densitometric analysis of three independent experiments. Data are the mean ± SD and are reported as protein expression normalized to the total protein content considering the intensity of all proteins in the lane. The increase of membrane-associated SK1 and SK2 induced by NPS was statistically significant (Student's *t*-test, *$P < 0.05$).

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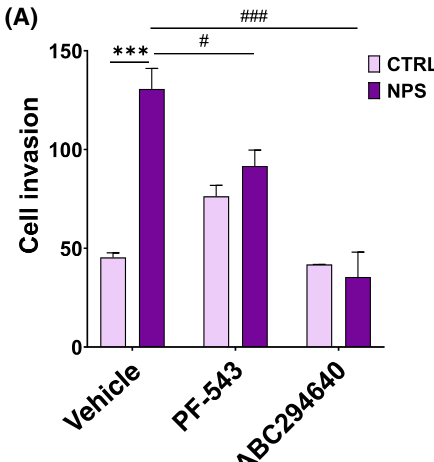

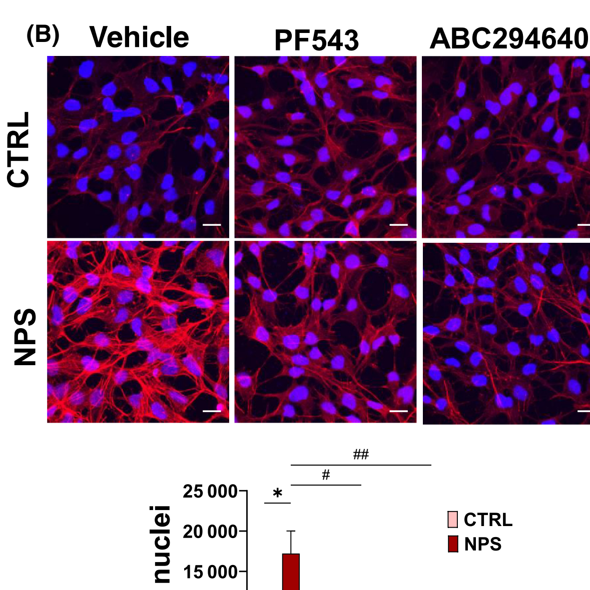

Fig. 3. SK inhibition prevents NPS-induced biological effects in endometriotic epithelial cells. (A) Cell invasion was determined in endometriotic epithelial cells pretreated or not with SK1 and SK2 specific inhibitors (1 µM PF-543 or 1 µM ABC294640, respectively) for 45 min and then challenged with 100 nM NPS for 6 h. Data are mean ± SD of three independent experiments performed in triplicate. The effect of SK1- or SK2-inhibition in NPS-induced invasion (***$P < 0.001$) was statistically significant by two-way ANOVA followed by Bonferroni's *post hoc* test ($^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$). (B) Endometriotic epithelial cells were seeded on matrix-coated glass coverslips and pre-incubated with SK1 and SK2 specific inhibitors (1 µM PF-543 and 1 µM ABC294640, respectively) for 45 min before being treated with NPS 100 nM for 45 min. Cells were fixed and stained with TRITC-labeled phalloidin and DAPI. Scale bar: 10 µm. The histogram represents the quantification of the intensity of TRITC-labeled phalloidin fluorescence normalized to the number of nuclei. Data are mean ± SD of eight fields quantified in three independent experiments. The effect of SK1- or SK2-inhibition in NPS-induced F-Actin polymerization (*$P < 0.05$) was statistically significant by two-way ANOVA followed by Bonferroni's *post hoc* test ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$). MFI, mean fluorescence intensity.

In order to get insight into the mechanism by which S1P signaling axis mediates NPS biological effects in endometriotic cells, the possible involvement of S1P receptors was examined. To this aim, $S1P_1$, $S1P_2$ and $S1P_3$ were individually knocked-down by specific siRNA and their efficacious specific downregulation was evaluated by RT-PCR (Fig. 5A). Interestingly, $S1P_1$- or $S1P_3$-siRNA significantly reduced cell invasion promoted by NPS (Fig. 5B). Notably, on the contrary, $S1P_2$ silencing exacerbated the NPS pro-migratory action, highlighting a negative role of this receptor isoform in NPS-induced cell invasion (Fig. 5B). The crucial role of $S1P_1$ and $S1P_3$ in mediating NPS-induced cell invasion and cytoskeletal remodeling was also confirmed by pharmacological approach. Indeed, cell pretreatment with VPC23019 (10 µM), a selective $S1P_1/S1P_3$ antagonist, abolished the promotion of cell invasion (Fig. 5C) and F-actin polymerization (Fig. 5D) elicited by the neuropeptide. These findings demonstrate that the biological action of NPS is mediated by $S1P_1$ and $S1P_3$ in endometriotic epithelial cells.

NPS biological effects rely on RhoA activation in endometriotic epithelial cells

Finally, to investigate the signaling pathways downstream to NPS action, the effect of the neuropeptide on the activation of the monomeric G-protein RhoA, crucially involved in cell invasion and cytoskeletal dynamics [22], was examined. Western blot analysis reported in Fig. 6A showed that 100 nM NPS at 15 and 30 min significantly increased the activation of RhoA, measured by its translocation to the membrane-enriched fraction [23]. We then investigated whether S1P receptors are involved in the activation of RhoA triggered by NPS. To this aim, endometriotic cells were pretreated with the selective $S1P_1/S1P_3$ antagonist VPC23019 (10 µM) before being challenged with 100 nM NPS for 15 min. As shown in Fig. 6B, the blockade of $S1P_1$ and $S1P_3$ totally impaired RhoA

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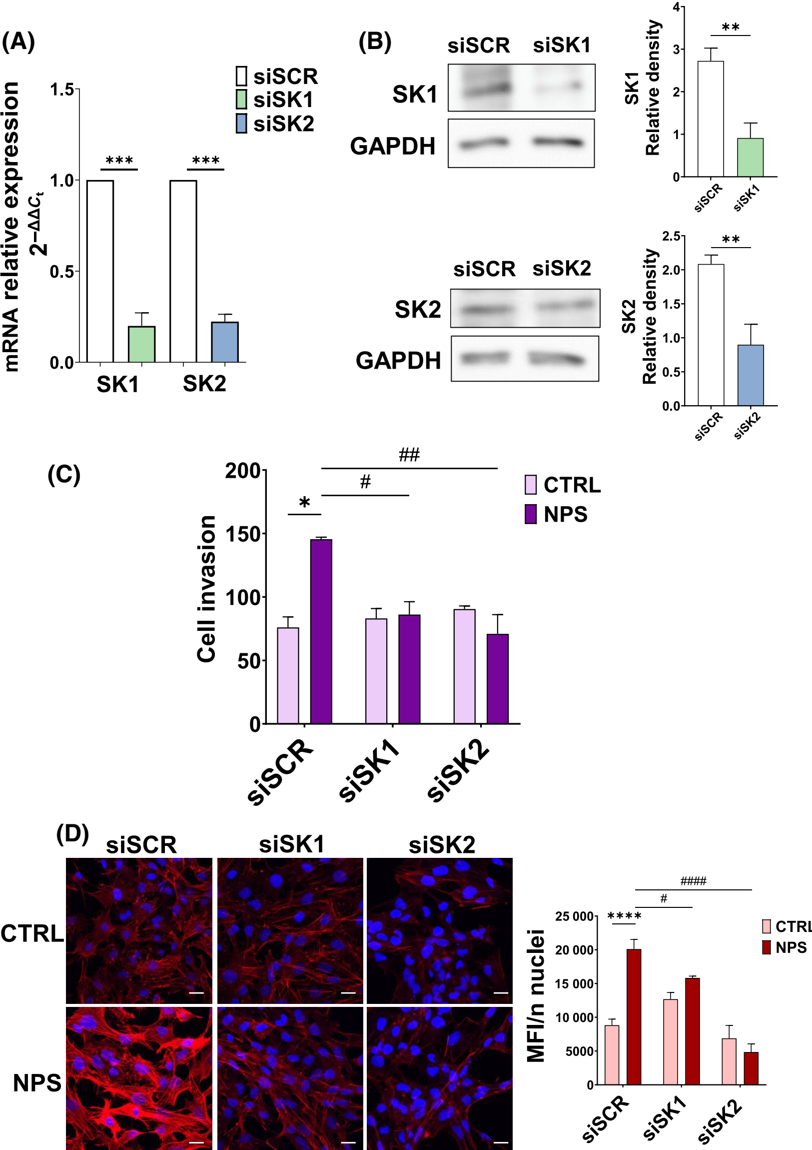

Fig. 4. SK downregulation impairs NPS-induced biological effects in endometriotic epithelial cells. (A) Endometriotic epithelial cells were transfected with SCR-, SK1- and SK2-siRNA and RT-PCR analysis was performed using TaqMan Gene Expression Assay probes specific for human SK1 and SK2. Results, analyzed with the $2^{-\Delta\Delta C_t}$ method, were normalized to β-actin housekeeping gene and expressed as fold change (specific siRNA vs SCR-siRNA). Data are mean ± SD of three independent experiments performed in triplicate. The effect of gene downregulation was statistically significant by Student's *t*-test (***$P < 0.001$). (B) Endometriotic epithelial cells were transfected with SCR-, SK1- and SK2-siRNA and western blot analysis was performed using specific anti-SK1 and anti- SK2 antibodies. A blot representative of three independent experiments with analogous results is shown. The histograms represent the densitometric analysis of three independent experiments. Data are the mean ± SD and are reported as protein expression normalized to GAPDH. The effect of gene downregulation was statistically significant by Student's *t*-test (**$P < 0.01$). (C) Endometriotic epithelial cells, transfected with SCR-, SK1- and SK2-siRNA, were serum-starved prior to be challenged with 100 nM NPS for 6 h for the invasion assay. Data are mean ± SD of three independent experiments performed in triplicate. The effect of SK1- or SK2-downregulation in NPS-induced invasion (*$P < 0.05$) was statistically significant by two-way ANOVA followed by Bonferroni's *post hoc* test ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$). (D) Endometriotic epithelial cells seeded in matrix-coated glass coverslips and transfected with SCR-, SK1- and SK2-siRNA were serum-starved prior to be treated with 100 nM NPS for 45 min. Cells were fixed and stained with TRITC-labeled phalloidin and DAPI. Scale bar: 10 μm. The histogram represents the quantification of the intensity of TRITC-phalloidin fluorescence normalized to the number of nuclei. Data are mean ± SD of eight fields of three independent experiments. The effect of SK1- or SK2-downregulation in NPS-induced F-actin polymerization (****$P < 0.0001$) was statistically significant by two-way ANOVA followed by Bonferroni's *post hoc* test ($^{\#}P < 0.05$; $^{\#\#\#\#}P < 0.0001$). MFI, mean fluorescence intensity.

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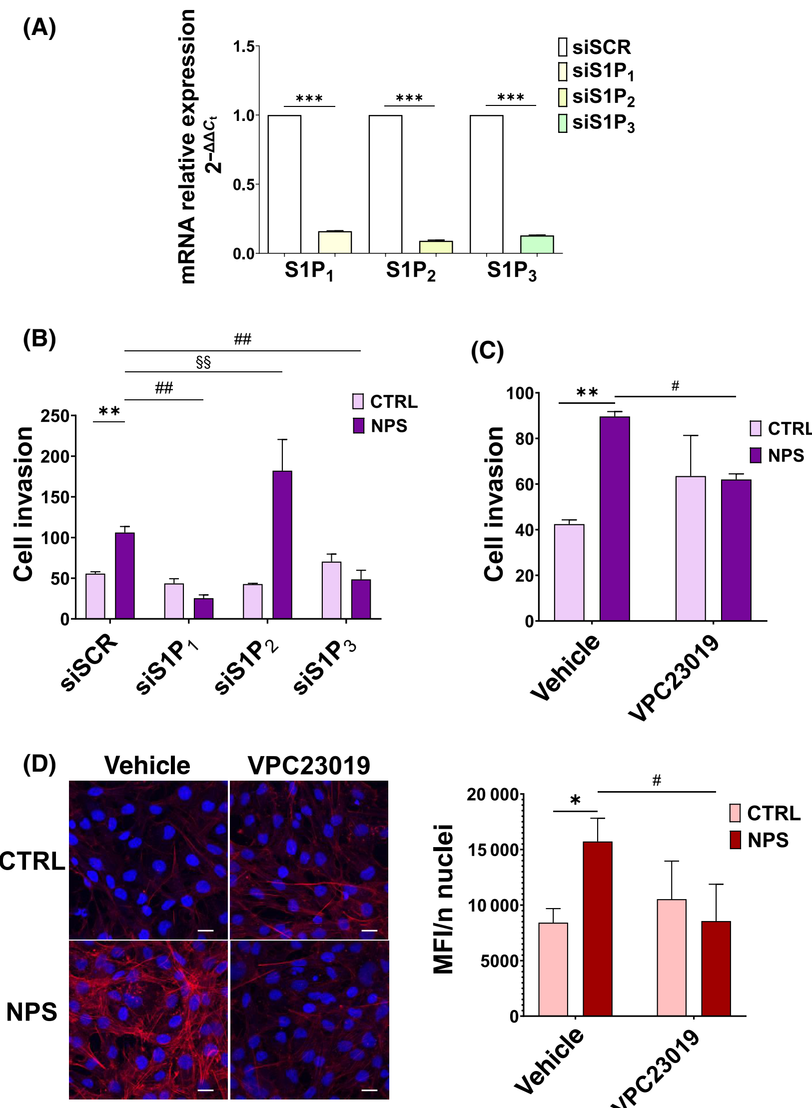

Fig. 5. NPS biological effects are mediated by $S1P_1/S1P_3$ in endometriotic epithelial cells. (A) Endometriotic epithelial cells were transfected with SCR-, $S1P_1$-, $S1P_2$- and $S1P_3$-siRNA and RT-PCR analysis was performed using TaqMan Gene Expression Assay probes specific for human $S1P_1$, $S1P_2$ and $S1P_3$. Results, analyzed with the $2^{-\Delta\Delta C_t}$ method, were normalized to β-actin housekeeping gene and expressed as fold changes (specific siRNA vs SCR-siRNA). Data are mean ± SD of three independent experiments performed in triplicate. The effect of gene downregulation was statistically significant by Student's *t*-test (***$P < 0.001$). (B) Endometriotic epithelial cells transfected with SCR-, $S1P_1$-, $S1P_2$- and $S1P_3$-siRNA were serum-starved prior to be challenged with 100 nM NPS for 6 h in the invasion assay. Data are mean ± SD of three independent experiments performed in triplicate. The effect of $S1P_1$ or $S1P_3$-downregulation in the reduction of NPS-induced invasion (**$P < 0.01$) was statistically significant by two-way ANOVA followed by Bonferroni's *post hoc* test (##$P < 0.01$). The effect of $S1P_2$ downregulation in the increase of NPS-induced invasion was statistically significant by two-way ANOVA followed by Bonferroni's *post hoc* test (§§$P < 0.01$). (C) Cell invasion was determined in endometriotic epithelial cells pretreated or not with $S1P_1/S1P_3$ specific antagonist (10 μM VPC23019) for 45 min and then challenged with 100 nM NPS for 6 h. Data are mean ± SD of three independent experiments performed in triplicate. The blockade of $S1P_1/S1P_3$ in NPS-induced invasion (**$P < 0.01$) was statistically significant by two-way ANOVA followed by Bonferroni's *post hoc* test (#$P < 0.05$). (D) Endometriotic epithelial cells were seeded in matrix-coated glass coverslips and pre-incubated with $S1P_1/S1P_3$ specific antagonist (10 μM VPC23019) for 45 min and then challenged with 100 nM NPS for 45 min. The cells were fixed and stained with TRITC-labeled phalloidin and DAPI. Scale bar: 10 μm. The histogram represents the quantification of the intensity of TRITC-labeled phalloidin fluorescence normalized to the number of nuclei. Data are mean ± SD of eight fields quantified in three independent experiments. The blockade of $S1P_1/S1P_3$ in NPS-induced F-Actin polymerization (*$P < 0.05$) was statistically significant by two-way ANOVA followed by Bonferroni's *post hoc* test (#$P < 0.05$). MFI, mean fluorescence intensity.

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membrane translocation elicited by NPS providing evidence that these two S1P receptor subtypes mediate NPS-induced RhoA activation in endometriotic cells. Finally, the involvement of RhoA activation in NPS action in endometriotic cells was assessed by examining the effect of NPS in cells where the main downstream target of RhoA, Rho kinase (ROCK), was pharmacologically inhibited. Indeed, pre-treatment with 10 μM Y27632, a specific ROCK inhibitor, significantly reduced the increase of endometriotic cell invasion (Fig. 6C) and microfilament organization and stress fibers formation (Fig. 6D) elicited by NPS. Altogether, these findings highlight a crucial role of RhoA/ROCK pathway, downstream to SK1 and SK2 activation and $S1P_1/S1P_3$ engagement, in mediating the NPS-induced invasive phenotype in endometriotic cells.

Discussion

Endometriosis is a chronic debilitating disease whose complex pathogenesis is not yet fully understood. Current treatments count on surgical removal of the lesions and/or hormonal medical therapies with high recurrence rate and relevant side effects [6], so it is imperative to investigate new possible therapeutic options, contemplating non-hormonal approaches.

Cellular invasion is one of the most important traits in endometriosis, accountable for dissemination of endometrial tissue outside the uterus and for the development of the lesion [3,4]. In this study, we identified NPS as a critical regulator for the acquisition of an invasive phenotype by endometriotic cells. In addition, the results presented here demonstrate, for the first time, that the S1P signaling pathway is required for the regulation of invasion and cytoskeletal remodeling induced by NPS. By genetic and pharmacologic approaches, it was demonstrated that the activation of both isoforms of the S1P-generating enzyme, SK1 and SK2, followed by transactivation of S1P receptors $S1P_1$ and $S1P_3$ elicited by the neuropeptide, is required for the induction of the invasive phenotype of endometriotic cells.

Previous studies reported that the genomic locus of NPSR1 links with many inflammatory diseases like asthma [24], inflammatory bowel disease [25] and rheumatoid arthritis [26]. Of note, NPSR1 has been recently identified to be critically implicated in endometriosis-associated inflammation [18] but, although NPSR1 is expressed in glandular epithelium of eutopic and ectopic endometrium [18], the biological action exerted by the neuropeptide on endometriotic cells is presently unknown. The here reported data demonstrate that NPS is a potent chemoattractant for epithelial endometriotic cells, suggesting a role for the neuropeptide in the establishment of endometriotic lesions. Although NPS has been reported to modulate focal adhesion [27] and to stimulate monocyte [18,28] and eosinophil chemotaxis [29], as key step in the evoked inflammatory response, this is the first evidence of a pro-migratory action of the neuropeptide in endometriotic cells, primary candidates to determine cell invasion of the extra-uterine tissues.

Notably, the molecular mechanism implicated in NPS biological action in endometriotic cells has been here elucidated, identifying a critical role for the signaling axis of the bioactive sphingolipid S1P. Despite the occurrence of a functional cross-talk between S1P signaling axis and multiple growth factors and cytokines has been extensively reported [30–32], here experimental evidence for the exploitation of S1P signaling by a neuropeptide is provided. These data add a piece of information on the emerging key role of S1P signaling in endometriosis demonstrating that the bioactive sphingolipid, known to modulate cellular migration and invasion both in physiological and pathological contexts [33,34], is involved in the establishment of endometriotic lesions by stimulating cellular invasion. We previously demonstrated that S1P metabolism and signaling are profoundly dysregulated in endometriosis being the expression of the enzyme SK1 and the receptor isoforms $S1P_1$, $S1P_3$ and $S1P_5$ up-regulated [10]. Of note, $S1P_3$, whose expression in endometriotic lesions positively correlates with endometriosis-associated fibrosis, is the receptor isoform found to be crucially involved in transmitting the pro-fibrotic effect of S1P in epithelial endometriotic cells [14]. Remarkably, S1P levels are augmented in the peritoneal fluid of women with endometriosis in comparison with healthy women [12]. Moreover, the pharmacological blockade of SK1 suppresses the development of endometriotic lesions in a murine model of endometriosis [13], underlining the involvement of S1P signaling in the establishment and progression of the disease. Furthermore, we recently showed that S1P induces a ROS-mediated proinflammatory response in human endometrial stromal cells [11], suggesting a key role of the bioactive sphingolipid in the inflammatory process associated with endometriosis.

Interestingly, in this study, we provide the first evidence of the involvement of SK2 in endometriosis. Indeed, although SK2 expression is not altered in endometriosis [10,19], here we demonstrate that SK2, similarly to the SK1 isoform, is required for transmitting the chemotactic effect of NPS, highlighting a key role of this enzyme isoform in the disease. Since the inhibition of SK1 or SK2 is responsible for the abolition of NPS action, these findings suggest that there is

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no functional redundancy between the two enzyme isoforms but both, presumably at different steps, are implicated in the onset of the biological effect of the neuropeptide. SK1 and SK2, characterized by different tissue distribution and subcellular localization, can modulate fundamental cellular processes, such as apoptosis and proliferation, even in opposite ways [35]. Our findings support the concept that SK isoforms may have overlapping functions, as already reported for the pro-myogenic action exerted by IGF [36] and for the modulation of electrophysiological properties and oxidative metabolism by adiponectin in skeletal muscle [37]. Of note, SK1 and SK2 are equally required for the chemotactic effect exerted by epidermal growth factor (EGF) in breast cancer cells [38] and by TGFβ in esophageal cancer cells [39].

The involvement of $S1P_1$ and $S1P_3$ in cellular migration has been established in different physiological and pathological conditions being $S1P_1$ essential for triple-negative breast cancer cell migration [40], $S1P_3$ critical for EGF-stimulated invasion of lung adenocarcinoma cells [41] and both receptor isoforms necessary for neural stem cells migration toward a site of spinal cord injury [42]. Of note, the two receptor isoforms here

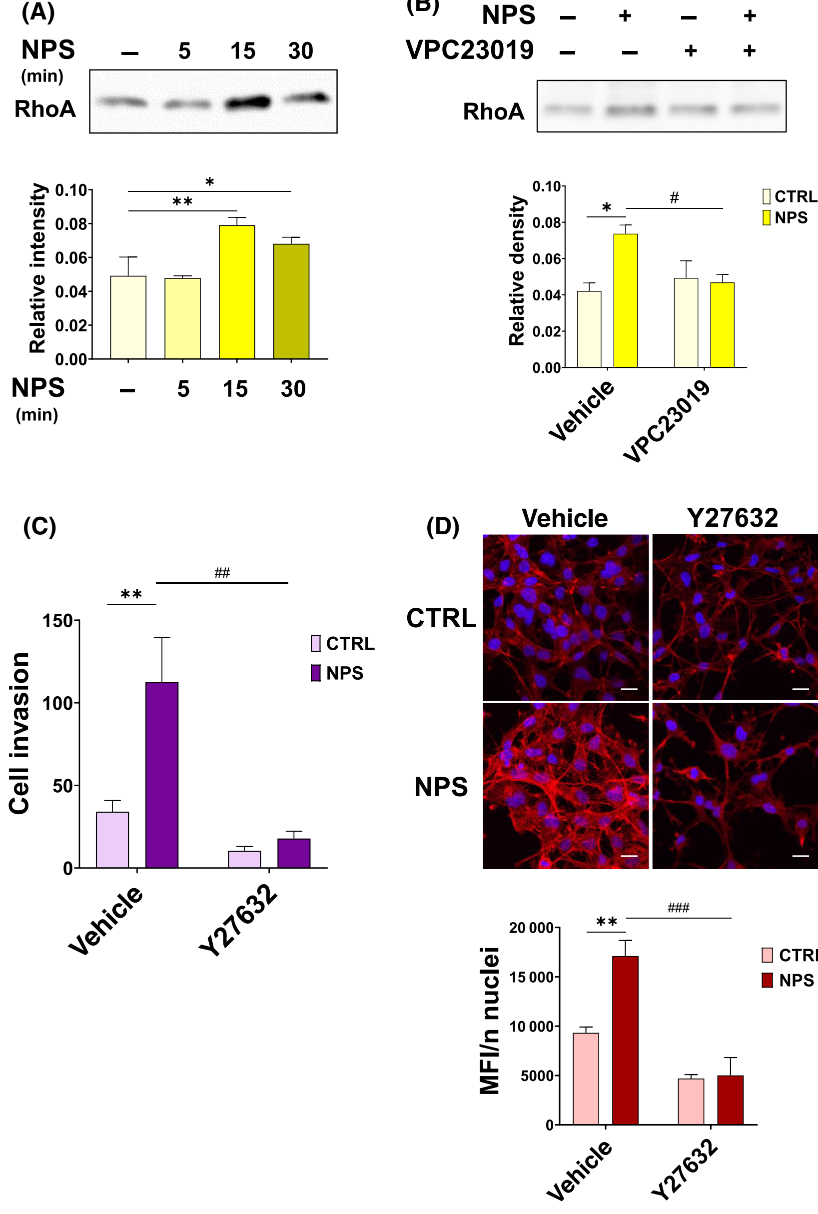

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Fig. 6. NPS biological effects rely on RhoA activation in endometriotic epithelial cells. (A) Serum-starved endometriotic epithelial cells were incubated with 100 nM NPS for the indicated time intervals (5–30 min). Western analysis of RhoA was performed in membrane fractions obtained as described in the method section. A blot representative of three independent experiments is shown. The histogram represents the densitometric analysis of three independent experiments. Data are the mean ± SD and are reported as protein expression normalized to the total protein content considering the intensity of all proteins in the lane. The increase of membrane-associated RhoA induced by NPS was statistically significant by one-way ANOVA (*$P < 0.05$; **$P < 0.01$). (B) RhoA membrane translocation was determined in endometriotic epithelial cells pretreated or not with $S1P_1/S1P_3$ specific antagonist (10 μM VPC23019) for 45 min and then challenged with 100 nM NPS for 15 min. Western analysis of RhoA was performed in membrane fractions. A blot representative of three independent experiments is shown. The histogram represents the densitometric analysis of three independent experiments. Data are the mean ± SD and are reported as protein expression normalized to the total protein content considering the intensity of all proteins in the lane. The blockade of $S1P_1/S1P_3$ in NPS-induced increase in membrane-associated RhoA (*$P < 0.05$) was statistically significant by two-way ANOVA followed by Bonferroni's *post hoc* test ($^{\#}P < 0.05$). (C) Serum-starved endometriotic epithelial cells were pre-incubated with the ROCK-specific inhibitor Y27632 (10 μM) before being challenged with 100 nM NPS for 6 h in the invasion assay. Data are mean ± SD of three independent experiments performed in triplicate. The effect of ROCK inhibition in NPS-induced invasion (**$P < 0.01$) was statistically significant by two-way ANOVA followed by Bonferroni's *post hoc* test ($^{\#\#}P < 0.01$). (D) Endometriotic epithelial cells were seeded in matrix-coated glass coverslips and pre-incubated with the ROCK specific inhibitor Y27632 (10 μM) for 45 min before being treated with NPS 100 nM for 45 min. The cells were fixed and stained with TRITC-labeled phalloidin and DAPI. Scale bar: 10 μm. The histogram represents the quantification of the intensity of TRITC-labeled phalloidin fluorescence normalized to the number of nuclei. Data are mean ± SD of eight fields quantified in three independent experiments. The effect of ROCK-inhibition in NPS-induced F-Actin polymerization (**$P < 0.01$) was statistically significant by two-way ANOVA followed by Bonferroni's *post hoc* test ($^{\#\#\#}P < 0.001$). MFI, mean fluorescence intensity.

shown to be implicated in transmitting the NPS-induced invasive phenotype were found to be up-regulated in endometriotic lesions [10], thus identifying $S1P_1$ and $S1P_3$ as possible new non-hormonal pharmacological targets for the treatment of endometriosis. On the contrary, the knocking-down of $S1P_2$ led to a dramatic increase of the invasive action of NPS, suggesting a negative role of this receptor isoform in NPS-induced invasion in endometriotic cells. These data are in agreement with earlier studies where $S1P_2$ was shown to negatively regulate chemotaxis of myoblasts [43], satellite cells [33] as well as in basophilic leukemia cells [44] and human thyroid cancer cells [45]. At odds with the present findings, previous reports support a role for $S1P_2$ in mediating the pro-migratory action of S1P in breast cancer cells [46], human lung fibroblasts [47] and in bone marrow-derived macrophages in mouse models of cholestatic liver injury [48]. Although not being investigated here, it can be speculated that the opposite role of $S1P_2$ in the modulation of chemotaxis could be ascribed to alternative G-protein coupling of the receptor in a cell type-specific manner.

The RhoA/ROCK pathway is known to be involved in cell invasion and cytoskeletal dynamics [22]. Interestingly, in our study, RhoA/ROCK activation was identified to be implicated in the induction of the invasive phenotype of endometriotic cells elicited by NPS, downstream to $S1P_1$ and $S1P_3$. These data are in agreement with previous studies in which RhoA/ROCK was found to be activated by S1P receptors to mediate cell migration and invasion [49]. Of note, RhoA has been recently linked to endometriosis: indeed, Huang *et al*. demonstrated a higher expression of both RhoA as well as ROCK in eutopic and ectopic endometrium of women affected by endometriosis compared to healthy endometrium. Furthermore, they highlighted the role of RhoA and ROCK in promoting epithelial-to-mesenchymal transition and proliferation of human eutopic endometrial epithelial cells [50]. Here, a link between NPS signaling and RhoA pathway is reported for the first time.

All together, these findings add new information to the understanding of the molecular mechanisms implicated in endometriosis pathogenesis and establish the rationale for the exploitation of innovative therapeutic targets for its treatment. Indeed, S1P signaling axis has been employed in innovative therapeutic approaches based on modulators of S1P receptors, such as fingolimod and ozanimod, already approved by FDA for the treatment of multiple sclerosis and other immune syndrome, respectively [51,52]. The repurposing of S1P receptor modulator compounds [53] could pave the way for non-hormonal endometriosis therapy in a short range of time.

Conclusions

The here presented data demonstrate that the induction of the invasive phenotype by the neuropeptide NPS relies on the engagement of $S1P_1/S1P_3$ signaling via a mechanism dependent on SK activation in endometriotic cells. Notably, genetic findings that link missense coding variants of NPSR1 to endometriosis [18] are strengthened by the here identified biological

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action exerted by the neuropeptide in endometriotic cells. On the whole, these findings reinforce the concept that the signaling axis of the bioactive sphingolipid S1P is crucially involved in endometriotic cell biology and endometriosis pathogenesis.

Materials and methods

Materials

Applied cell extracellular matrix was obtained from Applied Biological Materials Inc. (Richmond, BC, Canada). Bradford protein assay, Tris/Glycine/SDS, EveryBlot Blocking Buffer, Clarity western ECL substrate and trans-blot turbo PVDF membranes were purchased from Bio-Rad (Hercules, CA, USA). All biochemicals, TRI reagent, cell culture reagents, Dulbecco's Modified Eagle Medium (DMEM), Nutrient Mixture F-12 Ham (F12), fetal bovine serum (FBS), protease inhibitor cocktail, phosphatase inhibitor cocktail 3, bovine serum albumin (BSA), Fluoromount aqueous mounting medium, TRITC-phalloidin, DAPI, the specific SK1 inhibitor PF-543, the $S1P_1/S1P_3$ antagonist VPC23019, the human neuropeptide S (NPS) and the specific siRNA for SK1, SK2, $S1P_1$, $S1P_2$, $S1P_3$ and the scramble siRNA were purchased from Merck Life Sciences (Burlington, MA, USA). The selective SK2 inhibitor ABC294640 was obtained from Cayman Chemical (Ann Arbor, MI, USA). The specific Rho kinase (ROCK) inhibitor Y27632 was purchased from MedChemExpress (Monmouth Junction, NJ, USA). The Diff-Quick staining solution was obtained from Medion Diagnostic (Düdingen, Switzerland). Anti-SK2 (N-terminal region; catalog #SP4621), anti-SK1 (central region; catalog #SP1621), anti-phospho-SK2 (Thr578) (catalog #SP4631) and anti-phospho-SK1 (Ser225) (catalog #SP1641) antibodies were purchased from ECM Biosciences LLC (Versailles, KY, USA). Monoclonal anti-GAPDH (catalog #sc-32233) and anti-RhoA (catalog #sc-418) antibodies, as well as secondary antibody conjugated to horseradish peroxidase, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TaqMan Universal Master Mix II, TaqMan gene expression assays, polycarbonate filters (8 μm pores), Lipofectamine RNAiMAX, high-capacity cDNA reverse transcription kit and Geltrex LDEV-free reduced growth factor basement membrane matrix were obtained from Thermo Fisher Scientific INC (Waltham, MA, USA).

Cell culture

Authenticated human endometriotic epithelial 12Z cells (RRID:CVCL_0Q73) were purchased from Applied Biological Materials Inc. (Richmond, BC, Canada) and grown in a 1 : 1 mixture of DMEM : F12, supplemented with 10% FBS, 2 mm L-glutamine, 100 $U \cdot mL^{-1}$ penicillin, and 100 $\mu g \cdot mL^{-1}$ streptomycin as previously described [14]. Cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C and grown for a maximum of 20 passages, and routinely tested to ensure that they were free from mycoplasma contamination. For the experiments, cells were seeded and the following day were serum-starved overnight in medium without serum supplemented with 1 $mg \cdot mL^{-1}$ fatty acid-free BSA. To enhance cellular adhesion, the plates were coated with Applied cell extracellular matrix before use.

Western blot analysis

Cells were collected with the aid of a scraper and incubated for 30 min at 4 °C in 50 mm Tris, pH 7.5, 120 mm NaCl, 6 mm EGTA, 1 mm EDTA, 20 mm NaF, 15 mm $Na_4P_2O_7$, 1% Nonidet, with the addition of protease inhibitor cocktail and phosphatase inhibitor cocktail. They were then centrifuged for 15 min at 10 000 ***g*** at 4 °C, and the supernatant was collected for western blot analysis. For the analysis of membrane fraction-associated SK1/SK2 or RhoA, cells were collected in a buffer solution containing 20 mm HEPES, pH 7.4, 2 mm EGTA, 0.5 mm EDTA, SDS 0.1%, 250 mm sucrose, 5 mm NaN_3, 10 mm β-glycerophosphate, protease and phosphatase inhibitors and disrupted by a tissue grind pestle (100 strokes). Lysates were centrifuged (10 min, 800 ***g***) and the resulting supernatant was centrifuged again at 200 000 ***g*** for 1 h to separate cytosolic and membrane fraction [23,36]. The evaluation of membrane fraction-associated SK1/SK2 or RhoA was performed by western blot analysis of membrane fractions with specific antibodies. Normalization was performed by measuring total protein directly on the membrane used for western blot by means of stain-free technology [54] that uses a proprietary trihalo compounds in Mini Protean TGX Stain-free gels (Bio-Rad Laboratories) to enhance the fluorescence of tryptophan amino acids when exposed to UV light.

Cell transfection

Cells grown into tissue culture 6-well plates were transfected with siRNA duplexes using Lipofectamine RNAiMAX, according to the manufacturer's instructions as described in [55,56]. Briefly, lipofectamine RNAiMAX was incubated with siRNAs in DMEM : F12 without serum and antibiotics at room temperature for 20 min and then added to cells to a final concentration of 50 nm, in DMEM : F12 containing serum. After 30 h cells were serum-starved overnight and used for experiments 48 h after the beginning of transfection. The efficacy of specific gene knockdown was evaluated using real-time reverse-transcription polymerase chain reaction (RT-PCR).

RT-PCR

Total RNA was extracted using TRI reagent according to the manufacturer's instructions, and reverse transcribed

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with the high-capacity cDNA reverse transcription kit. The quantification of target mRNA expression through RT-PCR was performed in duplicate using TaqMan gene expression assays and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) [57]. Target sequences were simultaneously amplified together with the housekeeping gene β-actin. Relative quantification of mRNA expression was performed by $2^{-\Delta\Delta C_t}$ method [58].

Invasion

Cell invasion was measured using a modified Boyden chamber system as described previously [43]. Polycarbonate filters with 8 μm pores were coated with Geltrex matrix ($9\ \text{mg·mL}^{-1}$) for 60 min at 37 °C. One hundred nanomolar NPS was added to the lower chamber while 12Z cells, resuspended in DMEM : F12 containing $250\ \mu\text{g·mL}^{-1}$ heat-inactivated BSA, were placed into the upper well of the chamber and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 6 h. When requested, cells were pre-incubated with SK1 or SK2 specific inhibitors (1 μM PF-543 or 1 μM ABC294640, respectively), ROCK-specific inhibitor (10 μM Y27632) or $S1P_1/S1P_3$ antagonist (10 μM VPC23019) for 45 min. Alternatively, cells were transfected with scrambled (SCR)-siRNA or selective siRNA for SK1, SK2, $S1P_1$, $S1P_2$, $S1P_3$ and then used for the invasion assay. Polycarbonate filters were fixed with methanol for 10 min at RT and stained with Diff-Quick staining solution. Invasion was assessed by counting the number of migrated cells in six random fields per filter.

Confocal microscopy

Cells were seeded on glass coverslips and pre-incubated with SK1 or SK2 specific inhibitors (1 μM PF-543 or 1 μM ABC294640, respectively), ROCK specific inhibitor (10 μM Y27632) or $S1P_1/S1P_3$ antagonist (10 μM VPC23019) before being treated with 100 nM NPS for 45 min. Alternatively, cells were seeded on glass coverslips and transfected with SCR-siRNA or selective siRNA for SK1 and SK2 before being treated with 100 nM NPS for 45 min. Cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed with PBS and incubated with TRITC-phalloidin for 30 min to visualize F-actin filaments [59]. Cell nuclei were stained with DAPI. Fluorescence was assessed by confocal microscopy (Leica SP8; Leica Microsystems, Mannheim, Germany) with a 63× oil immersion-objective and quantified using the IMAGEJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data represent mean ± SD values calculated on at least three independent experiments. Statistical analysis and graphical representations of the data were performed using the GRAPHPAD PRISM 10 software (Dotmatics, Boston, MA, USA). P values were calculated using Student's t-test, one-way or 2-way ANOVA followed by Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

MP provided the methodology, investigation, visualization, writing the original draft, review, and editing. CB provided the conceptualization, methodology, investigation, visualization, formal analysis, funding acquisition, writing the original draft, review, and editing. IS, MRo, and MRa acquired the data, review, and editing. FC and FP provided the data analysis, funding acquisition, review, and editing. SV and MF provided the data analysis, review, and editing. PB provided the conceptualization, data analysis, funding acquisition, review, and editing. CD provided the conceptualization, visualization, formal analysis, funding acquisition, writing the original draft, review, editing, and supervision.

Data availability statement

The data that supports the findings of this study are available in the figures of this article.

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