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## **Substance P enhances keratocyte migration and neutrophil recruitment through interleukin-8**

Marta Słoniecka, Sandrine Le Roux, Qingjun Zhou, Patrik Danielson

Department of Integrative Medical Biology, Umeå University, Umeå, Sweden

(M.S.,S.LR.,Q.Z., P.D.); Department of Clinical Sciences, Ophthalmology, Umeå University, Umeå, Sweden (M.S.); Shandong Provincial Key Laboratory of Ophthalmology, Shandong Eye Institute, Qingdao, China (Q.Z.)

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**Running title:** Substance P enhances keratocyte migration

**To whom correspondence should be addressed:**

Professor Patrik Danielson, M.D., Ph.D.; Dept. of Integrative Medical Biology, Umeå

University, SE-901 87, Umeå, Sweden; Phone: +46 90 786 58 93; e-mail:

[patrik.danielson@umu.se](mailto:patrik.danielson@umu.se)

**Number of pages:** 43

**Number of tables:** 0

**Number of figures:** 7

**Number of references:** 58

**Word count in Abstract:** 240

**Word count in Introduction:** 728

**Word count in Discussion:** 1926

**Abbreviations:**  $\alpha$ -SMA, alpha smooth muscle actin; CXCR1, Interleukin 8 receptor, alpha; CXCR2, Interleukin 8 receptor, beta; DMEM/F12, Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; FBS, Fetal Bovine Serum; HBSS, Hanks' Balanced Salt Solution; IL-8, interleukin-8; HRP, horseradish peroxidase; L-733,060, (2*S*,3*S*)-3-[[3,5 *bis*(Trifluoromethyl)phenyl]methoxy]-2-phenylpiperidine hydrochloride; NK-1R, neurokinin 1 receptor; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate buffered saline; PI3K, Phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PVDF, polyvinylidene difluoride; Rac1, Ras-related C3 botulinum toxin substrate 1; RhoA, Ras homolog gene family, member A; RIPA, radioimmunoprecipitation buffer; RPMI, Roswell

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Park Memorial Institute medium; SP, substance P; TBS, TRIS-buffered saline; TBS-T, TRIS-buffered saline containing 0.1% Tween-20; TRITC, tetramethylrhodamine.

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## Abstract

Keratocytes are the resident cells of the corneal stroma responsible for maintaining the turnover of this tissue by synthesizing extracellular matrix components. When the cornea is injured, the keratocytes migrate to the wounded site and participate in the stromal wound healing. The neuropeptide substance P (SP), which is also known to be produced by non-neuronal cells, has previously been implicated in epithelial wound healing after corneal injury. As corneal scarring, which occurs in the stroma when the process of wound healing has malfunctioned, is one of the major causes of preventable blindness, this study aimed to elucidate the potential role of SP in keratocyte migration and therefore in stromal wound healing. We report that the expression and secretion of SP in human keratocytes were increased in response to injury *in vitro*. Moreover, SP enhances the migration of keratocytes by inducing the actin cytoskeleton reorganization and focal adhesion formation through the activation of the phosphatidylinositol 3-kinase and Rac1/RhoA pathway. Furthermore, SP stimulation leads to upregulated expression of the proinflammatory and chemotactic cytokine interleukin-8 (IL-8), which also contributes significantly to SP-enhanced keratocyte migration and is able to attract neutrophils. In addition, the preferred SP receptor, the neurokinin-1 receptor, is necessary to induce keratocyte migration and IL-8 secretion. In conclusion, we describe new mechanisms by which SP enhances migration of keratocytes and recruits neutrophils, two necessary steps in the corneal wound healing process, which are also likely to occur in other tissue injuries.

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## Introduction

The cornea is the transparent front part of the eye and it is responsible for the majority of the eye's total optical power. It comprises three functionally distinct layers: An outer epithelial layer, a middle stromal layer, and an inner endothelial layer. The stroma accounts for up to 90 percent of the corneal thickness and it consists of regularly packed collagen fibrils arranged into lamellae (Benedek, 1971; Farrell et al., 1973; Hassell and Birk, 2010; Maurice, 1957; Ruberti et al., 2011). Sparsely distributed between the lamellae, the quiescent cells of the stroma, keratocytes, are found. They have a dendritic morphology and are responsible for maintenance and repair of the stroma. Keratocytes produce lumican and keratocan, which regulate corneal transparency and shape (Musselmann et al., 2005). The cornea is protected from injury both by physical and molecular barriers. However, under some circumstances, such as trauma, infection or surgery, the cornea may get injured. When the cornea gets injured a healing response is activated, which ideally leads to a healed, healthy cornea (Lim et al., 2003). The corneal wound healing process is a complex interplay between the epithelium, the stroma, and the immune system, and it is mediated by various cytokines and growth factors. Briefly, after an injury occurs, keratocytes adjacent to the wound undergo apoptosis. As apoptosis results in an area devoid of keratocytes, the remaining cells start to proliferate and migrate towards the wound. Part of the mitotic keratocytes generate myofibroblasts, which are mainly responsible for producing new extracellular matrix components (Wilson et al., 2001). At 12-24 hours after injury there is an influx of inflammatory cells to the stroma (O'Brien et al., 1998). Cells such as macrophages, monocytes, and neutrophils scavenge cellular components released from dying keratocytes. In the final stage of corneal wound healing, restoration of the quiescent state of the keratocytes and remodeling of any disordered collagen takes place (Wilson et al., 2001).

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However, when the process of the wound healing is incomplete or disturbed, corneal scarring may occur, transforming the transparent cornea to an opaque tissue with consequent visual impairment. Despite extensive research on corneal wound healing, corneal scarring remains a major cause of preventable blindness (Stepp et al., 2014). More in-depth understanding of this process is still needed.

The migration of keratocytes is an important step towards proper wound closure and healing. The process of migration involves reassembly of the actin cytoskeleton, and in this step a small G protein called Rac1 is one of the main regulators (Ridley, 2001). Moreover, phosphoinositide 3-kinases (PI3Ks) are the key players in maintaining cell polarity and defining the leading edge of the migrating cell (Cain and Ridley, 2009). Well-coordinated cooperation of Rac1, RhoA and PI3Ks is needed for a successful cell migration and thus wound closure.

Substance P (SP) is a neuropeptide well-known for its role in the sensory nervous system, including that it has been detected in sensory nerve fibers of the cornea (Muller et al., 2003). Nevertheless, it has been shown that SP can also be produced by non-neuronal cells, such as corneal epithelial cells and keratocytes (Watanabe et al., 2002). SP acts through binding to G protein-coupled receptors; the neurokinin 1 receptor (NK-1R) being the subtype with the highest affinity for SP (Regoli et al., 1994). SP activates the phospholipase C (PLC) (Nakajima et al., 1992), protein kinase C (PKC), and PI3K (Sun et al., 2009) signaling pathways to initiate diverse physiological functions such as modulation of immune cell activity (Maggi, 1997), vasodilation of blood vessels (Baluk et al., 1997), and contraction of smooth muscles (Lundberg and Saria, 1982). Moreover, SP has been shown to sensitize corneal epithelial cells to migrate (Yamada et al., 2005; Yang et al., 2014), and to stimulate synthesis of the proinflammatory cytokine interleukin 8 (IL-8), which recruits and activates

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neutrophils during inflammation (Hammond et al., 1995; Tran et al., 2000). Importantly, recent studies have demonstrated that increased SP levels accelerate corneal wound healing in an alkali burn model in rabbits (Hong et al., 2009). Considering all the reports on SP and the fact that most corneal wound healing research is done on corneal epithelial cells, we aimed to explore the role of SP in keratocyte migration and therefore in stromal wound healing. The results demonstrate that SP enhances keratocyte migration through activation of the PI3K/Rac1/RhoA pathways and via IL-8, and therefore that it may facilitate corneal wound healing.

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## **Materials and Methods**

### **Collection of human corneas**

Healthy human corneas from deceased individuals who had chosen, when alive, to donate their corneas post-mortem for transplantation and research, according to Swedish law, were kept in a corneal biobank at the University Hospital of Umeå, Sweden. If these healthy donated corneas were not used for transplantation after their collection, they were delivered to the laboratory for research purpose. If corneas were used for transplantation, some or all of the transplantation graft leftovers were retrieved for study purpose: the donor healthy limbal part or the donor healthy anterior or posterior central lamella. The study was vetted by the Regional Ethical Review Board in Umeå (2010-373-31M) without objections. The study was performed according to the principles of the Declaration of Helsinki.

### **Isolation and primary culture of human keratocytes**

Healthy human corneas were obtained from donated transplantation grafts or graft leftovers, as described in the previous section. Primary culture of human keratocytes was established. Samples were scraped using a sterile scalpel to remove any remaining epithelial or endothelial cells before being washed in sterile Hanks' Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, CA, USA, # 14170). In cases of whole corneal samples, the central part was separated from the peripheral part using a scalpel. Each part was then minced with a scalpel and digested in 2mg/ml collagenase (Clostridopeptidase A, Sigma, St. Louis, MO, USA, # C-1030) diluted in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM / F-12, Gibco, Carlsbad, CA, USA, # 31330-095) O/N at 37°C. The samples were then transferred and cultured in DMEM / F-12 medium supplemented with 2% Fetal Bovine Serum (FBS; Gibco, # 10082-147), 1% penicillin-streptomycin (Gibco, # 15410-122). Medium was replaced every second to third day until the cells reached confluence. Confluent



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cells were detached with 0.05% Trypsin-EDTA (Gibco, # 15400-054) and split in a 1:2 ratio. Cells from the central cornea in passages 4 to 5 were used for experiments. Corneas were assessed individually.

### **Cell culture and treatments**

In order to maintain keratocyte phenotype, central keratocytes were cultured in DMEM/F12 medium supplemented with 2% FBS (instead of 10% FBS, which transforms keratocytes into fibroblasts and activates them) and 1% penicillin-streptomycin. Culturing keratocytes in medium supplemented with 2% FBS maintains their proper phenotype: cells express keratocan and lumican, markers of keratocytes, and only small amounts of  $\alpha$ -SMA, a marker of activated keratocytes (Sloniecka et al., 2015). For experiments cells were seeded in either 6 well plates (Sarstedt, Helsingborg, Sweden, # 83.1839):  $0.25 \times 10^6$  cells per well (western blot and RT- qPCR),  $0.4 \times 10^6$  cells per well (scratch and wound healing assay),  $1 \times 10^6$  cells per well (scratch assay for SP EIA) or in 96 well plates (Sarstedt, # 83.1835): 8000 cells per well (IL-8 ELISA) in DMEM/F12 medium containing 0.1% FBS (unless stated otherwise) and allowed to adhere overnight. Vehicle controls (DMSO for wortmannin [concentration 0.00495%], water for SP and L-733,060; referred to as controls, as no differences between controls and vehicle controls were found) were included. In order to block NK-1 receptor, cells were incubated with L-733,060 (Tocris, Bristol, UK, # 1145) for 30 minutes at 37°C. Afterwards cells were treated with two different concentrations of SP:  $10^{-7}$ M and  $10^{-5}$ M (Sigma, # S6883). To confirm IL-8 induced migration, either IL-8 neutralizing antibody ( $3\mu\text{g/ml}$ ; Pepro Tech, # 500-P28) was added to the cells 8h after SP treatment, or cells were treated with various concentrations of recombinant human IL-8 (Pepro Tech, #200-08). Normal rabbit IgG antibody was used as an isotype control (R&D Systems, Minneapolis, MN, USA, #AB-105-C) at a concentration of  $3\mu\text{g/ml}$ . Wortmannin (Sigma, # 681675) was

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used to inhibit PI3K. Cells were pretreated with 100nM wortmannin for 1h at 37°C and afterwards they were treated with SP. Various concentrations of L-733,060, SP and wortmannin were tested regarding effect on keratocyte viability, and concentrations which were non-toxic to the cells were chosen for the subsequent experiments. CXCR1 and CXCR2 antibodies (R&D Systems, #MAB330 and #MAB331-100, respectively) were used to block IL-8 receptors. Cells were incubated with 2µg/ml of CXCR1 and CXCR2 antibodies for 1h at 37°C. Mouse IgG2A antibody was used as an isotype control (R&D Systems, #MAB003) at a concentration of 2µg/ml. At specified time points supernatants were collected, cells were either lysed in RLT buffer or freeze/thawed and lysed in RIPA buffer or fixed and immunostained. Images of the cells (migration scratch assay and wound healing assay) were taken at specified time points.

### **Isolation of human neutrophils**

Blood was collected from three healthy donors and anticoagulated in glass aprotinine K3EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA, #BDAM361017). Polymorphprep™ (Axis-Shield, Rodelokka, Norway, #1114683) was used to isolate neutrophil polymorphonuclear granulocytes (PMNs). 5 ml of blood was layered over 5 ml of separation media and centrifuged at 500 RCF for 35 minutes at RT. After separation into six distinct bands, the top three bands were disposed and the layer of neutrophils and isolation media beneath it was placed into a clean centrifuge tube, resuspended in HBSS without  $\text{Ca}^{2+}/\text{Mg}^{2+}$  (Invitrogen, #14175-053) and centrifuged at 350 RCF for 10 minutes. Next, residual red blood cells were lysed with red blood cell lysis buffer. Afterwards, cells were centrifuged at 250 RCF for 5 minutes and washed with HBSS without  $\text{Ca}^{2+}/\text{Mg}^{2+}$  two times. Neutrophils were counted, adjusted to desired concentration and used within 2 hours after

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isolation. Isolated neutrophils were primed with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, #P8139)

### **RT-qPCR Assay**

IL-8, TAC1 (SP) and TACR1 (NK-1R) mRNA levels were assessed by RT-qPCR. Total RNA was isolated from keratocytes by RNeasy Mini Kit (Qiagen, Venlo, The Netherlands, #74104) according to the manufacturer's protocol and reverse transcribed into cDNA with High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA, # 4368814). To determine IL-8, TAC1 and TACR1 gene expression, TaqMan® Gene Expression Assay (Applied Biosystems, Carlsbad, USA, # Hs00174103\_m1, # Hs00243225\_m1, # Hs00185530\_m1, respectively) were used. cDNA transcribed from 40ng of RNA was run in duplicates by ViiA™ 7 Real-Time PCR system (Applied Biosystems), with 18S as an internal control (Life Technologies, #4333760F), and analyzed with ViiA™ 7 Software (Applied Biosystems).

### **Western blot analysis**

Keratocytes were washed with PBS and freeze/thawed 3 times. Cells were further lysed in RIPA buffer supplemented with 0.5% Proteinase inhibitor cocktail (Sigma, # P1860). Protein concentration was assessed by Bradford assay (Bio-Rad, Hercules, CA, USA, # 500-0006). Total proteins were separated by sodium dodecyl sulfate / polyacrylamide gel electrophoresis and transferred to PVDF membranes (GE Healthcare, Little Chalfont, UK, # GEHERPN303F). Membranes were blocked with 5% (w/v) BSA (Sigma, # A9647) or 5% non-fat milk in TRIS-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for one hour at room temperature and incubated overnight at 4°C with primary antibodies: Anti-NK-1R from R&D Systems, Minneapolis, MN, USA (#MAB6687), anti-RhoA, anti-SAPK/JNK, anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-NF-κB p65, anti-phospho-NF-κB p65

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(Ser536), and anti- $\beta$ -actin from Cell Signaling, Leiden, The Netherlands (# 2117, # 9252, # 9251, # 8242, #3033, and # 4967, respectively), and anti-CXCR1 and anti-CXCR2 from Abcam, Cambridge, UK (# ab60254 and # ab14935, respectively). After washing, HRP-conjugated secondary antibody was added and incubated for 1h at room temperature. For detection of Rac1, membranes were incubated with Rac1 antibody (Cytoskeleton, Denver, CO, USA, # ARC03) for one hour at room temperature in TBS-T, washed and incubated with HRP-conjugated secondary antibody for 30 minutes at room temperature in TBS-T. Images were taken by Odyssey® Fc imaging system (LI-COR, Lincoln, NE, USA). Densitometry was performed using Image J analysis software (NIH). Densitometry analysis of phosphorylated NF- $\kappa$ B and SAPK/JNK was calculated as follows: Intensity of the phosphorylated protein of interest was divided by the intensity of  $\beta$ -actin. Intensity of total protein of interest was divided by the intensity of  $\beta$ -actin. Ratio of phosphorylated protein of interest was divided by ratio of the total protein of interest.

### **ELISA/EIA**

Supernatants collected from scratch assay were subjected to Substance P EIA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA, # EK-061-05) in order to measure SP secretion, according to the manufacturer's protocol. IL-8 secretion was measured with Human CXCL8/IL-8 DuoSet (R&D Systems, # DY208) according to the manufacturer's protocol.

### **Immunofluorescence staining**

$10^4$  cells per well were seeded in an 8 well chamber slides (Corning, Corning, NY, USA # 354118). After treatments, medium was removed and cells were washed twice with PBS. Keratocytes were fixed with 3.7% paraformaldehyde in 1xPBS for 20 minutes at room temperature, permeabilized with 0.1% Triton X-100 in 1xPBS for 5 minutes, then blocked for 30 minutes with 1% BSA in PBS containing 0.1% Tween-20 (PBS-T). Cells were incubated

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with anti-vinculin antibody (Sigma, #V9131) for 1 hour. After washing, secondary antibody labelled with TRITC (Dako, Glostrup, Denmark, #R0270) was added together with BODIPY®FL phalloidin (Life Technologies, #B607) for 30 minutes. Finally, cells were mounted in ProLong® Diamond Antifade Mountant with DAPI (Life Technologies, #P36962). A control well was also prepared for secondary antibody by replacing the primary antibody with PBS. A Zeiss Axioskop 2 plus microscope equipped with epifluorescence and an Olympus DP70 digital camera were used for analysis.

### **Scratch Assay**

$10^6$  keratocytes were seeded into 6 well plates in DMEM /F12 medium supplemented with 0.1% FBS and allowed to adhere overnight. Next morning, medium was replaced with fresh one, and bottom of the well was scratched with 200 $\mu$ l pipette tip, leaving control wells not scratched. Cells were washed with PBS and fresh medium was added. Supernatants were collected (for SP EIA) at 8 and 24 hours, cells were lysed in RLT buffer (for TAC1, TACR-1 RT-qPCR) at 8h.

For migration scratch assay,  $0.4 \times 10^6$  keratocytes were seeded into 6 well plates in DMEM/F12 medium without FBS and allowed to adhere overnight. Next morning, medium was replaced with fresh one, and bottom of all wells was scratched with 200 $\mu$ l pipette tip, creating a wound field. Cell were washed with PBS and treated as described in cell culture and treatments section. Images of the wounds were taken at same spots, at times 0, 8h, 24h and 48h using Motic AE31 Trinocular inverted microscope. Cell migration was quantified by counting cells which migrated into the created wound field at 0h, 8h, 24h and 48h and compared to 0h (the number of cells that had migrated into the wound field at a specified time point was divided by the number of cells that were in the wound field at time 0).. Counting was done with ImageJ software.

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### **Chemotaxis**

CytoSelect™ 24-Well Cell Migration Assay (Cell Biolabs, #CBA-103 and # CBA-101) (transwell assay) was used to assess SP induced chemotaxis, involvement of PI3K in SP enhanced migration, and role of IL-8 in keratocyte migration. Isolated human neutrophils and keratocytes were used as migratory cell populations. Two different membrane pore sizes were used: 3µm for neutrophils and 8µm for keratocytes. The assay was used according to manufacturer's protocol. Briefly: 500µl of supernatants collected from cell treatments or fresh medium supplemented with SP or different concentrations of IL-8, was added to lower well of the migration plate. Suspension of either  $1.5 \times 10^6$  neutrophils or  $0.4 \times 10^6$  keratocytes in serum free medium was added to the inside of each insert. Plates were incubated for 18h in a cell culture incubator. After incubation time, media from the inside of the insert was aspirated and the insert was transferred to a clean well containing Cell Detachment Solution and incubated for 30 minutes at 37°C. Cells were dislodged from the underside of the membrane by tilting the insert several times in the detachment solution. Medium from the lower well of the plate was transferred to the well containing Cell Detachment Solution for the same migration assay sample (only neutrophils assay). 180µl of the mixture was transferred to a 96 well plate and treated with lysis buffer and CyQuant® GR dye solution for 20 minutes at room temperature. Mixture was transferred into 96 well plate suitable for fluorescence measurement (Corning, Corning, NY, USA, # 734-4122) and fluorescence was read with a Synergy HT plate reader at 480/520nm (BioTek, Winooski, VT, USA).

### **Cell proliferation assay**

3,000 keratocytes per well were seeded into 96 well plate in DMEM/F12 medium containing 0.1% FBS and allowed to adhere overnight. Cell were treated with two concentrations of SP

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( $10^{-7}$ M and  $10^{-5}$ M) or the following concentrations of IL-8: 0.25 ng/ml, 0.5 ng/ml, 0.75 ng/ml, 1 ng/ml, 10 ng/ml, 50 ng/ml and 100 ng/ml. Cell proliferation was measured with CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) according to manufacturer's instructions. Proliferation was measured at 0h, 8h, 24h, 48h and 72h after stimulation.

### **Statistical analysis**

Statistical analyses were performed by using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Data were expressed as means  $\pm$  standard deviation (SD). Statistical analysis was performed with one-way or two-way ANOVA and Bonferroni post hoc test. A *p* value of  $<0.05$  was predetermined as a statistically significant difference. All experiments were successfully performed at least 3 times. (i.e. at least three separate experiments were performed with cells isolated from different patients). All samples were prepared in triplicates.

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## Results

### Scratch injury increases SP production by keratocytes cells *in vitro*

To test whether corneal injury has an effect on SP production by keratocytes, primary human keratocytes were subjected to a scratch assay. Cells were grown in 6 well plates and a scratch injury was created by using a 200 $\mu$ l pipette tip. Cells were lysed 8h after induction of the injury and analyzed for TAC1 (SP) and TACR1 (NK-1R) gene expression quantification. Both TAC1 and TACR1 genes were upregulated after scratch injury (Fig. 1A). Moreover, supernatant from injured cells was collected and cells were lysed at 8h and 24h after injury. SP secretion and NK-1R protein expression were then measured by SP EIA and western blot, respectively. The analysis revealed that the levels of secreted SP were significantly elevated at 8h and 24h after injury (Fig. 1B). Expression of NK-1R was significantly increased at both 8h and 24h in cells subjected to scratch injury (Fig. 1C).

### SP increases migration of keratocytes *in vitro* through NK-1R

To investigate the possible effect of SP on migration of keratocytes, cells were subjected to scratch injury, and treated with two different concentrations of SP ( $10^{-7}$  and  $10^{-5}$ M). To evaluate if SP acts through its preferred receptor, NK-1R, cells were pretreated with the NK-1R antagonist L-733,060 ( $10^{-6}$ M) after the scratch injury before treatment with SP. Migration of keratocytes was observed under microscope and pictures were taken at 0h, 8h, 24h and 48h (Supplemental Figure 1). Cells were counted inside the created injury and compared to time 0. Cells treated with  $10^{-5}$ M SP showed enhanced migration towards the center of the scratch injury at 24h after injury, and at 48h both concentrations of SP significantly enhanced keratocyte migration (Fig. 2A). SP was shown to act through NK-1R, as the SP-induced increased migration of the cells was abolished at both 24h and 48h after L-733,060 pretreatment (Fig. 2A). To further quantify SP induced migration, we used CytoSelect™ cell



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transwell migration assay. SP in fresh medium served as a chemoattractant and keratocytes as migratory cells. Cells were allowed to migrate for 18h. Both concentrations of SP ( $10^{-7}$  and  $10^{-5}$ M) had a significantly positive effect on cell migration and this effect was dose dependent (Fig. 2B). Pretreatment of keratocytes with L-733, 060 ( $10^{-6}$ M) abolished the effect of SP on their migration (Fig. 2B). Additionally, SP had no proliferative effect on keratocytes (data not shown).

**SP induces actin cytoskeleton reorganization and formation of focal adhesion points in keratocytes *in vitro* by activation of PI3K**

Cytoskeletal changes and formation of focal adhesion points after cells were stimulated with SP were studied. Cytoskeleton changes occurred in cells stimulated with SP ( $10^{-5}$ M) and could be observed 8h and 48h after stimulation. As seen in Fig. 3, in control (untreated) cells actin filaments (F-actin) were well organized and distributed throughout the cell. The control cells had no formation of focal adhesion points (vinculin) and their shape was elongated. However, after stimulation with  $10^{-5}$ M SP (8h and 48h), spike-like filopodia were observed and lamellipodium could be found at the leading edges of the migrating cells. Prominent stress fibers appeared. The migrating cells also showed formation of numerous focal adhesion points. To confirm that SP acts through NK-1R in this regard, cells were pretreated with L-733,060 ( $10^{-6}$ M) and subsequently treated with  $10^{-5}$ M SP. Actin reorganization could then not be observed, the cells did not form a leading edge with lamellipodia, and no formation of focal adhesion points could be seen. The cells remained in their resting state. The same effect was observed for SP at a concentration of  $10^{-7}$ M (Supplemental Figure 2).

Furthermore, we studied PI3K involvement in actin reorganization and formation of focal adhesion points (Fig. 3). The PI3K inhibitor wortmannin treatment reduced formation of actin stress fibers. The cells did not have a leading edge, and no filo- or lamellipodia. A slight

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membrane ruffling could be observed. The cells had a spindle-like shape and did not form focal adhesion points at either 8h or 48h.

### **SP upregulates Rac1 and RhoA through activation of PI3K**

The expression of Rac1 and RhoA, the two proteins involved in regulation of cytoskeleton, in keratocytes was evaluated by western blotting at 8h and 48h after SP stimulation ( $10^{-7}$  and  $10^{-5}$ M) (Fig. 4A). The results showed that expression of both Rac1 and RhoA was upregulated in SP treated cells at both 8h and 48h. When cells were pretreated with L-733,060 ( $10^{-6}$ M) before SP-treatment, Rac1 and RhoA expression were at the same levels as for the control group (except for cells pretreated with L-733,060 together with  $10^{-5}$ M SP at 8h, in which both Rac1 and RhoA were upregulated). To examine whether PI3K is involved in SP-enhanced migration, we treated cells with 100nM wortmannin (a PI3K inhibitor) and SP, and found out that SP was not able to induce upregulation of either Rac1 or RhoA (Fig. 4A). To confirm PI3K importance in SP enhanced migration, keratocytes were pretreated with 100nM wortmannin and then subjected to chemotaxis in which SP  $10^{-7}$ M or SP  $10^{-5}$ M served as chemoattractant. Cells pretreated with wortmannin and subsequently treated with SP showed significantly decreased migration as compared cells treated with SP alone (Fig. 4B). Moreover, keratocytes pretreated with wortmannin in the scratch injury model, migrated significantly less when compared to cells treated with only SP at 8h, 24h and 48h (Fig. 4C).

### **SP increases IL-8 gene expression and secretion**

As it has been reported that SP is able to stimulate cells to produce IL-8, we wanted to see if SP would have this effect on keratocytes. First of all, IL-8 gene expression was assessed by RT qPCR after stimulation with two concentrations of SP ( $10^{-7}$  and  $10^{-5}$ M) (Fig. 5A). SP stimulation increased IL-8 gene expression in a dose dependent manner; higher concentration being more potent. The maximum expression occurred at 6h after stimulation, with a sharp

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decline at 12h. Next we aimed to explore the mechanism by which SP induced IL-8 expression. Two pathways which control IL-8 transcription (SAPK/JNK, NF- $\kappa$ B) and one pathway which control post-transcriptional changes to IL-8 mRNA (p38 MAPK) were analyzed. SP stimulation ( $10^{-7}$ M and  $10^{-5}$ M) led to phosphorylation of SAPK/JNK (p46) after 1 and 5 minutes for SP  $10^{-5}$ M and after 5 minutes for SP  $10^{-7}$ M. SP stimulation also led to phosphorylation of NF- $\kappa$ B p65 with a peak at 15 and 30 minutes for both concentrations of SP. Additionally, a second peak of phosphorylated NF- $\kappa$ B p65 could be observed at 180 minutes for both concentrations of SP (Fig. 5B and C). SP stimulation did not result in p38 MAPK phosphorylation (data not shown). Next, to check if SP stimulation leads to increased IL-8 secretion, we treated cells with two different concentrations of SP ( $10^{-7}$  and  $10^{-5}$ M) and performed ELISA (Fig. 5D). IL-8 secretion was seen to also be dose dependent. Higher concentration of SP had a significant effect on IL-8 secretion already at 8h after stimulation, whereas lower concentration only at 48h. There was a significant difference between the two concentrations used. However, at 72h after stimulation, this difference could not be observed anymore. To check NK-1R involvement in SP induction of IL-8 secretion, cells were pretreated with two concentrations of L-733,060 ( $10^{-7}$  and  $10^{-6}$ M) and subsequently treated with SP ( $10^{-7}$  and  $10^{-5}$ M) for 24h. IL-8 secretion was measured by ELISA (Fig. 5E). The results showed that IL-8 secretion was decreased when pretreated with both concentrations of L-733,060 as compared to SP alone, indicating that SP acts through NK-1R in order to stimulate IL-8 expression.

### **IL-8 secreted by keratocytes after SP stimulation increases their migration**

It is known that IL-8 induces cell migration. Therefore, we wanted to determine whether IL-8 secreted after SP stimulation has an effect on keratocytes migration. First, expression of IL-8 receptors CXCR1 and CXCR2 in keratocytes was confirmed by western blot (Supplemental

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Figure 3). To test whether IL-8 is involved in keratocyte migration, a scratch assay was performed. Cells were scratched with a 200µl pipette tip and treated with  $10^{-7}$ M and  $10^{-5}$ M SP. 8h after stimulation IL-8 neutralizing antibody or normal rabbit IgG isotype control were added (3µg/ml). Images were taken at 0h, 8h, 24h and 48h to observe cell migration towards the center of the scratch (Supplemental Figure 4). Cells were counted inside the created injury and compared to time 0. Keratocytes treated with IL-8 neutralizing antibody showed decreased migration when compared to cells treated with either SP or isotype control and SP. This effect could be observed at 24h and 48h after SP stimulation (Fig. 6A). To test if cell migration was dependent on IL-8 concentration, CytoSelect™ cell transwell migration assay was used. Keratocytes served as migratory cells. As a chemoattractant we used supernatants collected from keratocytes untreated (control), treated with SP ( $10^{-7}$ M and  $10^{-5}$ M) or pretreated with L-733,060 ( $10^{-6}$ M) before SP treatment. IL-8 concentrations were measured in these supernatants (Supplemental Table 1). Cells were allowed to migrate for 18h. Migration of keratocytes, for which supernatant collected from SP treated cells served as a chemoattractant, was increased when compared to the control. Cell migration was, however, not enhanced when supernatant from L-733,060 and SP treated cells was used (Fig. 6B). An enhanced migration was corresponding to higher concentration of IL-8 (Supplemental Table 1). To further confirm IL-8 involvement in keratocyte migration, we used supernatants from keratocytes that were untreated or treated with SP ( $10^{-5}$ M) and/or IL-8 neutralizing antibody and/or normal rabbit IgG control (3µg/ml) as chemoattractants. Again, IL-8 concentrations were measured in these supernatants (Supplemental Table 2). Keratocytes served as migratory cells. Migration of keratocytes was increased with supernatant collected from SP and isotype control + SP treated cells, whereas cell migration was significantly decreased when supernatant collected from SP + IL-8 neutralization treated cells was used as a chemoattractant (Fig. 6C). Interestingly, supernatant collected from cells treated with IL-8

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neutralization antibody alone, also decreased cell migration when compared to supernatant of untreated cells. Again, enhanced migration was corresponding to higher concentration of IL-8. Finally, IL-8 receptors (CXCR1 and CXCR2) were blocked on keratocytes and these cells were allowed to migrate towards supernatant collected from cells treated with SP ( $10^{-5}$ M) (in which IL-8 concentration was measured (Supplemental Table 3)). Blockage of IL-8 receptors significantly decreased cell migration when compared to cells with IL-8 receptors not blocked or cells treated with mouse IgG2A isotype control, indicating that IL-8 is partially involved in SP induced migration (Fig. 6D). Moreover, to confirm that IL-8 is able to induce keratocyte migration, we tested seven concentrations (0.25ng/ml, 0.5ng/ml, 0.75ng/ml, 1ng/ml, 10ng/ml, 50ng/ml and 100ng/ml) of recombinant human IL-8 and performed scratch assay and chemotaxis assay. Cells were scratched with a 200 $\mu$ l pipette tip and treated with various concentrations of IL-8. Images were taken at 0h, 8h, 24h and 48h to observe cell migration towards the center of the scratch. Cells were counted inside the created injury and compared to time 0. At 8h, all concentrations of IL-8 except 0.25 ng/ml and 100 ng/ml significantly increased keratocyte migration. 24h after scratch injury all concentrations of IL-8 had positive effect on cell migration. At 48h only 0.5 ng/ml, 0.75 ng/ml and 1 ng/ml of IL-8 significantly increased keratocyte migration (Fig. 6E). CytoSelect™ cell transwell migration assay was used to test if various concentrations of IL-8 are able to attract keratocytes. Keratocytes were allowed to migrate for 18h. 0.5ng/ml, 0.75ng/ml and 1ng/ml of IL-8 significantly enhanced keratocyte migration, whereas 0.25 ng/ml, 10ng/ml, 50ng/ml and 100ng/ml had a positive effect on the migration, although not statistically significant (Fig. 6F). Additionally, IL-8 had no effect on keratocyte proliferation (data not shown).

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### **SP increases neutrophil migration indirectly through IL-8**

To test whether IL-8 secreted by keratocytes cells after SP stimulation is able to attract neutrophils, or if SP is able to attract neutrophils directly, CytoSelect™ cell transwell migration assay was used. Isolated human neutrophils were used as migratory cells. Cells were allowed to migrate for 18h. First, supernatants collected from keratocytes untreated (control), treated with either SP ( $10^{-7}$ M and  $10^{-5}$ M) or L-733,060 ( $10^{-6}$ M) and SP ( $10^{-7}$ M and  $10^{-5}$ M), were used as chemoattractant. Concentrations of IL-8 were measured in these supernatants (Supplemental Table 4). The results showed that isolated human neutrophils migrated significantly more towards the supernatants collected from SP treated keratocytes than towards control. Supernatants collected from L-733,060 and SP treated cells did not enhance cell migration (Fig. 7A). Again, enhanced migration was corresponding to higher concentration of IL-8. Next, we tested if SP is able to attract neutrophils directly. Fresh medium supplemented with two different concentrations of SP ( $10^{-7}$  and  $10^{-5}$ M) was used as a chemoattractant. Neither concentration of SP had a positive effect on isolated human neutrophils migration. Moreover, when neutrophils were pretreated with L-733,060 ( $10^{-6}$ M) and subsequently treated with SP, no migratory effect was observed (Fig. 7B), indicating that SP induced neutrophil migration through IL-8 but not directly.

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## Discussion

The cornea is the major refractive unit of the eye. Although relatively well protected from injury, when injured, a proper wound healing response in the cornea is needed in order to avoid scarring and subsequent visual loss or even blindness. SP and its receptor NK-1R, have been identified in rat and rabbit corneal epithelial cells in which they are believed to play a role in maintaining corneal integrity and wound healing (Nakamura et al., 2003; Nakamura et al., 1997; Yamada et al., 2005). It has also been shown that human stromal keratocytes express SP and NK-1R (Watanabe et al., 2002), which has also been confirmed in our laboratory for the keratocyte cell culture model used in the present study (Sloniecka et al., 2015). However, little is known about the role of SP and its receptor in corneal stroma wound healing. In this study, we show that injured cultured human keratocytes produce more SP than resting cells and that they are able to respond to SP stimulation with increased migration and with expression of the proinflammatory and chemotactic protein IL-8, which further stimulates the cells to migrate and also attracts neutrophils. Two different concentrations of SP were tested. The concentrations used were tested for toxicity and found not to be toxic to keratocytes. As there is no available data on endogenous SP levels *in vivo* in the human cornea, it is not evident what concentrations should be used in *in vitro* studies. In other species, such as for example mouse and rabbit, the endogenous SP levels are 40.5-68.6 pg/cornea and 5.1pmols/g, respectively (Keen et al., 1982; Stjernschantz et al., 1982). There is data available for SP levels in human tear fluid:  $306 \pm 96.5$  pg/ml (Yamada et al., 2002). When compared to the concentrations of SP we have used in our experiments, the SP concentration in tear fluid is about 400 (for SP  $10^{-7}$ M) and 40,000 (for SP  $10^{-5}$ M) times less. This is reasonable, as SP in the tear fluid would be much diluted as compared to SP in the interstitial extracellular fluid around the keratocytes that produce and secrete SP. Moreover, the source of SP in the cornea *in vivo* is far more than keratocytes themselves, which makes it

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likely that the endogenous levels around keratocytes *in vivo* are far greater than what is produced in culture dishes *in vitro*. For instance, nerves, such as the trigeminal nerve, also produce SP (Garcia-Hirschfeld et al., 1994; Ko et al., 2014; Kowtharapu et al., 2014), and corneal epithelial cells also have been found to produce it (Watanabe et al., 2002). The choice of the two concentrations which have been used in this study is based on other studies in which wide range of SP concentrations have been used, including the two concentrations used in this study (Backman and Danielson, 2013; Koon et al., 2004; Koon et al., 2007; Villablanca et al., 1994; Yang et al., 2014).

Both concentrations enhanced keratocytes migration in a dose dependent manner. Cell migration is initiated in response to various signals coming from cells or extracellular matrix (Ridley et al., 2003). Actin cytoskeleton reorganization, which includes formation of filopodia, lamellipodia, and stress fibers, are essential for successful cell motility (Lamallice et al., 2007). We found that treatment of keratocytes with SP leads to formation of filo- and lamellipodia on the leading edge of the cells and it increased actin stress fibers. Moreover, expression of focal adhesion protein, vinculin, was observed in the migrating cells. The cytoskeleton changes induced by SP lasted for a long time, suggesting that the SP effect on cell migration is relatively persistent. Expression of Rac1 and RhoA (Ridley, 2001), two proteins involved in regulation of cytoskeleton, was increased in cells stimulated with SP. Their expression was diminished in cells for which the SP receptor was blocked, indicating that SP induced cell migration through its preferred receptor (NK1-R). PI3K is a major regulator of key cellular functions, such as cell growth, aging and cell motility (Cain and Ridley, 2009), and it has been shown to activate Rac1 at the leading edge of the migrating cells (Welch et al., 2003). It is also involved in a SP signaling pathway leading to proinflammatory responses in mouse macrophages (Sun et al., 2009). In this study we showed that PI3K is critical for SP induced cell migration by inhibiting PI3K with



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wortmannin. By using western blot analysis we found that SP was not able to increase Rac1 and RhoA expression when PI3K was inhibited. Moreover, the changes of cytoskeleton induced by SP were no longer observed when cells were pretreated with wortmannin. Taken together, our data suggests that SP enhances keratocyte migration through PI3K, which also acts as an upstream activator of Rac1 and RhoA.

It has been reported that SP stimulates human colonic epithelial cells (Koon et al., 2005), human astrocytoma cells (Palma and Manzini, 1998), and human corneal epithelial cells (Tran et al., 2000) to produce the chemotactic protein IL-8. It has furthermore been shown that SP regulates the synthesis of this protein post-transcriptionally, making IL-8 transcripts more stable in human astrocytoma cell lines and human corneal epithelial cells (Palma and Manzini, 1998; Tran et al., 2000). Our results showed that SP upregulates IL-8 gene expression in keratocytes, with a peak at 6h and the mechanism behind this IL-8 gene increase seems to involve phosphorylation of both SAPK/JNK and NF- $\kappa$ B, which have been shown to control IL-8 gene expression at transcription level (Baud et al., 1999), rather than phosphorylation of p38 MAPK, which controls the IL-8 expression at post-transcriptional level (Holtmann et al., 1999; Winzen et al., 1999). Although the p38 pathway contributes to IL-8 expression, it is not essential, whereas NF- $\kappa$ B is (Feoktistov et al., 1999). In order to have significant increase in IL-8 expression, activation of NF- $\kappa$ B and at least one other pathway is needed (Hoffmann et al., 2002). However, maximal amounts of IL-8 can only be achieved if NF- $\kappa$ B pathway, SAPK/JNK pathway, and p38 MAPK are all activated (Hoffmann et al., 2002). The lack of p38 MAPK kinase activation after SP stimulation of keratocytes in the present study, might explain the significant but not robust increase in IL-8 gene secretion and expression. SP treated cells secreted more IL-8 protein than controls and this effect was dose dependent. Additionally, NK-1R was necessary to convey SP effect on IL-8 production. IL-8 is a chemoattractant but it has also been shown to stimulate cell

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migration in endothelial cells (Lai et al., 2011) and cancer cells (Yin et al., 2014). Our results suggest that IL-8 secreted by keratocytes due to SP stimulation is responsible for SP enhanced keratocyte migration. This could be established by the fact that neutralization IL-8 abolished the SP effect on keratocyte migration. Furthermore, using supernatants collected from keratocytes treated with SP and with measured IL-8 concentrations, we could see that increased keratocyte migration correlates to higher IL-8 concentrations in the supernatant. Interestingly, IL-8 neutralization revealed that endogenous levels of IL-8 produced by keratocytes make the cells more motile, since when control cells (not treated with SP) were treated with IL-8 neutralizing antibody, their migration was lower than control cells without IL-8 neutralization. Lastly, when IL-8 receptors CXCR1 and CXCR2 were blocked, migration of keratocytes was decreased, confirming that IL-8 is important in the SP enhanced migration. However, also other cytokines may be produced by keratocytes due to SP stimulation and thereby contribute to the enhanced migration, as we did observe that recombinant IL-8 alone was not as effective in enhancing migration as SP or supernatants collected from SP treated cells. In fact, we have found that at least two other cytokines are produced after SP treatment: MCP-1 (Monocyte Chemoattractant Protein-1) and RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) (unpublished data).

The issue of the high basal IL-8 secretion in untreated keratocytes of this study should be addressed. One possibility is that the cultured keratocytes are in an activated state, due to for example presence of FBS in the culture. However, in a recent study of ours (Sloniecka et al., 2015), we have examined the phenotype of the cells used in this study. As FBS is known to alter the phenotype of keratocytes and activate them, we have compared keratocyte markers in keratocytes grown with or without 2% FBS. Our results showed that the keratocytes grown both with and without FBS express keratocyte markers (such as keratocan, lumican, CD34,

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and ALDH), and express only very low amounts of e.g.  $\alpha$ -SMA (a marker of myofibroblasts), and therefore they are not in an activated state (Sloniecka et al., 2015). The reason for high basal IL-8 level remains unknown. However, we have observed that the basal IL-8 level is not passage dependent. It seems to be a cumulative event, i.e. the longer the cells are in culture (at a specified passage) the more IL-8 is secreted. It has been shown that growing keratocytes in medium containing low glucose concentrations (1,000 mg/L) has been beneficial for maintaining keratocyte phenotype. Therefore, we have tested if medium containing low glucose concentration has an effect on IL-8 secretion by keratocytes (unpublished data). Our results have shown that there is a slight, but not significant, decrease in IL-8 secretion in keratocytes grown in the low glucose medium when compared to the IL-8 secretion from cells grown in high glucose medium (3,151 mg/L). We have also tested IL-8 secretion in keratocytes grown on collagen I (as the cornea is composed of collagen I). Surprisingly, keratocytes grown on collagen I secrete almost twice as much IL-8 when compared to keratocytes grown on uncoated surface (both for high and low glucose media; unpublished data). There is no data indicating how much IL-8 is produced by keratocytes or other cells in the healthy cornea *in vivo*. However, studies show that IL-8 in tear fluid in healthy individuals oscillate between 148-414 pg/ml (Carreno et al., 2010).

Neutrophils play an important role in the cornea protection. They provide protection against pathogens and foreign substances on the eye surface (Waring and Rodrigues, 1987). However, as the cornea has no blood supply or lymphatic drainage, the nearest source of neutrophils can be found in blood vessels of the corneal limbus (Van Buskirk, 1989), i.e. in the periphery adjacent to the tunica conjunctiva. Therefore, a powerful chemokine is needed to attract neutrophils to the cornea, such as IL-8 which is known to be able to attract neutrophils into inflamed tissues (Baggiolini et al., 1994). On the other hand, neutrophil

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infiltration in an injured cornea needs to be tightly controlled, otherwise the release of various proteases by neutrophils may result in stromal degradation and ulceration, which in turn might lead to corneal opacity and neovascularization (Brown et al., 1970), causing decreased vision or even blindness. SP has been reported to chemoattract rabbit neutrophils (Marasco et al., 1981), activate human neutrophils (Serra et al., 1988), and increase neutrophil adhesion to bovine bronchial epithelial cells (DeRose et al., 1994). However, our results show that SP is not able to attract human neutrophils directly, but that it does so by stimulating keratocytes to produce IL-8, which then attracts neutrophils.

In summary, we conclude that SP, binding to its preferred receptor NK-1R, through activation of IP3 kinases and the Rac1/RhoA pathway is able to enhance keratocyte migration after injury, thus facilitating wound healing. Moreover, SP stimulates keratocytes to produce IL-8, which further enhances cell migration and, as this IL-8 was also shown to be a chemoattractant of human neutrophils, it is possible that SP might help initiate acute inflammation within the corneal stroma after injury due to trauma or infection.

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## **Acknowledgements**

The authors thank Dr. Ludvig Backman, Dr. Jialin Chen, and Dr. Gabor Borbely for technical and scientific advice. We also thank Dr. Maria Brohlin and Ms. Randi Elstad for help in providing the donated corneas from the biobank, as well as to all the OR-staff at the Ophthalmic Surgery Clinic, especially Dr. Berit Byström, of the University Hospital of Umeå for deliverance of graft leftovers.

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### **Authorship Contributions**

*Participated in research design:* Słoniecka and Danielson

*Conducted experiments:* Słoniecka

*Performed data analysis:* Słoniecka, Le Roux, and Danielson

*Wrote or contributed to the writing of the manuscript:* Słoniecka, Zhou, and Danielson

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## Footnotes

This work was supported by the national Swedish Research Council [Grant 521-2013-2612]; the J.C. Kempe and Seth M. Kempe Memorial Foundations [Grant JCK-1222]; the Swedish Society of Medicine [Grants SLS-176511, SLS-248321, SLS-329341, SLS-410021]; the Cronqvist foundation [Grants SLS-120651, SLS-249071, SLS-329561]; the foundation Kronprinsessan Margaretas Arbetsnämnd för synskadade [Grants 2010/30, 2012/26, 2013/10]; the foundation Ögonfonden; Västerbotten County Council 'Spjutspetsmedel'; and a regional agreement (ALF) between Umeå University and Västerbotten County Council.

Preliminary data from this work were presented by Słoniecka et al. in poster sessions at the following meetings: The ARVO Annual Meeting 2014 May 4-8, Orlando, FL, USA, and the Wound Healing Society Annual Meeting, San Antonio, TX, USA, 2015 April 29 - May 3.

These studies are part of the PhD-thesis work of Marta Słoniecka, Umeå University, December 2015.

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## Figure Legends

**Fig. 1.** Scratch injury upregulates TAC1 and TACR1 gene expression, as well as substance P (SP) secretion and NK-1R expression, in human keratocytes. (A) RT qPCR showed that genes for both SP (TAC1) and its receptor, the neurokinin-1 receptor (TACR1), were upregulated in human keratocytes 8h after a scratch injury was induced *in vitro*. (B) SP secretion, as quantified by EIA, was significantly increased in human keratocytes at 8h and 24h after scratch injury. (C) NK-1R expression, analyzed by western blot and densitometry was significantly increased at 8h and 24h after scratch injury. Values are means  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

**Fig. 2.** SP enhances migration of keratocytes through the neurokinin-1 receptor (NK-1R). (A) Quantification of keratocyte migration after scratch injury. Scratch injury was introduced to keratocytes *in vitro*. Cells were pretreated with NK-1 R antagonist L-733,060 ( $10^{-6}$ M). Afterwards two different concentrations of SP ( $10^{-7}$ M and  $10^{-5}$ M) were tested. Cells were counted inside the created injury and compared to time 0 at 8h, 24h and 48h after scratch. SP  $10^{-5}$ M strongly enhanced cell migration at 24h. At 48h after introduction of scratch injury both concentrations of SP significantly enhanced keratocyte migration. Pretreating cells with L-733,060 abolished the SP enhanced migration. (B) Quantification of a keratocyte transwell migration assay, in which SP served as a chemoattractant. Two concentrations of SP were used ( $10^{-7}$ M and  $10^{-5}$ M). Both SP  $10^{-7}$ M and SP  $10^{-5}$ M had a positive effect on cell migration. Cell pretreatment with L-733,060 abolished that effect. Values are means  $\pm$  SD. *n.s.* (not significant)  $p > 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

**Fig. 3.** SP induces the actin cytoskeleton reorganization and formation of focal adhesions through the NK-1R and phosphoinositide 3-kinase (PI3K) activation. Cells were pretreated with the NK-1R antagonist L-733,060 or wortmannin, a PI3K inhibitor, and then SP ( $10^{-5}$ M)

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for 8h or 48h. Cells were fixed and immunostained with BODIPY FL phalloidin (F-actin; green), vinculin (red) and DAPI to visualize nuclei (blue). Filopodia (arrows) and lamellipodia (arrowheads) could be observed at the leading edge of migrating cells treated with SP at 8h and 48h (green). Prominent stress fibers were induced, especially at 48h (intracellularly; green). Cells treated with SP showed expression of focal adhesion protein (arrows), vinculin, at 8h and 48h (red). Cells pretreated with L-733,060 showed no formation of filopodia and lamellipodia, nor focal adhesion points or stress fibers. Cells pretreated with wortmannin did not show actin reorganization (no formation of stress fibers, leading edge, filopodia and lamellipodia) and did not express the focal adhesion protein (vinculin) either at 8h or at 48h.

**Fig. 4.** SP induces keratocyte migration through activation of phosphoinositide 3-kinase (PI3K) and the Rac1/RhoA pathway. (A) Cells were left untreated (ctrl) or treated with two different concentrations of SP ( $10^{-5}$ M and  $10^{-7}$ M). L-733,060 ( $10^{-6}$ M) was used as a NK-1R antagonist and wortmannin (100nM) was used to inhibit PI3K. Protein expression was assessed by western blot at 8h and 48h. Both Rac1 (22 kDa) and RhoA (21 kDa) levels were higher in SP treated cells. Pretreatment of cells with L-733,060 before SP prevented the increased Rac1 and RhoA levels in  $10^{-7}$ M SP treated cells. However, no such inhibitory effect of the NK-1R antagonist was present when cells were treated with  $10^{-5}$ M SP. Also wortmannin treatment prevented SP from acting and thus both Rac1 and RhoA protein levels were not increased.  $\beta$ -actin was used as a loading control (45 kDa). (B) Quantification of transwell migration assay in which keratocytes were pretreated with 100nM wortmannin for 1h and SP ( $10^{-7}$ M or  $10^{-5}$ M) was used as a chemoattractant. Pretreatment of keratocytes with wortmannin significantly decreased migration. (C) Quantification of keratocyte migration after scratch injury. Scratch injury was introduced to keratocytes. Cells were treated with



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100nM wortmannin for 1h or left untreated. Cells were subsequently treated with two different concentrations of SP ( $10^{-7}$ M or  $10^{-5}$ M). Cells were counted inside the created injury and compared to time 0 at 8h, 24h and 48h after scratch. Keratocytes treated with wortmannin showed decreased migration.

**Fig. 5.** SP upregulates IL-8 gene expression and protein secretion in keratocytes. (A) Stimulation of keratocytes with SP upregulated IL-8 gene expression in a dose dependent manner with a peak at 6h, as quantified by RT qPCR. (B and C) Western blot and densitometry analysis of keratocytes stimulated with SP ( $10^{-7}$ M and  $10^{-5}$ M). SP stimulation resulted in phosphorylation of SAPK/JNK (46 kDa) with a peak at 1 and 5 minutes after stimulation and phosphorylation of NF- $\kappa$ B (65 kDa) with a peak at 15, 30 and 180 minutes after stimulation. SAPK/JNK (46, 54 kDa) and NF- $\kappa$ B (65 kDa) served as unphosphorylated controls.  $\beta$ -actin was used as a loading control (45 kDa). (D) SP stimulation increased IL-8 secretion by keratocytes in a dose dependent manner, as assessed by ELISA. (E) Pretreatment of cells with the NK-1R inhibitor L-733,060 significantly decreased the SP-enhanced IL-8 secretion, assessed 24h after treatment by ELISA. Values are means  $\pm$  SD. *n.s.* (not significant)  $p > 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

**Fig. 6.** SP induced migration is mediated through IL-8. (A) Quantification of keratocyte migration after scratch injury. Scratch injury was introduced to keratocytes. Cells were treated with two different concentrations of SP ( $10^{-7}$ M and  $10^{-5}$ M). After 8h, IL-8 neutralizing antibody or normal rabbit IgG, as isotype control, were added (3 $\mu$ g/ml). Cells were counted inside the created injury and compared to time 0 at 8h, 24h and 48h after scratch. Treatment of cells with IL-8 neutralizing antibody significantly reduced SP enhanced migration after 24h and 48h. Isotype control alone had no effect. (B) Quantification of transwell migration assay, in which a supernatant collected from keratocytes treated with

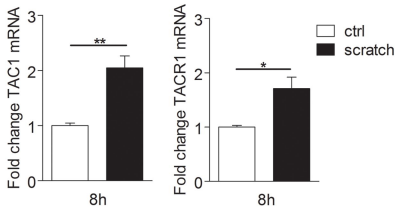
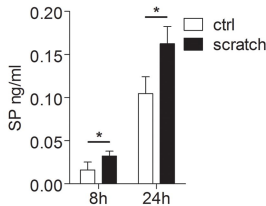
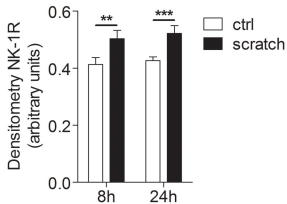
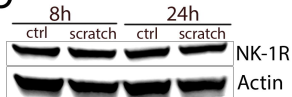
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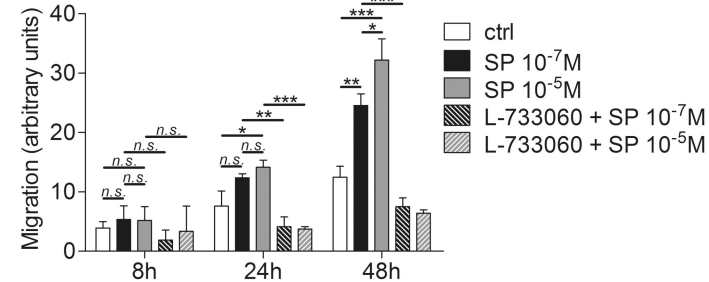
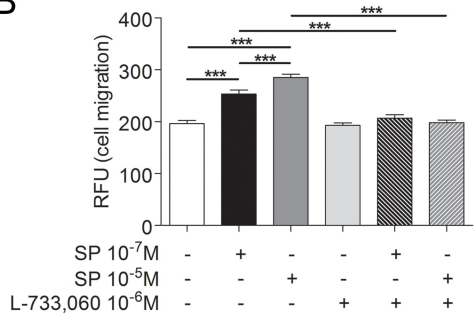
either SP ( $10^{-7}$ M and  $10^{-5}$ M) or L-733,060 ( $10^{-6}$ M) and SP, was used as a chemoattractant. Supernatant collected from cells treated with SP had a positive effect on cell migration, whereas supernatant collected from cell treated with L-733,060 and SP had no effect on cell migration. (C) Quantification of transwell migration assay, in which a supernatant collected from keratocytes treated with SP ( $10^{-5}$ M) and/or with rabbit IgG control and/or with IL-8 neutralizing antibody was used as a chemoattractant. Supernatants from cells treated with IL-8 neutralizing antibody and SP significantly reduced cell migration as compared to supernatant from cells treated with SP alone. Isotype control had no effect. (D) Quantification of transwell migration assay, in which CXCR1 and CXCR2 receptors were blocked on keratocytes. Supernatant collected from keratocytes treated with SP ( $10^{-5}$ M) was used as a chemoattractant. Mouse IgG2A served as isotype control. Blocking CXCR1 and CXCR2 significantly reduced cell migration. (E) Quantification of keratocyte migration after scratch injury. Scratch injury was introduced to keratocytes. Cells were treated with various concentrations of IL-8. Cells were counted inside the created injury and compared to time 0 at 8h, 24h and 48h after scratch. At 8h all concentrations of IL-8 except 0.25 ng/ml and 100 ng/ml significantly increased keratocyte migration. 24h after scratch injury all concentrations of IL-8 had positive effect on cell migration. At 48h only 0.5 ng/ml, 0.75 ng/ml and 1 ng/ml of IL-8 significantly increased keratocyte migration. (F) Quantification of transwell migration assay, in which different concentrations of IL-8 were used as a chemoattractant. 0.5ng/ml, 0.75 ng/ml and 1 ng/ml of IL-8 significantly enhanced keratocyte migration. Values are means  $\pm$  SD. *n.s.* (not significant),  $p > 0.05$ ,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ .

**Fig. 7.** SP has a chemotactic effect on neutrophils via IL-8. Quantification of transwell migration assay of isolated human neutrophils. (A) Supernatant from keratocytes with known concentrations of secreted IL-8 after either SP or L-733,060 and SP treatment was used as a

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chemoattractant. Neutrophils migrated significantly more when supernatant collected from SP treated keratocytes was used. Supernatant collected from keratocytes treated with L-733,060 and SP had no effect on neutrophils migration. (B) SP was used as a direct chemoattractant in fresh medium. SP had no direct effect on neutrophils migration. Pretreating cells with L-733,060 and SP had no effect on neutrophils migration either. Values are means  $\pm$  SD. *n.s.* (not significant) \*\*\* $p < 0.001$ .

**A****B****C****Figure 1**

**A****B****Figure 2**

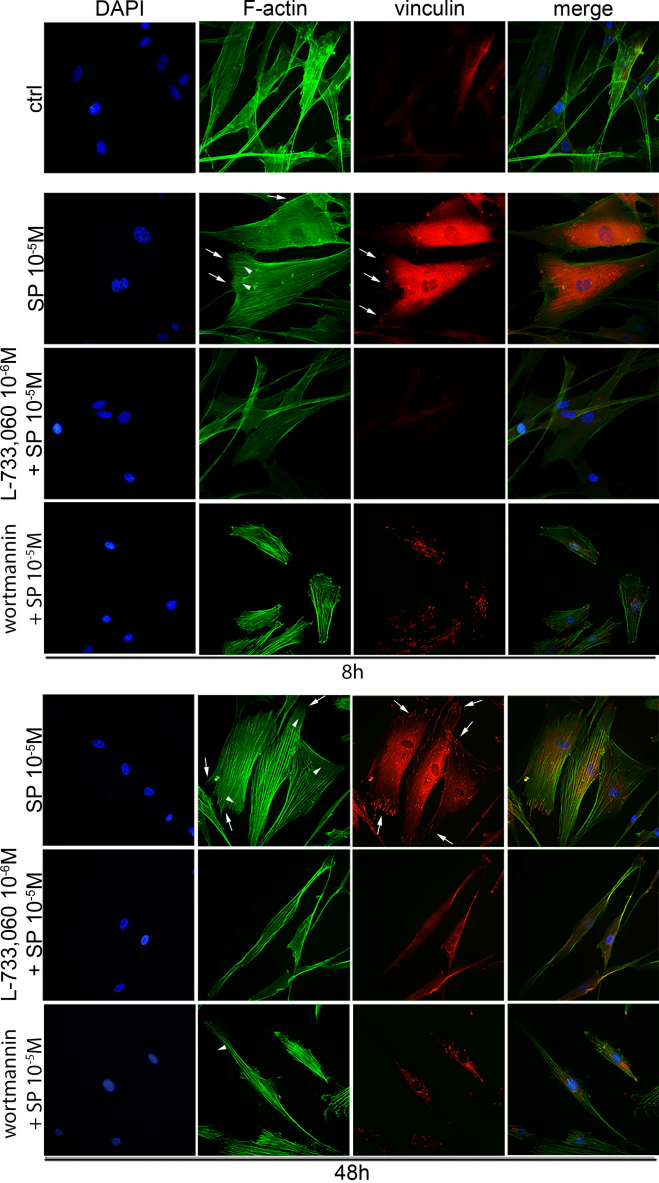
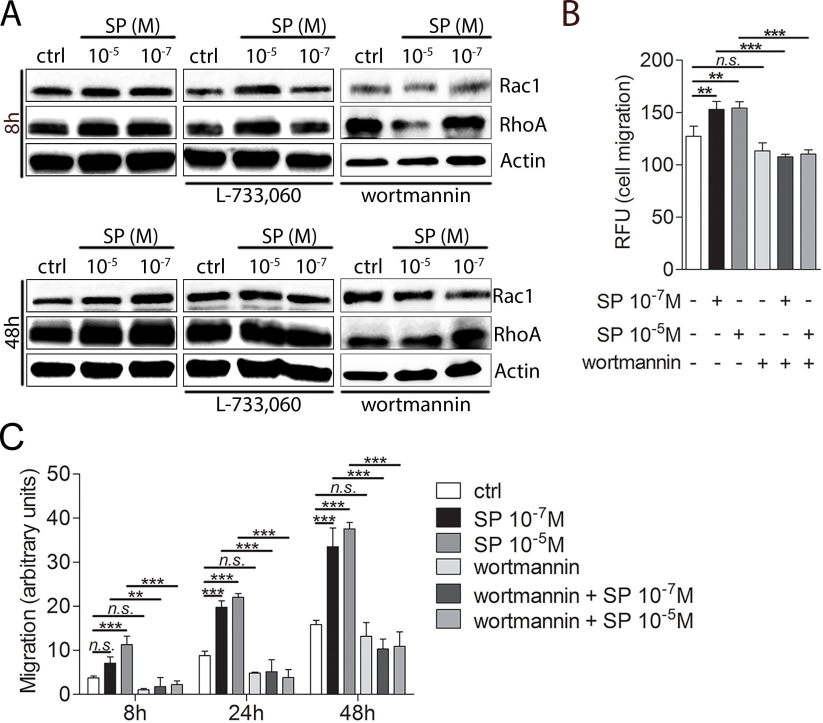


Figure 3



**Figure 4**

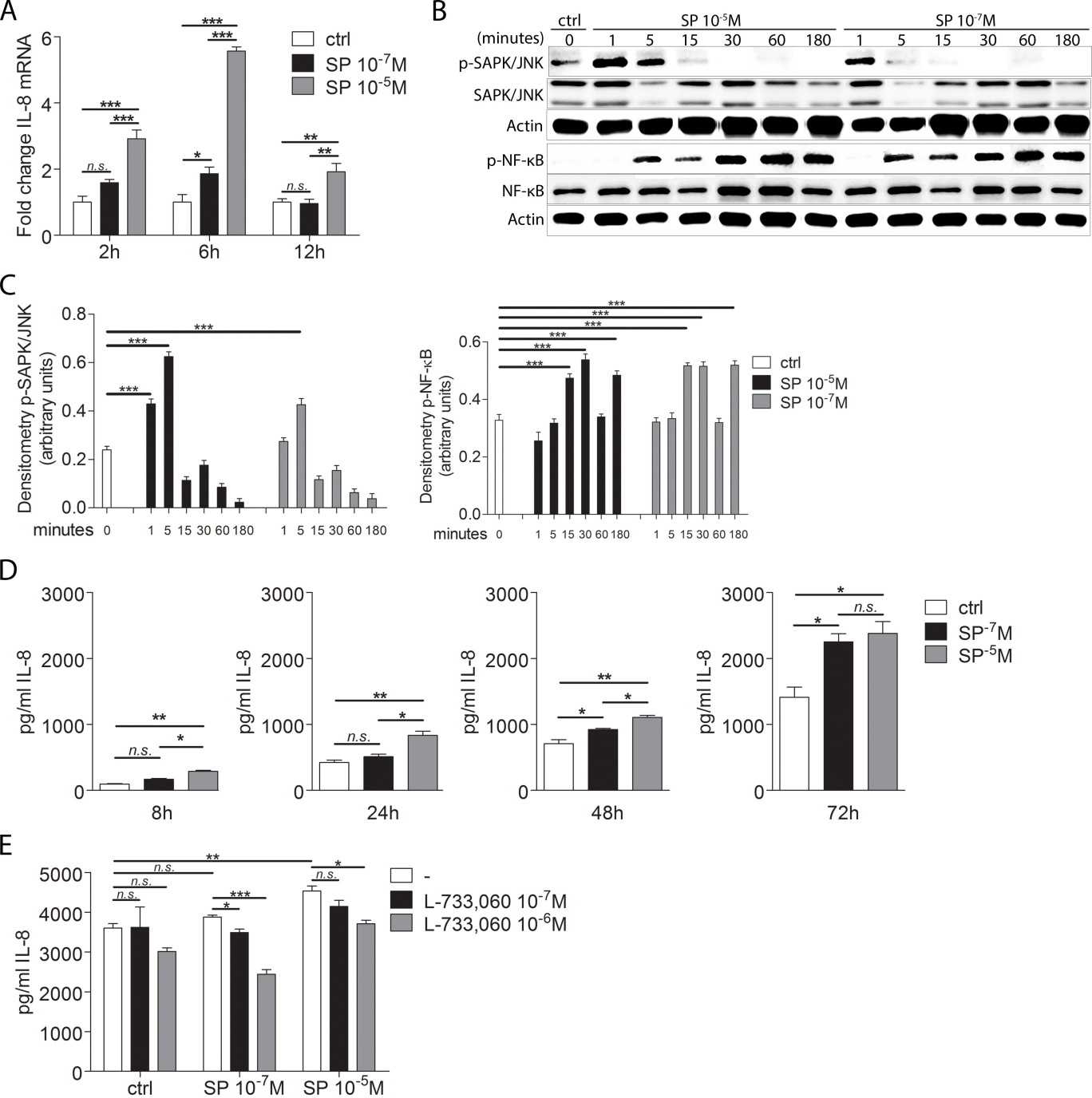
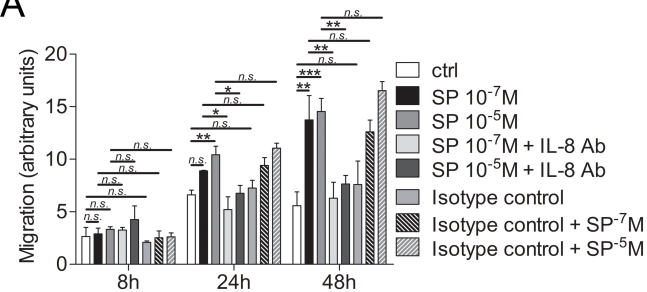
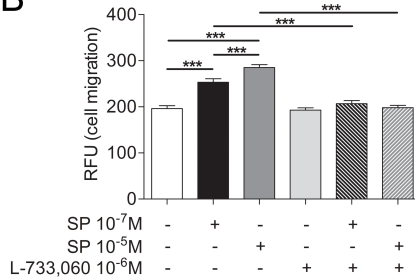
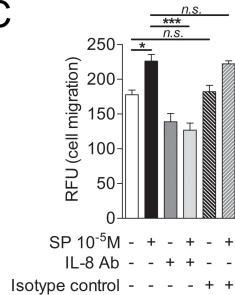
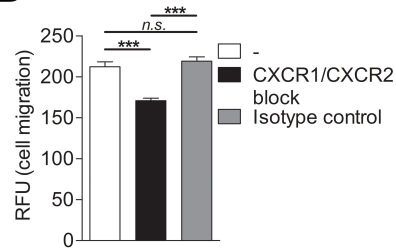
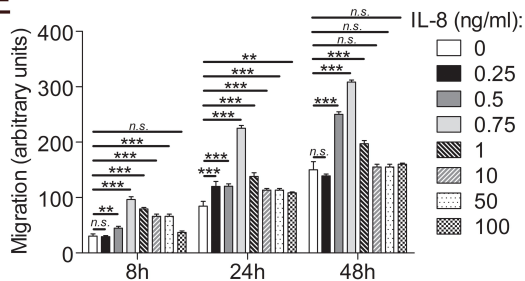
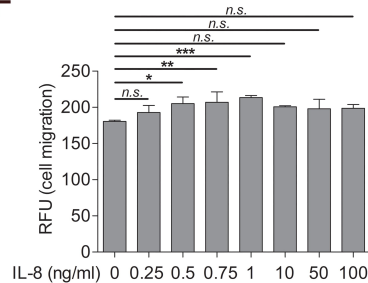


Figure 5



**A****B****C****D****E****F****Figure 6**

A

B

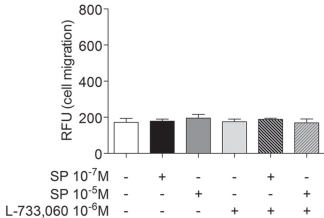
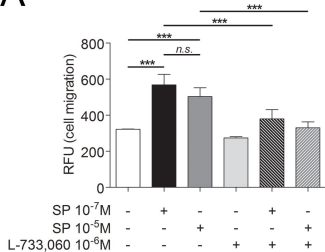


Figure 7

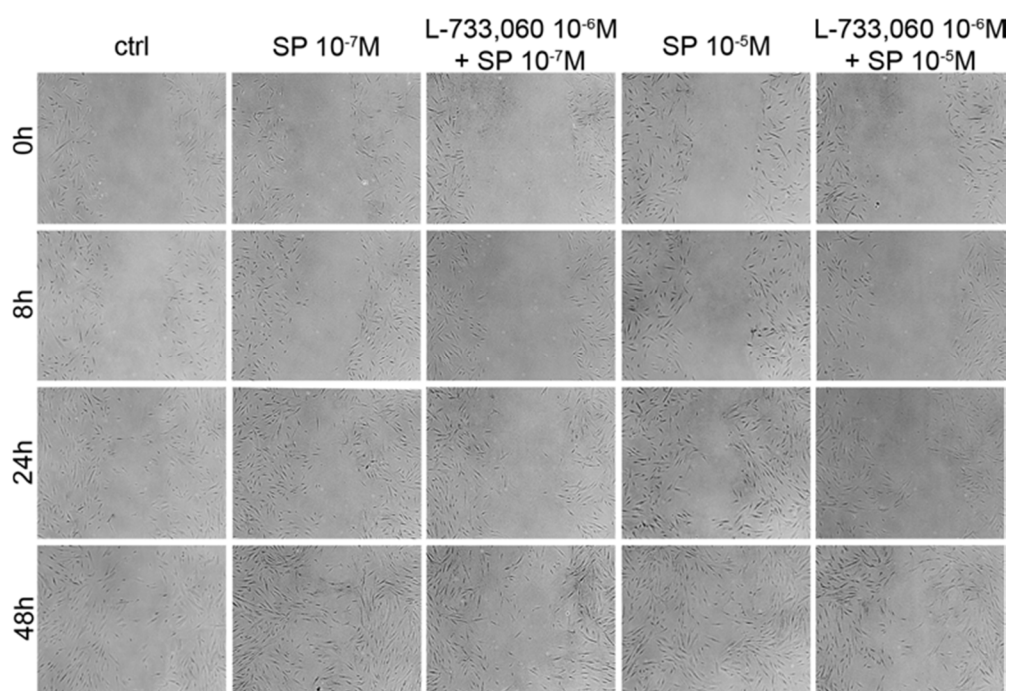
**Supplemental Information, Figures and Tables, for:**

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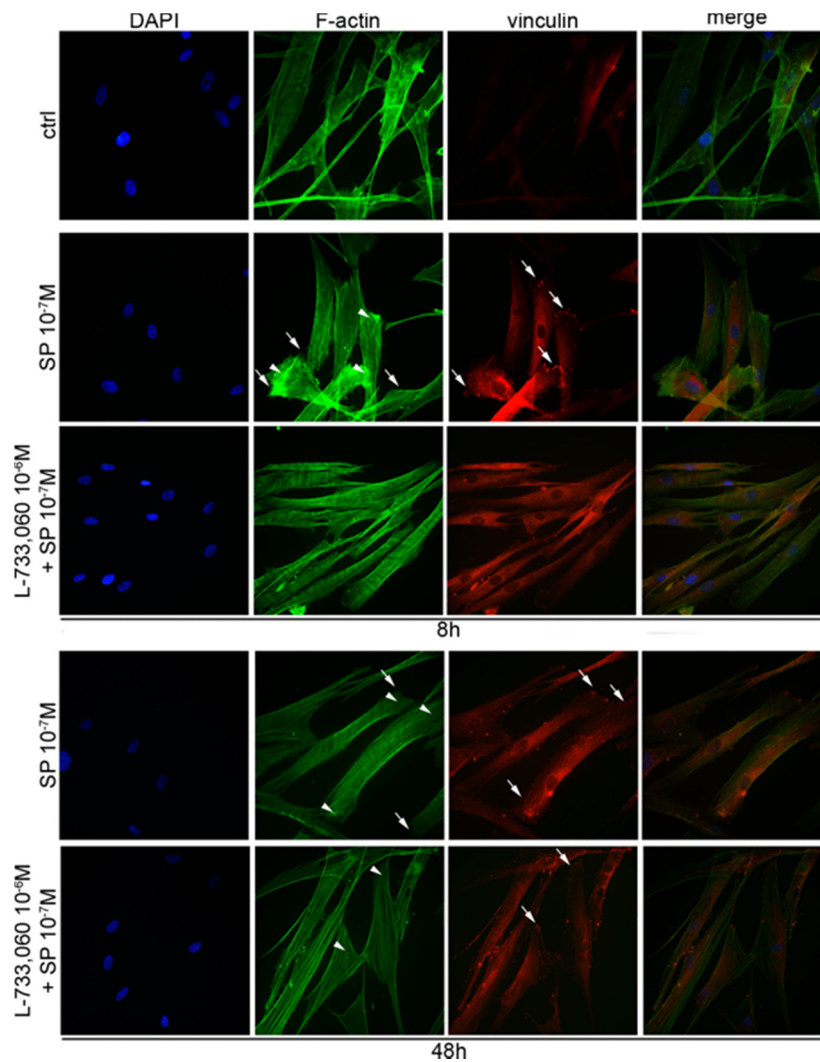
**Substance P enhances keratocyte migration and neutrophil recruitment through interleukin-8**

Marta Słoniecka, Sandrine Le Roux, Qingjun Zhou, Patrik Danielson

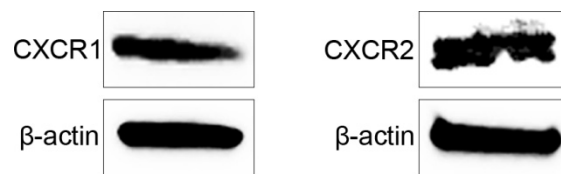
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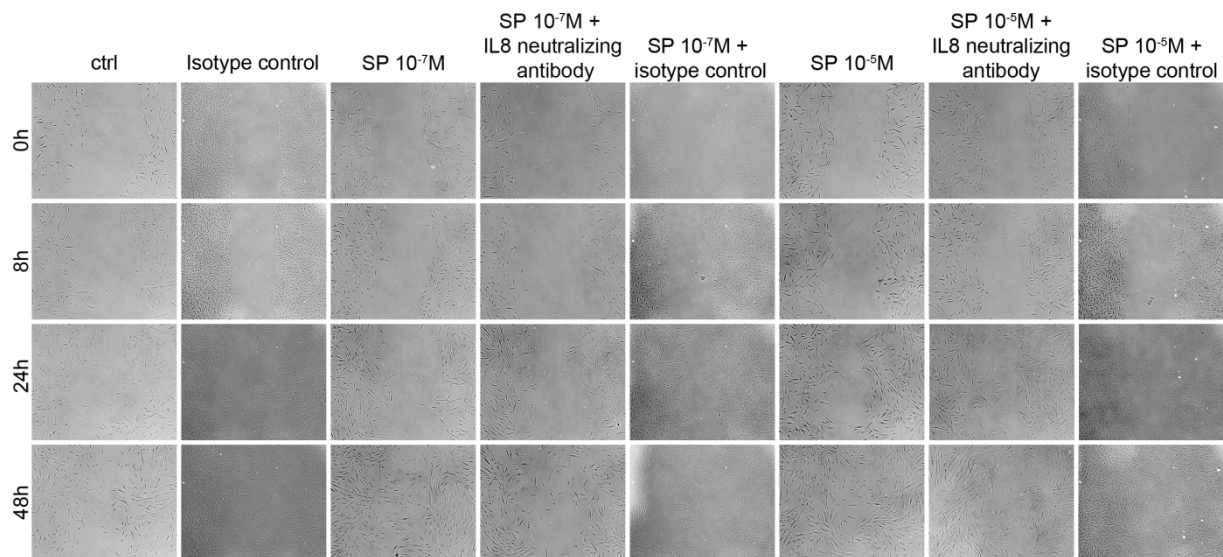
**Supplemental Figure 1.** Substance P (SP) enhances migration of keratocytes through the neurokinin-1 receptor (NK-1R). Representative pictures of keratocytes after scratch injury, untreated, treated with two concentrations of SP ( $10^{-7}$ M and  $10^{-5}$ M) or pretreated with the NK-1R antagonist L-733,060 ( $10^{-6}$ M) before SP-treatment at 0h, 8h, 24h and 48h. Both concentrations of SP strongly enhanced cell migration 48h after introduction of scratch injury. Pretreating cells with L-733,060 reduced the SP enhanced migration.



**Supplemental Figure 2.** SP induces the actin cytoskeleton reorganization and formation of focal adhesions through NK-1 receptor activation. Cells were treated with L-733,060 and/or SP (10<sup>-7</sup>M) for 8h or 48h. Cells were fixed and immunostained with BODIPY FL phalloidin (F-actin; green), vinculin (red) and DAPI (blue). Filopodia (arrows) and lamellipodia (arrowheads) could be observed at the leading edge of migrating cells treated with SP at 8h and 48h (green). Prominent stress fibers were induced, especially at 48h (intracellularly; green). Cells treated with SP showed expression of focal adhesion protein (arrows), vinculin at 8h and 48h (red). Cells pretreated with L-733,060 showed no formation of filopodia and lamellipodia, nor focal adhesion points or stress fibers 8h after SP stimulation, however after 48h few filopodia and focal adhesion points could be observed.



**Supplemental Figure 3.** Keratocytes express IL-8 receptors CXCR1 and CXCR2. Expression of CXCR1 (40 kDa) and CXCR2 (55 kDa) in resting keratocytes was assessed by western blot.  $\beta$ -actin (42 kDa) was used as a loading control.



**Supplemental Figure 4.** SP induced migration is partially mediated through IL-8. Representative pictures of keratocytes after scratch injury, untreated, treated with two different concentrations of SP ( $10^{-7}\text{M}$  and  $10^{-5}\text{M}$ ) and treated with both, SP and IL-8 neutralizing antibody ( $3\mu\text{g/ml}$ ) at 0h, 8h, 24h and 48h. Both concentrations of SP strongly enhanced cell migration 48h after introduction of scratch injury. Treatment with IL-8 neutralizing antibody decreased SP induced cell migration.

**Supplemental Table 1.** Concentrations of IL-8 in supernatants used for keratocyte chemotaxis induction

	ctrl	SP 10 <sup>-7</sup> M	SP 10 <sup>-5</sup> M	L-733,060 10 <sup>-6</sup> M	L-733,060 10 <sup>-6</sup> M + SP 10 <sup>-7</sup> M	L-733,060 10 <sup>-6</sup> M + SP 10 <sup>-5</sup> M
IL-8 (pg/ml)	490	540	713.33	520	433.33	450

**Supplemental Table 2.** Concentrations of IL-8 in supernatants used for IL-8 neutralizing antibody keratocyte chemotaxis induction

	ctrl	SP 10 <sup>-5</sup> M	IL-8 Ab	SP 10 <sup>-5</sup> M + IL-8 Ab	Isotype control	SP 10 <sup>-5</sup> M + isotype control
IL-8 (pg/ml)	312	827	0	0	297	672

**Supplemental Table 3.** Concentration of IL-8 in supernatants used for CXCRs blocked keratocytes chemotaxis induction

IL-8 (pg/ml)
837

**Supplemental Table 4.** Concentrations of IL-8 in supernatants used for HL-60 cells chemotaxis induction

	ctrl	SP <sup>-7</sup> M	SP <sup>-5</sup> M	L-733,060 10 <sup>-6</sup> M	L-733,060 10 <sup>-6</sup> M + SP <sup>-7</sup> M	L-733,060 10 <sup>-6</sup> M + SP <sup>-5</sup> M
IL8 (pg/ml)	274.83	522.65	841.69	373.07	306.57	311.35