Cell migration towards CXCL12 in leukemic cells compared to breast cancer cells

Running title: CXCL12 induced migration in cancer cells

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Abbreviations

DMEM – Dulbecco's Modified Eagle's Medium

EDTA - Ethylenediaminetetraacetic acid

FCS - Foetal calf serum

GPCR – G protein coupled receptor

mAB – monoclonal antibody

n.s. – non significant

PBS - Phosphate buffered saline

PKC - Protein kinase c

S.E.M. – Standard Error of Means

Abstract

Chemotaxis or directed cell migration is mediated by signalling events initiated by binding of chemokines to their cognate receptors and the activation of a complex signalling cascade. The molecular signalling pathways involved in cell migration are important to understand cancer cell metastasis. Therefore we investigated the molecular mechanisms of CXCL12 induced cell migration and the importance of different signalling cascades that become activated by CXCR4 in leukemic cells versus breast cancer cells. We identified Src kinase as being essential for cell migration in both cancer types, with strong involvement of the Raf/MEK/ERK1/2 pathway. We did not detect any involvement of Ras or JAK2/STAT3 in CXCL12 induced migration in Jurkat cells. Preventing PKC activation with inhibitors does not affect migration in Jurkat cells at all, unlike in the adherent breast cancer cell line MCF-7 cells. However in both cell lines, knock down of PKCα prevents migration towards CXCL12, whereas the expression of PKCζ is less crucial for migration. PI3K activation is essential in both cell types, however LY294002 usage in MCF-7 cells does not block migration significantly. These results highlight the importance of verifying specific signalling pathways in different cell settings and with different approaches.

1. Introduction

Tumour metastasis is the major cause of death in cancer patients who have suffered a primary solid tumour or haematological malignancy. The chemokine CXCL12 along with Src and P13K/Akt signalling appears to be implicated in both breast cancer metastatic progression to bone and other tissues, and leukaemia reoccurrence [1-4]. Metastasis results from a sequential series of processes, in which tumour cells undergo epithelial-mesenchymal transition. Cells detach from the original tumour tissue, intravasate into blood vessels, survive and travel along the circulation, extravasate to secondary organs, transform back to the epithelial state, and proliferate at their new location [5-7]. Recent data indicates that chemotactic signalling plays a crucial role in tumour invasion and spreading [8-14]. Chemotaxis is mediated by signalling events initiated by binding of chemokines to their cognate receptors, and involves re-arrangement of the actin cytoskeleton. CXCL12 can bind the G-protein coupled receptor CXCR4 causing Gαi triggered adenylate cyclase inhibition and hence a reduction in cAMP levels in the cell. This mediates the Src kinase phosphorylation cascade, which leads to ERK activation, Rho triggered actin rearrangement, cell polarisation and finally migration down a chemokine gradient [15]. Chemokine induced chemotaxis is key in homeostasis; for example CXCL12 is essential in lymph tissues and for movement of haematopoietic cells between blood and bone marrow [15, 16]. CXCR4 has been the subject of much scrutiny, since it has been implicated in the metastasis of various cancers (1,7,8,). For example, CXCL12 has been shown to be detrimental in the movement of blasts in leukaemia [17] and CXCL12+ cells are implicated in the formation of bone metastasis following breast cancer [18]. However the signal networks that are important for chemokine receptor triggered cell migration and metastasis are not yet completely understood due to their complexity. Nevertheless for CXCR4 signalling, concentrations and gradients of CXCL12 are purportedly important. Excess CXCL12 may further damage tissues suffering insult from ischaemia, toxins, chemotherapeutic agents and atherosclerosis [15]. CXCL12 levels tend to rise with age [19] and excess concentrations may inhibit metastasis [20]. Many chemokines bind several chemokine receptors and CXCL12 binds CXCR4 and CXCR7. It appears CXCR7

activities include acting as a scavenger, modulating the levels of CXCL12 in the vicinity of cells carrying the receptor, and that binding of CXCL12 to CXCR7 may cause internalisation of the receptor without resulting in downstream signalling [21-23]. Over the years various signalling molecules that are involved in CXCR4 triggered migration have been identified, however there is still some uncertainty about which pathways are directly involved in cell migration. For CXCR4 it has been shown that migration under certain circumstances is dependent on β-arrestins as well as filamin-A, a protein, which can bind actin and interacts with β-arrestins [24-26]. Several groups have shown that ERK1/2 or p38 MAPK activation is important for cell migration as well [13, 27]. Similarly, Protein Kinase Cε (PKCε) activation has been shown to be implicated in the movement of T cells [28] and atypical Protein Kinase Cζ (PKCζ) is directly involved in CXCL12 signalling in immature human CD34(+)-enriched cells and in leukemic pre-B acute lymphocytic leukaemia (ALL) G2 cells [29]. There is still some discussion whether JAK kinase activity is needed for migration or not, with some reports showing that in murine neural progenitor cells JAK activation is not necessary [30] whereas in metastatic T-lymphoma JAK activation is essential for migration [31]. Of particular interest in this respect is a study by Pfeiffer *et al* [32] where the JAK2 inhibitor AG490 only inhibited CXCL12 induced adhesion in NCI-H82 and not in NCI-H69 cells. These data show that the signalling networks which are used by CXCL12 and its receptor CXCR4 probably vary between different species and cell types. Subsequently, we determined in this study whether the pharmacological blockade of different signalling cascades like the MEK/ERK1/2 kinase cascade or JAK/STAT differentially block CXCL12 induced cell migration in leukemic cells versus adherent breast cancer cells.

2. Materials and Methods

2.1 Cells and materials

The leukemic cell line Jurkat was obtained from the ATCC and grown in RPMI containing 10% FCS and 2 mM L-glutamine. The breast cancer cell line MCF-7 was obtained from the ATCC and grown in DMEM containing 10% FCS and 2 mM L-glutamine. The chemokine CXCL12 was obtained from Peprotech. JAK inhibitor 2 (1,2,3,4,5,6-Hexabromocyclohexane, JAK-2) and STAT3 inhibitor VIII, 5,15-DPP (STAT3 VIII) were from Calbiochem. LY294002, AG490, Bosutinib, Rottlerin, GF109203X, Staurosporine and CID755673 were purchased from Tocris. Farnesyl thiosalicylic acid (FTS), SB203580, PD98059, L775450, FH535 and SL327 were from Abcam. Cells were treated with 10 μM LY294002, 50 μM JAK2, 50 μM STAT3 VIII, 5 μM GF109203X, 10 nM Staurosporine, 11 μM CID755673, 10 μM AG490, 5 μM Bosutinib, 12.5 μM FTS, 10 μM SB203580, 12.5 μM & 25 μM PD98059, 0.5 & 1 μM L775450, 1 μM FH535, 1 μM SL327 and 4μM Rottlerin for 30 minutes before induction of chemotaxis. Anti-CXCR4 antibody 12G5 was from Santa Cruz and the corresponding goat anti-mouse FITC labelled secondary antibody came from Sigma Aldrich. Anti-PKCα (H-7), anti-PKCζ and β-actin antibodies were purchased from Santa Cruz. Anti-Src and anti-Pi3K p85 were from Biotechne and the mouse peroxidase labelled secondary antibodies were from Sigma Aldrich. All other chemicals were obtained from Fisher Scientific.

2.2 Chemotaxis Assays

Cells were harvested and then resuspended at a concentration of $25x10^4$ cells mL^{-1} in serum-free RPMI 1640 containing 0.1% BSA. Cells were loaded in a total volume of 20 μL into the upper compartment of a microchemotaxis chamber (Receptor Technologies, Adderbury, UK). For inhibitor treatment, cells were incubated for 30 minutes with the relevant inhibitors or vehicle control before loading onto the membrane. Chemoattractants at a concentration of 1 nM were loaded in a final volume of 31 μL at indicated concentrations in the lower compartment. The two compartments were separated by a polyvinylpyrollidone-free

polycarbonate filter with 5 µm pores. The chemotaxis chamber was incubated at 37°C, 100% humidity, and 5% CO_2 for 4 h. The filter was then removed, and the number of cells migrating into each bottom compartment was counted using a haemocytometer. In all experiments, each data point was performed in duplicate.

2.3 Wound healing assays:

MCF-7 cells were seeded onto 24 well plates overnight. After 24 hours, the cells were washed once in DMEM without supplements and incubated in DMEM without supplements. A scratch was introduced to the monolayer with 200 µL pipette tips (time point 0). Inhibitors were added to the cells and incubated for 30 minutes at 37°C, 100% humidity, and 5% CO_2. Chemokines or vehicle controls were added to the cells and pictures were taken at timepoint 0 and after 24 hours using an inverted Leica microscope. Images were analysed and the width of the wound was measured for control and with inhibitor treatment (with and without chemokine) at 0 hours and 24 hours. The ratio of the width of the wound after 24 hours divided by the width of the wound at 0 hours can then be used compare the effectiveness of treatments in preventing migration, where a number of 1 denotes no migration and a number smaller than 1 denotes migration of cells.

2.4 Cell Viability Studies

MTS assays were performed using a CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega). 100 µL wells containing $5x10^5$ cells mL^{-1} in complete RPMI supplemented with the test compounds at working concentrations were maintained at 37°C and 5% CO_2 for 2 hours in a humidified atmosphere. After incubation, cell viability was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) tetrazolium compound is bioreduced by cells into a coloured formazan product that is soluble in tissue culture medium [33]. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells [34]. Aliquots of 10

µL of the CellTiter 96® AQueous One Solution Reagent were added directly to the wells and the plates were incubated for 4 hours at 37 °C in a humidified atmosphere, 5% CO_2 and then absorbance at 490 nm was read with a 96-well plate reader. The quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture. The inhibitors were used at concentrations which did not show any toxic effects over a 5 hour incubation period.

2.5 Immunofluorescence staining

The Jurkat cell line was washed in PBS and re-suspended at a concentration of 5 x 10^6 cells/ml in PBS and were incubated at 4°C with CXCR4 mAB 12G5 or isotype control for 1 h. Cells were washed twice in cold PBS staining was performed using fluorescein isothiocyanate (FITC) labelled donkey anti-mouse immunoglobulin secondary antibodies (1:100, Sigma Aldrich) for 1 h at 4°C. Cells were then washed in PBS and dropped onto a glasslide and pictures taken with an inverted Leica DMII fluorescence microscope. MCF-7 cells were seeded on coverslips overnight, washed with PBS and stained with 12G5 or isotype control as described above.

2.6 siRNA transfection

PKCα, PKCζ siRNA and scrambled siRNA were obtained from Qiagen (Hilden, Germany), PI3K and Src siRNA were obtained from GE Healthcare (UK) and diluted to working concentrations in RNAse free water. Jurkat cells were transfected with 50 nM scrambled siRNA, 50 nM PKCα or PKCζ siRNA or vehicle, respectively, using the Amaxa Nucleofector according to manufacturer's instructions. In short 3X10^6 cells per cuvette were used for the transfection and after 48 hours a chemotaxis assay was performed. The MCF-7 cells were transfected using Lipofectamine RNAi (ThermoFisher Scientific) according to manufacturer's instruction.

2.7 SDS–PAGE and Western blot.

Cells were harvested and then resuspended in Mammalian Protein Extraction Buffer (GE Healthcare) at 4 °C for 40 minutes with gentle mixing. Analysis of the proteins on SDS–PAGE was done as described [35]. Antibodies were removed from the membrane before a second stain by incubation with Millipore Stripping Solution (Millipore, Temecula California) at room temperature for 15 minutes before blocking and reprobing.

2.8 Analysis of data

Data were analysed using GraphPad Prism (GraphPad Software). Statistical analyses were performed using an One-way ANOVA with a Bonferroni Multiple Comparison test as post-test with a p value <0.05. Data represent the mean $\pm$ s.e.m. of at least three independent experiments.

3. Results

3.1 PKC activation is vital for cell migration in breast cancer cell, but less so in leukemic cells

CXCR4 induced cell migration is depending on a plethora of intracellular proteins, which need to become activated to allow the cell to move towards the chemokine stimulus. Here we investigated different signalling cascades and whether they are implicated in CXCR4 migration in suspension cells versus adherent cells. We have recently shown that PKC activation is not essential for CCL3 induced migration of the suspension cell line THP-1 [36]. In a similar fashion, we used two PKC inhibitors which block a wide variety of PKC isoforms (Staurosporine and GF109203X) as well as the more specific PKD inhibitor CID755673 and Rottlerin, which has been described as a selective PKCδ, but has since been found to block other kinases and to uncouple mitochondria [37]. All four inhibitors used did not block migration in Jurkat cells induced by CXCL12 (Figure 1A - D). The only significant effect was observed with Rottlerin, which actually increases the number of cells migrating. However in wound

healing assays on the breast cancer cell line MCF-7, both Rottlerin and GF109203X exhibit the opposite effect. In these cells, they prevent migration of cells into the wound effectively after 24 hours (Figure 1E, F). Expression of CXCR4 in both cell lines was confirmed using a monoclonal antibody against CXCR4 (Figure 1G). siRNA knockdown of PKCα and PKCζ proteins in MCF-7 cells confirmed the importance of PKC for migration in these cells (Figure 2 A,C), where the loss of PKCα and PKCζ completely abolishes any migration towards CXCL12. Whereas transfection of PKCα and PKCζ siRNA into Jurkat cells allows us to differentiate between the use of different PKC isoforms. PKCα knockdown leads to a loss of about half the migratory response, whereas the PKCζ knockdown has less impact. In both cases, there are still a robust number of cells migrating, even though the migration is significantly lower than in control cells (transfected with scrambled siRNA) (Figure 2 B,D), unlike the MCF-7 cells (Figure 2 A,C), where the knockdown of PKCα and PKCζ completely prevents movement of cells into the wound. The success of knockdown was confirmed by Western blot analysis (Figure 2 E, F, G).

3.2 JAK2 and STAT3 activation is not necessary for migration of leukemic cells.

We then investigated whether there are other pathways that are important in cell migration. We used inhibitors to block JAK2 and STAT3 activation (Figure 3 A, B), but none blocked CXCL12 induced migration in Jurkat cells. Similarly, a Ras inhibitor, Farnesylthiosalicyllic acid, did not affect Jurkat migration towards CXCL12 (Figure 3 C). The Ras inhibitor shows toxicity in MCF-7 cells after 24 hours (data not shown) and is therefore not suitable for wound healing assays, even at lowered concentrations. Similarly the JAK2 and STAT3 inhibitors affect cell viability over 24 hours at 10 µM, at the lower concentration of 1 µM both inhibitors do not show any toxicity. Whereas the STAT3 inhibitor does not affect cell migration into the wound, the effect of the JAK2 inhibitor is less clear; there seems to be a slight trend to block migration, but it was not significant (Figure 3 D).

3.3 Src activation is vital for both breast cancer cell as well as leukemic cell migration.

Another signalling cascade, which has been highlighted as being involved in CXCR4 induced migration, is the Raf/MEK/ERK network. Cells were pre-treated with SB203580, an inhibitor of p38 MAPK, PD98059, a small molecule inhibitor targeting MEK specifically or Bosutinib, a Src inhibitor. Blocking p38 MAPK, MEK and Src resulted in around 50% reduction in migration (Figure 4). This reduction in migrating cells is not a consequence of any toxicity of Bosutinib, as shown by MTS assays (Figure 4 D). Again, there are significant differences in MCF-7 cells. Even though the Src inhibitor Bosutinib completely blocked any migration of MCF-7 cells into the wound, both the ERK1/2 inhibitor PD98059 and the p38 MAPK inhibitor SB203580 did not show a pronounced blocking of cell migration, however the wound in the SB203580 treated cells remained significantly larger than in the control cells (Figure 4 E, F). We verified the results obtained with Bosutinib with an siRNA approach. A knock down of Src prevents migration of Jurkat cells as well as the migration of MCF-7 cells in a wound-healing assay (Figure 5 A, B). The efficiency of knockdown was confirmed by Western blot analysis (Figure 5 E).

3.4 PI3K activation is important for leukemic cell migration but not in wound-healing assays.

To further evaluate which signalling partners are involved in transducing receptor activation to cell migration, we used a second MEK inhibitor (SL327) as well as the well-established PI3K inhibitor (LY294002), Raf (L779450) and β-catenin (FH535) inhibitors and we also knocked down p85 PI3K using an siRNA approach (Figure 5 C,D,E,F). Whereas the knock down of PI3K in Jurkat and MCF-7 cells prevented migration significantly, the results with a small molecule antagonist LY294002 were less clear. The blockade of PI3K completely abolishes any migration in Jurkat cells and the other inhibitors led to a small, but significant decrease of migrating suspension cells (Figure 6). In the adherent MCF-7 cells there are some differences. Whereas the effect of blocking migration by L775450 is much more pronounced, LY294002

fails to show a significant effect on these cells (Figure 6 F). We verified that in Jurkat cells the migration towards CXCL12 is in response to CXCR4 with the use of a monoclonal antibody directed against CXCR4 which blocks migration (Figure 6 H).

4. Discussion

Chemokine receptor induced cell migration is a crucial step in metastasis of cancer as well as the inflammatory response [23]. Understanding the mechanisms of migration therefore can potentially provide novel therapeutic targets to prevent undesirable cell migration. The chemokine receptor CXCR4 has been of interest for a number of years, as it has been shown to be up-regulated in several cancers and its activation can lead to cancer cell metastasis [23, 38-41]. Although numerous studies have investigated different aspects of the signalling cascades which are involved in the cell migration, some questions still remain unanswered. Furthermore there is conflicting evidence in the literature about the importance or involvement of downstream signalling partners in different systems [11, 27-29, 38, 42-44]. One problem is that most studies only characterised a small number of signalling molecules at any one time and since a whole variety of cell types/read-out systems and approaches were used, it can be expected that some of the data may contradict each other. We therefore set out to investigate the main signalling molecules that are thought to be of importance in CXCL12 induced migration in adherent cells versus suspension cells. One of the findings in our study is that there are indeed differences in which signalling molecules are important for cell migration of leukemic cells compared to adherent breast cancer cells. Both cell types used (the leukemic cell line Jurkat and the breast cancer cell line MCF-7) express CXCR4 and migrate towards CXCL12, however the signalling molecules show some intriguing differences. The two cell lines used express CXCR4 and blocking of CXCL12 binding to CXCR4 with the aid of a CXCR4 specific antibody prevents migration of cells, showing that CXCR4 is the main receptor, if not the only one on these cells to induce migration in response to CXCL12.

Protein Kinase C (PKC) has been shown to have central roles in signalling in response to many extracellular ligands, and can influence many aspects of cell behaviour. Several groups

have shown that receptor desensitization is not necessarily based only on phosphorylation of agonist- occupied receptors by G-Protein coupled receptor kinases (GRK) but also can be caused by phosphorylation of receptors by second messenger-activated kinases such as Protein Kinase C, to attenuate receptor interaction with G-Proteins. Oppermann *et al.* [45] have shown the equivalence in importance of both GRK and second messengers PKC in phosphorylation of receptors. Second messenger-activated kinases, Protein Kinase A (PKA) and PKC potentially phosphorylate both the ligand bound GPCR and multiple other receptors in a heterologous manner [46]. There are a variety of studies that show that PKC isoforms are also important for cancer cell migration [10, 47] and indeed, a pan PKC inhibitor GF109203X completely blocks breast cancer cell migration towards CXCL12; however at the same concentration there is no effect on the migration of the leukemic suspension cells towards CXCL12. Instead GF109203X leads to a slight, if not significant, increase in migration. This agrees with a previous study of our lab, where we showed that the same PKC inhibitor does not block CCL3 induced migration in the suspension cells THP-1 [36]. We used a siRNA approach to confirm the results obtained with the small molecule antagonists, and indeed, a knock down of PKCα and PKCζ in MCF-7 cells completely abrogates any movement of the cells into the wound, confirming the results obtained with the PKC inhibitors. The picture in Jurkat cells is slightly more complicated. Both PKCα and PKCζ knockdowns result in a significant loss of migratory cells which is in stark contrast to the PKC inhibitor studies. We therefore speculate that there is a difference in usage of PKC in the two cell types. Whereas the MCF-7 cells need the catalytic activity of PKC (hence a small molecule antagonist as well as knock down prevents migration) in Jurkat cells it seems plausible, that it is not the kinase activity of PKC which is implicated in cell migration, it is rather the functionality of the other PKC domains. For example PKCs localizes in cells with cytoskeletal proteins (such as actin and tubulin) and true scaffolding proteins (such as caveolin) and might therefore be implicated in migration. It has also been shown that PKCs can be cleaved by caspases, generating a catalytically active kinase domain and a freed regulatory domain fragment that can act both as an inhibitor of the full-length enzyme and as an activator of certain signalling responses

[48]. Altogether the data in Jurkat cells are more complex, but they show a difference towards PKC use in MCF-7 cells and warrant further investigation.

The JAK/STAT pathway has been implicated for being an essential pathway in cell migration for some time [49], however recent studies showed that some of the results could be due to the effects some inhibitors have on actin dynamics in cells [50, 51] or that JAK activation is not important for chemokine induced activation after all [30]. Using a variety of different JAK2/STAT3 inhibitors, we did not detect a significant effect of JAK2/STAT3 inhibition on migration in both leukemic or breast cancer cells.

In keeping with the findings of other groups studying aspects of CXCL12 signalling in a variety of cell types [42, 52] Src kinase activation is critical migration in both leukemic and breast cancer cells. We have also confirmed that the Raf/MEK/ERK1/2 pathway plays an important role in leukemic cell migration with a somewhat diminished importance in breast cancer cells. Unlike Sobolik et al., who showed that in a 3D model, inhibition of PI3K reversed the aggressive phenotype of MCF-7 [53], we did not observe a significant effect of PI3K inhibition on wound-healing in a 2D model when using LY294002. Knock down of PI3K p85 expression in MCF-7 cells, abrogates any migration towards CXCL12 in the wound healing assay, confirming published studies. In Jurkat cells, PI3K inhibition significantly reduces cell migration, whether it is the use of LY294002 or the knock down of protein expression.

Our study highlights that the cellular background can be important for the distinct signalling pathways used by the CXCR4 receptor and therefore a generalisation of how CXCR4 induces migration in different cell types and species should be avoided. There are quite a few similarities between the different cell types, however some subtle differences mean that there is the potential to block migration of specific cancer cell types when targeting metastases.

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6. Figures and legends

Figure 1: **PKC and PKD activation is not essential for CXCL12 induced migration**. A) Shows migratory response of Jurkat cells towards 1nM CXCL12 in untreated control cells or Staurosporine pre-treated cells. B) Cell migration towards 1nM CXCL12 in untreated control cells or GF109203x pre-treated cells. C) Cell migration towards 1nM CXCL12 in untreated control cells or CID755673 pre-treated cells. D) Cell migration towards 1nM CXCL12 in untreated control cells or Rottlerin pre-treated cells. E) Wound healing assay on MCF-7 cells in the presence or absence of Rottlerin or GF109203X. Cell migration was induced with 10 nM CXCL12 and measured after 24 hours. F) Quantification of migration of cells into the wound. A number of 1 denotes no migration occurred whereas a number < 1 denotes cell migration. * denotes a significant difference towards to corresponding control ($p \leq 0.05$, One-way ANOVA with a Bonferroni Multiple Comparison test as post-test). G) CXCR4 was visualised on Jurkat and MCF-7 cells by 12G5 mAb staining. Data shown are the mean ± SEM of at least 3 independent experiments.

Figure 2: siRNA transfection into MCF-7 cells and Jurkat cells A) Wound healing assay on MCF-7 cells after transfection with scrambled control siRNA (sc siRNA) or PKCζ siRNA. Cell migration was induced with 10 nM CXCL12 and measured after 24 hours. B) Cell migration towards 1 nM CXCL12 in Jurkat cells after transfection with scrambled control siRNA (sc siRNA) or PKCζ siRNA. C) Wound healing assay on MCF-7 cells after transfection with scrambled control siRNA (sc siRNA) or PKCα siRNA. Cell migration was induced with 10 nM CXCL12 and measured after 24 hours. D) Cell migration towards 1 nM CXCL12 in Jurkat cells after transfection with scrambled control siRNA (sc siRNA) or PKCα siRNA. E, F) Western blot analysis of PKCζ and PKCα expression in MCF-7 after knockdown, β-actin acts as loading control. G) Western blot analysis of PKCα expression in Jurkat cells after knockdown, β-actin acts as loading control. Quantification of migration of cells into the wound. A number of 1 denotes no migration occurred whereas a number < 1 denotes cell migration. ** denotes a

significant difference towards the corresponding control (**=p≤0.01, ***=p≤0.001, One-way ANOVA with a Bonferroni Multiple Comparison test as post-test). Data shown are the mean ± SEM of at least 3 independent experiments.

Figure 3: **Jak/STAT activation is not essential for CXCL12 induced migration**. A) Shows migratory response of Jurkat cells towards 1nM CXCL12 in untreated control cells or AG490 pre-treated cells, there is no significant differences between the inhibitor treated cells and control cells in the presence of CXCL12. B) Cell migration towards 1nM CXCL12 in untreated control cells or Stat VIII or Jak-2 pre-treated cells. C) Cell migration towards 1 nM CXCL12 in untreated control cells or Farnesylthiosalicylic acid pre-treated cells. Data shown are the mean ± SEM of at least 3 experiments.

Figure 4: **CXCL12 induced migration depends on Src, p38 MAPK and MEK activation**. A) Shows migratory response of Jurkat cells towards 1 nM CXCL12 in untreated control cells or SB203580 pre-treated cells. B) Cell migration towards 1 nM CXCL12 in untreated control cells or 25 µM PD98059 pre-treated cells. C) Cell migration towards 1 nM CXCL12 in untreated control cells or 2.5 µM Bosutinib pre-treated cells. Statistical analyses were performed using a one-way ANOVA with a Bonferroni multiple comparison test as post-test with *** showing a p value of ≤ 0.001. D) MTS assay in Jurkat cells with different concentrations of Bosutinib, as indicated. E) Wound healing assay on MCF-7 cells in the presence or absence of PD98059, SB203580 or Bosutinib. Cell migration was induced with 10 nM CXCL12 and measured after 24 hours. F) Quantification of migration of cells into the wound. 1 denotes no migration occurred whereas a number < 1 denotes cell migration. * denotes a significant difference towards to inhibitor treated/untreated cells in the presence of CXCL12, (p≤0.05, One-way ANOVA with a Bonferroni Multiple Comparison test as post-test)Data shown are the mean ± SEM of at least 3 experiments.

Figure 5: **siRNA transfection into MCF-7 cells and Jurkat cells** A) Wound healing assay on MCF-7 cells after transfection with scrambled control siRNA (sc siRNA) or Src siRNA. Cell migration was induced with 10 nM CXCL12 and measured after 24 hours. B) Cell migration towards 1 nM CXCL12 in Jurkat cells after transfection with scrambled control siRNA (sc siRNA) or Src siRNA. C) Wound healing assay on MCF-7 cells after transfection with scrambled control siRNA (sc siRNA) or PI3K siRNA. Cell migration was induced with 10 nM CXCL12 and measured after 24 hours. D) Cell migration towards 1 nM CXCL12 in Jurkat cells after transfection with scrambled control siRNA (sc siRNA) or PI3K siRNA. E, F) Western blot analysis of Src and PI3K expression in MCF-7 after knockdown, β-actin acts as loading control. G) Western blot analysis of Src expression in Jurkat cells after knockdown, β-actin acts as loading control. Quantification of migration of cells into the wound. A number of 1 denotes no migration occurred whereas a number < 1 denotes cell migration. ** denotes a significant difference towards the corresponding control (**=p≤0.01, ***=p≤0.001, One-way ANOVA with a Bonferroni Multiple Comparison test as post-test). Data shown are the mean ± SEM of at least 3 independent experiments.

Figure 6: **CXCL12 induced migration depends on Raf, MEK, PI3K and β-catenin activation**. A) Shows migratory response of Jurkat cells towards 1 nM CXCL12 in untreated control cells or L779450 pre-treated cells. B) Cell migration towards 1 nM CXCL12 in untreated control cells or SL327 pre-treated cells. C) Cell migration towards 1 nM CXCL12 in untreated control cells or FH535 pre-treated cells. E) Cell migration towards 1 nM CXCL12 in untreated control cells or LY294002 pre-treated cells F) Wound healing assay on MCF-7 cells in the presence or absence of L779450 and LY294002. Cell migration was induced with 10 nM CXCL12 and measured after 24 hours. G) Quantification of migration of cells into the wound. 1 denotes no migration occurred whereas a number < 1 denotes cell migration. *** denotes a significant difference towards to inhibitor treated/untreated cells in the presence of CXCL12, (p≤0.001, n.s is not significant, One-way ANOVA with a Bonferroni Multiple Comparison test as post-test). H) Incubation with CXCR4 specific mAB before inducement of migration towards

1nM CXCL12 significantly blocks migration. Statistical analyses were performed using a one-way ANOVA with a Bonferroni multiple comparison test as post-test with *** showing a p value of ≤ 0.001. Data shown are the mean ± SEM of at least 3 independent experiments.

Figure 7: Overview of signalling cascade in Jurkat and MCF-7 cells

Schematic overview of signalling cascades involved in migration of cells towards CXCL12.

7. References

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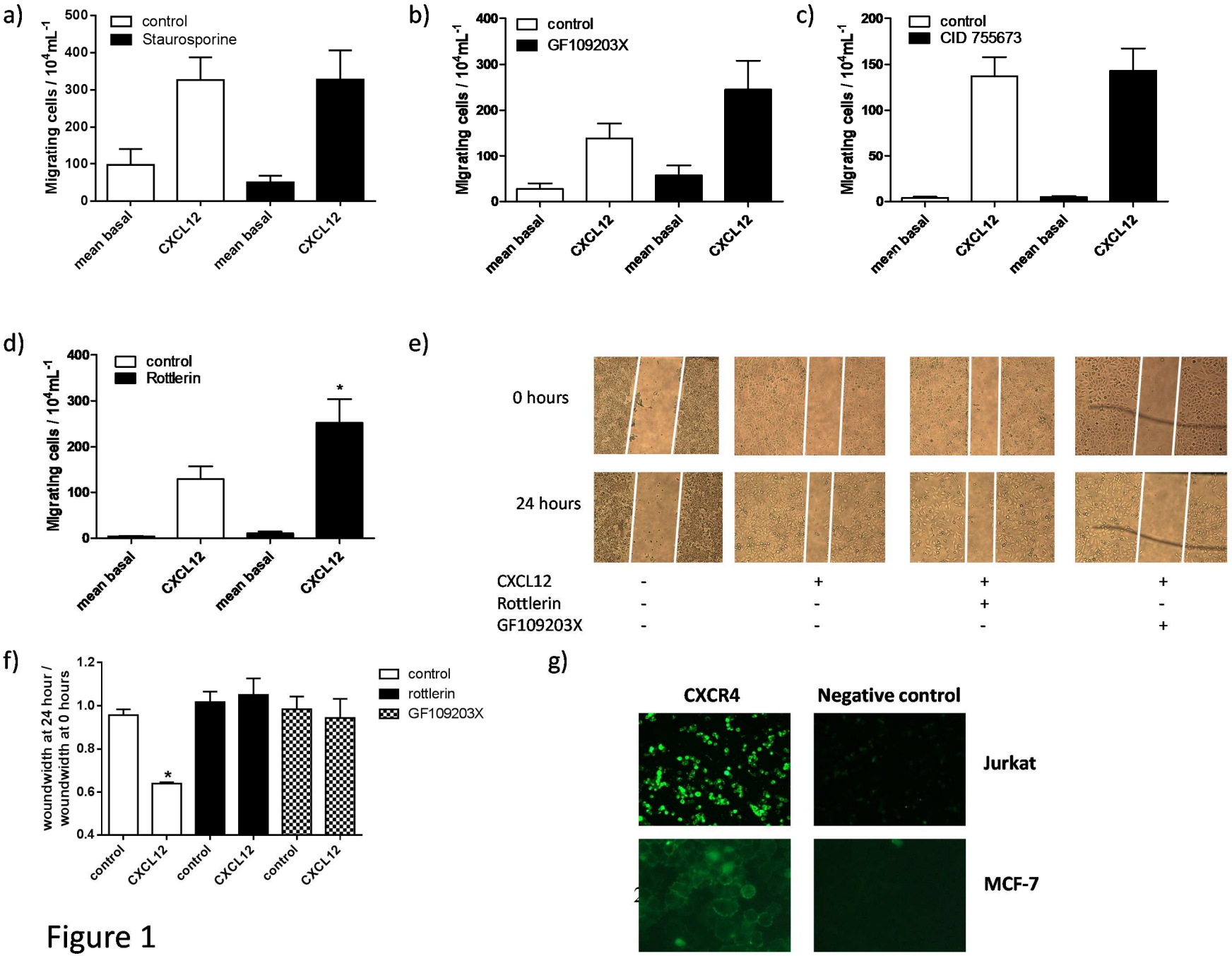

a)
Migrating cells / 10^4mL^-1
control
Staurosporine
mean basal
CXCL12
mean basal
CXCL12
b)
Migrating cells / 10^4mL^-1
control
GF109203X
mean basal
CXCL12
mean basal
CXCL12
c)
Migrating cells / 10^4mL^-1
control
CID 755673
mean basal
CXCL12
mean basal
CXCL12
d)
Migrating cells / 10^4mL^-1
control
Rottlerin
*
mean basal
CXCL12
mean basal
CXCL12
e)
0 hours
24 hours
CXCL12 - + + +
Rottlerin - - + -
GF109203X - - - +
f)
woundwidth at 24 hour / woundwidth at 0 hours
control
rottlerin
GF109203X
*
control
CXCL12
control
CXCL12
control
CXCL12
g)
CXCR4
Negative control
Jurkat
MCF-7

Figure 1

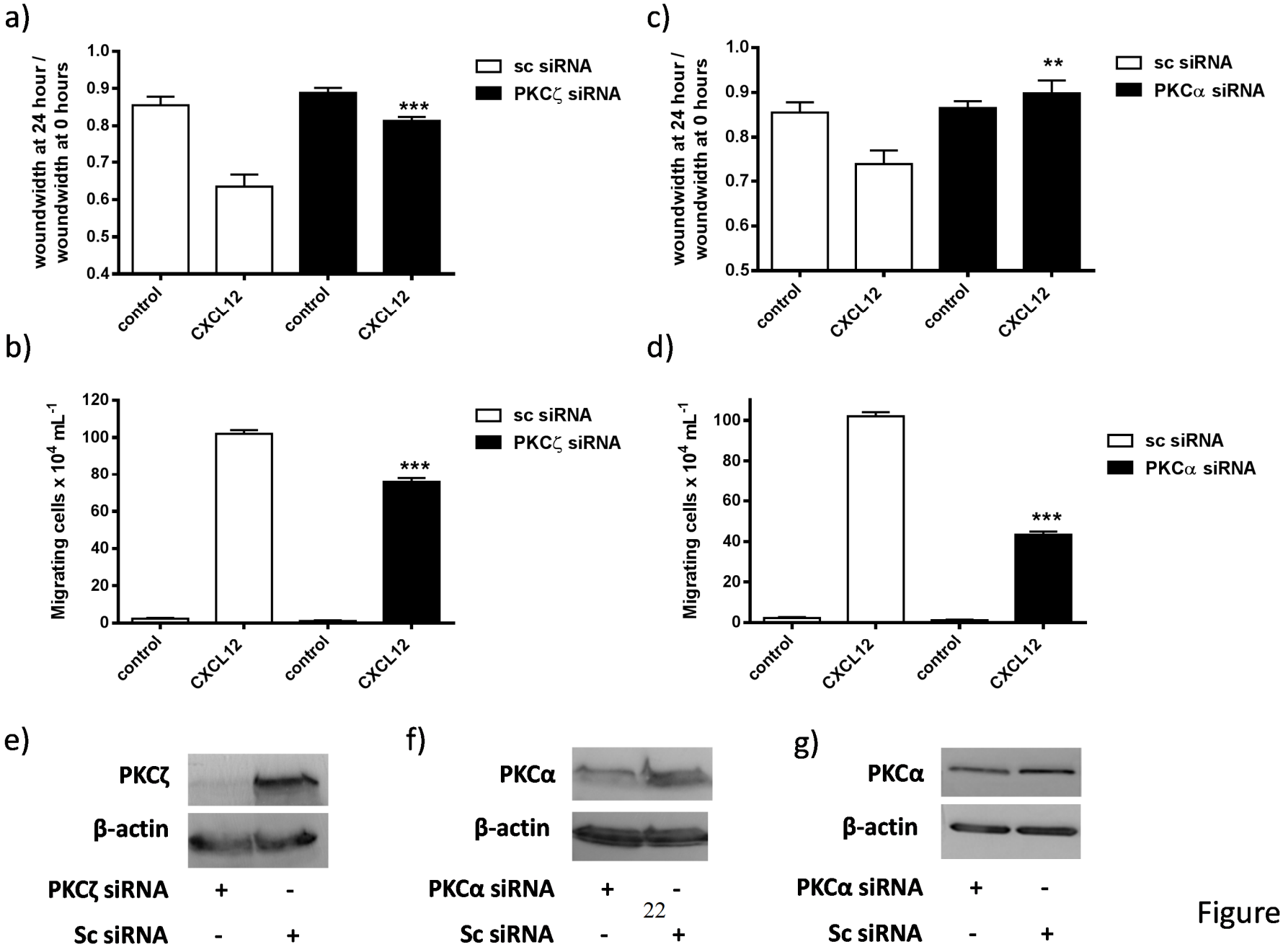

a)
woundwidth at 24 hour / woundwidth at 0 hours
1.0
0.9
0.8
0.7
0.6
0.5
0.4
***
sc siRNA
PKCζ siRNA
control
CXCL12
control
CXCL12
c)
woundwidth at 24 hour / woundwidth at 0 hours
1.0
0.9
0.8
0.7
0.6
0.5
**
sc siRNA
PKCα siRNA
control
CXCL12
control
CXCL12
b)
Migrating cells x 10^4 mL^-1
120
100
80
60
40
20
0
***
sc siRNA
PKCζ siRNA
control
CXCL12
control
CXCL12
d)
Migrating cells x 10^4 mL^-1
100
80
60
40
20
0
***
sc siRNA
PKCα siRNA
control
CXCL12
control
CXCL12
e)
PKCζ
β-actin
PKCζ siRNA + -
Sc siRNA - +
f)
PKCα
β-actin
PKCα siRNA + -
Sc siRNA - +
g)
PKCα
β-actin
PKCα siRNA + -
Sc siRNA - +

Figure 2

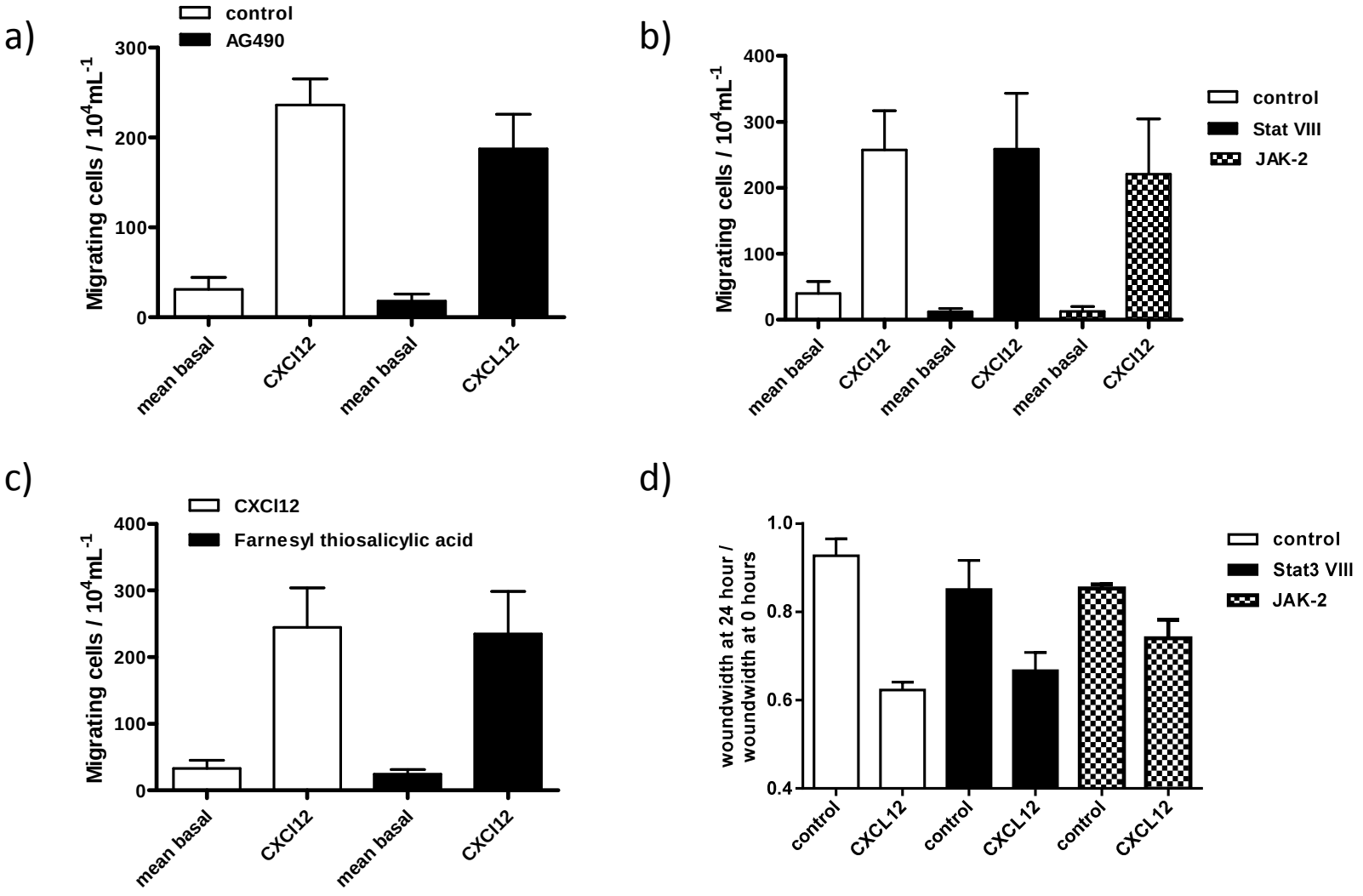

a)
control
AG490
Migrating cells / 10^4mL^{-1}
300
200
100
0
mean basal
CXCl12
mean basal
CXCL12
b)
Migrating cells / 10^4mL^{-1}
400
300
200
100
0
control
Stat VIII
JAK-2
mean basal
CXCl12
mean basal
CXCl12
mean basal
CXCl12
c)
CXCl12
Farnesyl thiosalicylic acid
Migrating cells / 10^4mL^{-1}
400
300
200
100
0
mean basal
CXCl12
mean basal
CXCl12
d)
woundwidth at 24 hour / woundwidth at 0 hours
1.0
0.8
0.6
0.4
control
Stat3 VIII
JAK-2
control
CXCL12
control
CXCL12
control
CXCL12

Figure 3

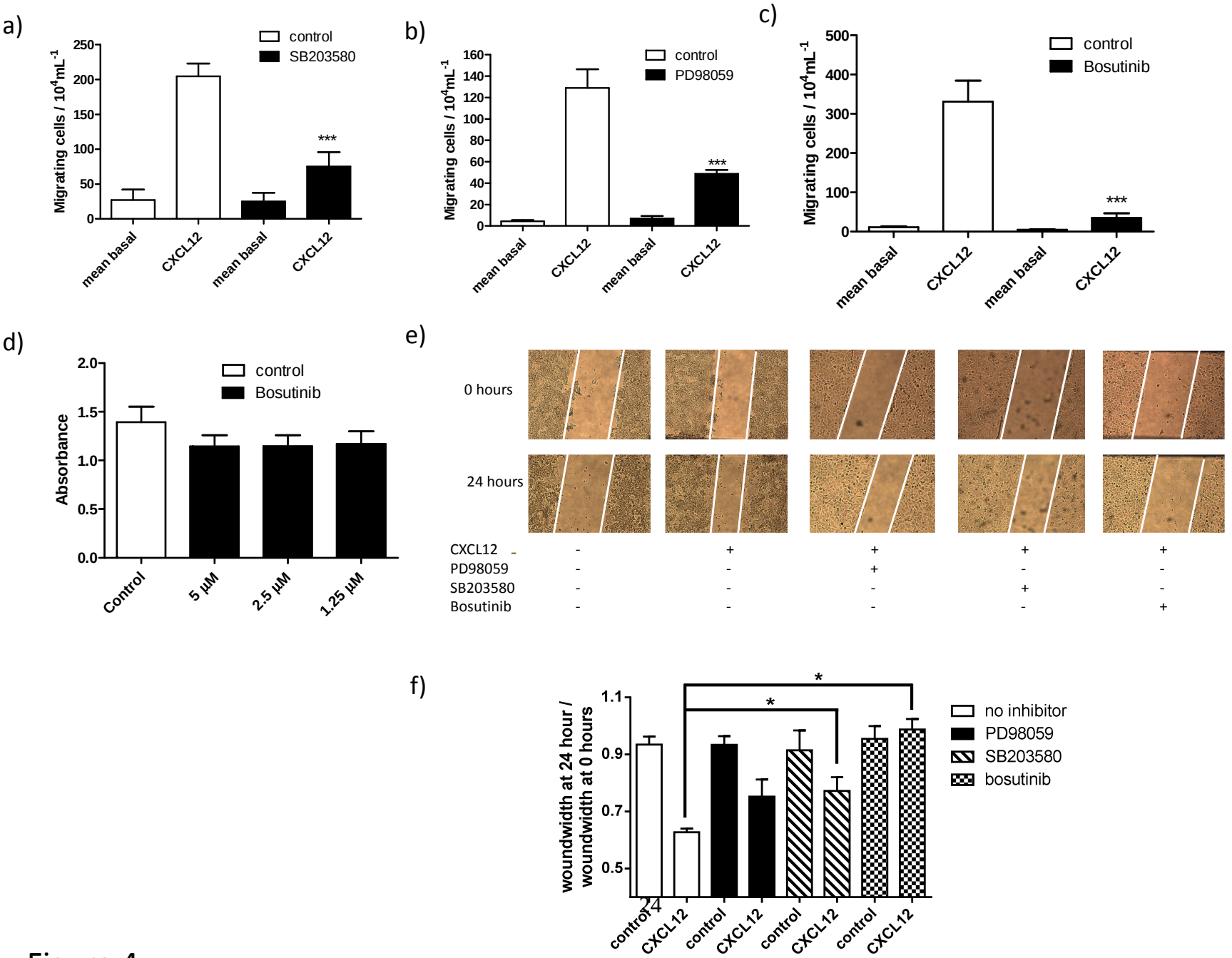

a)
control
SB203580
Migrating cells / 10^4mL^-1
250
200
150
100
50
0
***
mean basal
CXCL12
mean basal
CXCL12
b)
control
PD98059
Migrating cells / 10^4mL^-1
160
140
120
100
80
60
40
20
0
***
mean basal
CXCL12
mean basal
CXCL12
c)
control
Bosutinib
Migrating cells / 10^4mL^-1
500
400
300
200
100
0
***
mean basal
CXCL12
mean basal
CXCL12
d)
control
Bosutinib
Absorbance
2.0
1.5
1.0
0.5
0.0
Control
5 µM
2.5 µM
1.25 µM
e)
0 hours
24 hours
CXCL12 - + + + +
PD98059 - - + - -
SB203580 - - - + -
Bosutinib - - - - +
f)
woundwidth at 24 hour / woundwidth at 0 hours
1.1
0.9
0.7
0.5
*
*
no inhibitor
PD98059
SB203580
bosutinib
control
CXCL12
control
CXCL12
control
CXCL12
control
CXCL12

Figure 4

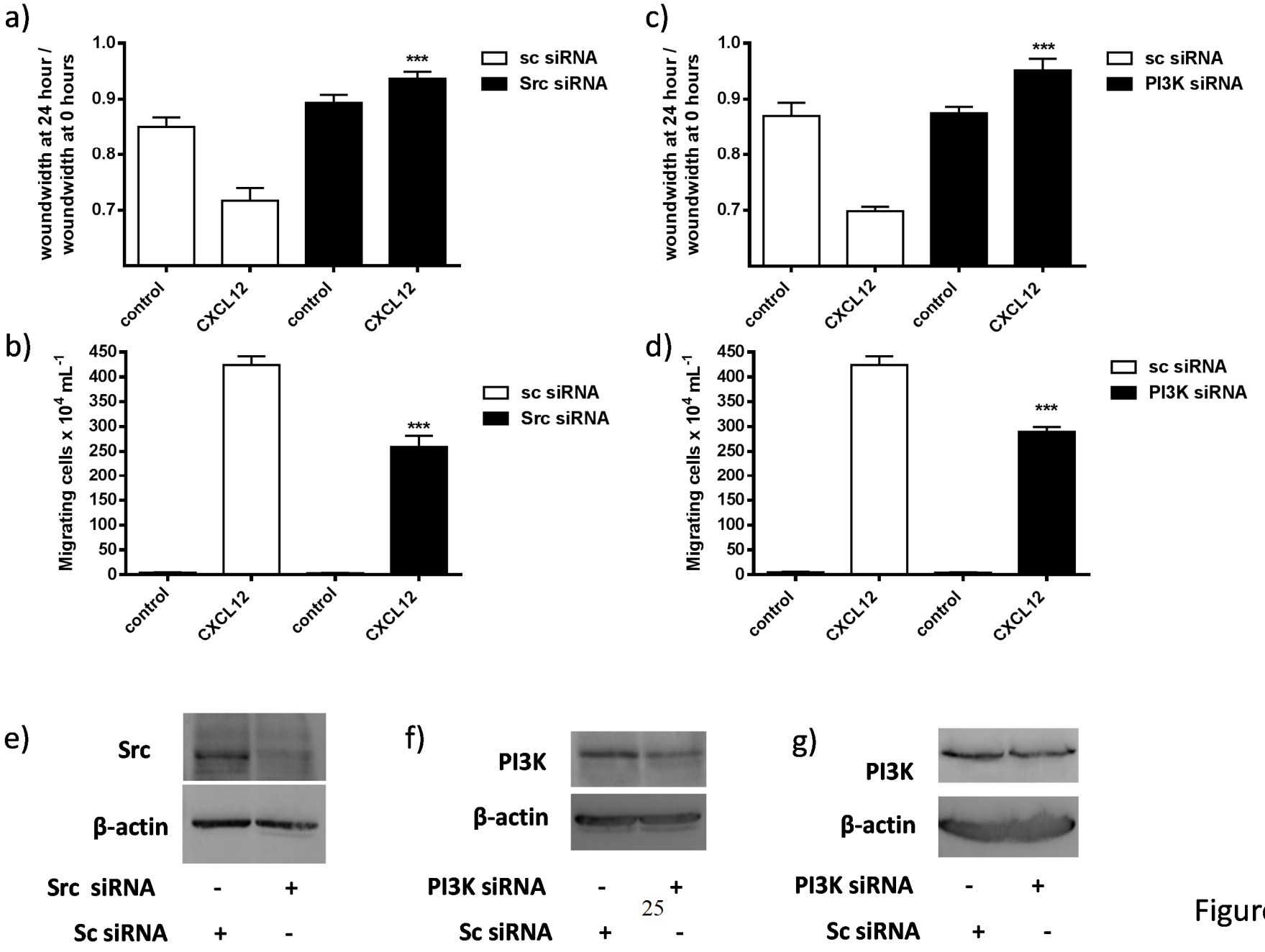

a)
woundwidth at 24 hour / woundwidth at 0 hours
1.0
0.9
0.8
0.7
***
sc siRNA
Src siRNA
control
CXCL12
control
CXCL12
b)
Migrating cells x 10^4 mL^-1
450
400
350
300
250
200
150
100
50
0
***
sc siRNA
Src siRNA
control
CXCL12
control
CXCL12
c)
woundwidth at 24 hour / woundwidth at 0 hours
1.0
0.9
0.8
0.7
***
sc siRNA
PI3K siRNA
control
CXCL12
control
CXCL12
d)
Migrating cells x 10^4 mL^-1
450
400
350
300
250
200
150
100
50
0
***
sc siRNA
PI3K siRNA
control
CXCL12
control
CXCL12
e)
Src
β-actin
Src siRNA - +
Sc siRNA + -
f)
PI3K
β-actin
PI3K siRNA - +
Sc siRNA + -
g)
PI3K
β-actin
PI3K siRNA - +
Sc siRNA + -

Figure 5

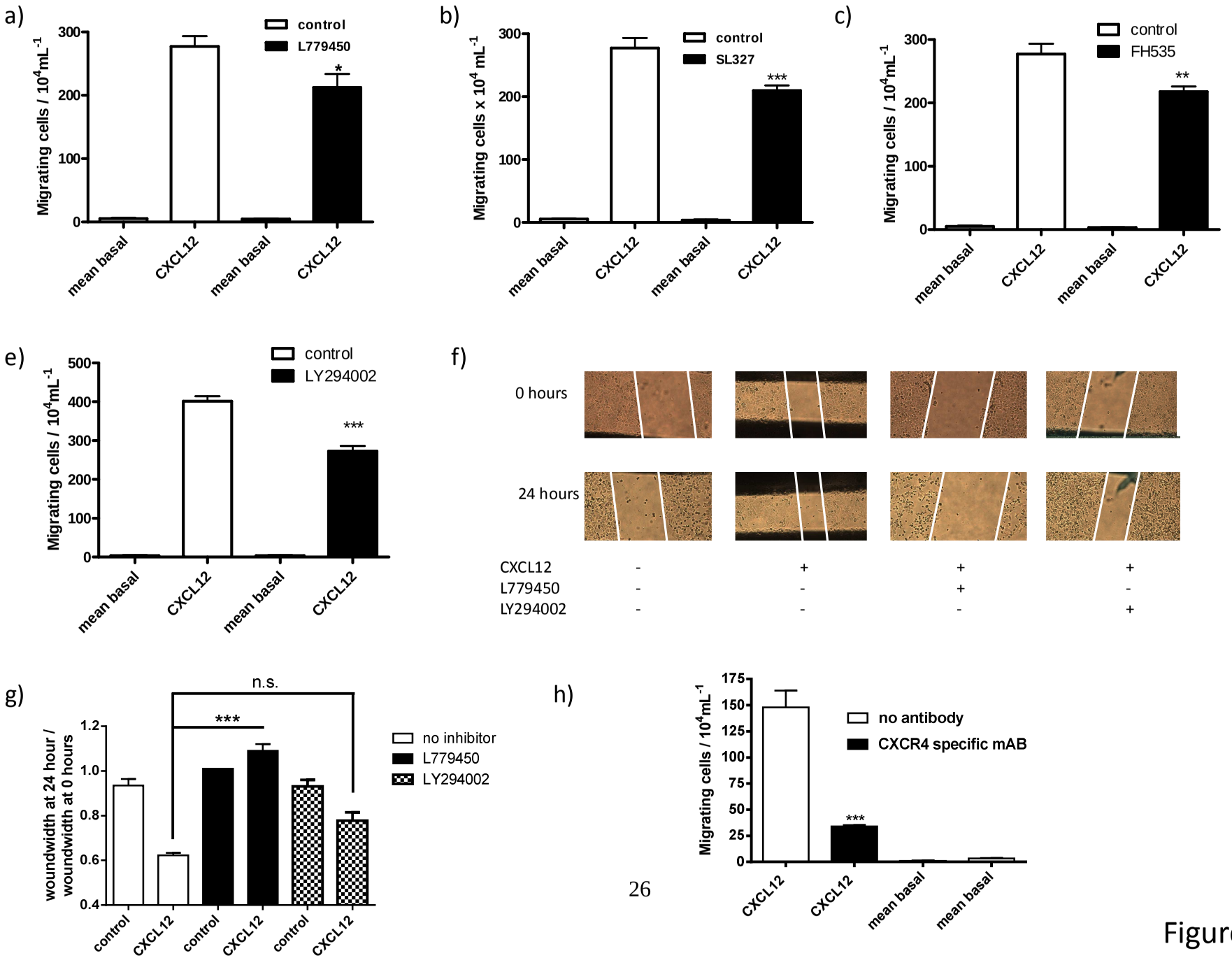
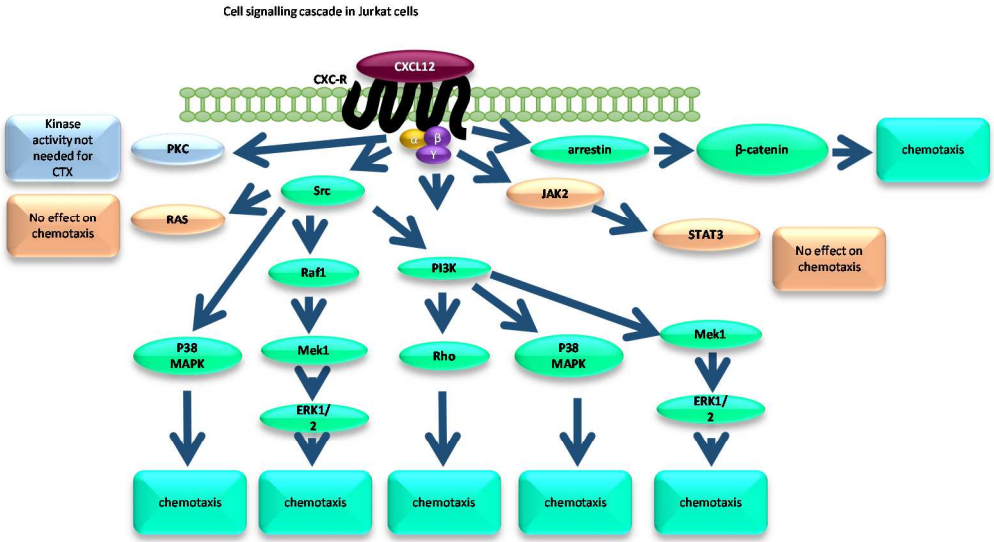

a)
Migrating cells / 10^4mL^-1
control
L779450
*
mean basal
CXCL12
mean basal
CXCL12
b)
Migrating cells x 10^4 mL^-1
control
SL327
***
c)
Migrating cells / 10^4mL^-1
control
FH535
**
e)
Migrating cells / 10^4mL^-1
control
LY294002
***
f)
0 hours
24 hours
CXCL12 - + + +
L779450 - - + -
LY294002 - - - +
g)
n.s.
***
woundwidth at 24 hour / woundwidth at 0 hours
no inhibitor
L779450
LY294002
control
CXCL12
h)
Migrating cells / 10^4mL^-1
no antibody
CXCR4 specific mAB
***
CXCL12
CXCL12
mean basal
mean basal

Figure 6


Cell signalling cascade in Jurkat cells
CXC-R
CXCL12
Kinase activity not needed for CTX
PKC
No effect on chemotaxis
RAS
Src
Raf1
PI3K
P38 MAPK
Mek1
Rho
P38 MAPK
Mek1
ERK1/ 2
ERK1/ 2
JAK2
arrestin
β-catenin
chemotaxis
STAT3
No effect on chemotaxis
chemotaxis
chemotaxis
chemotaxis
chemotaxis
chemotaxis

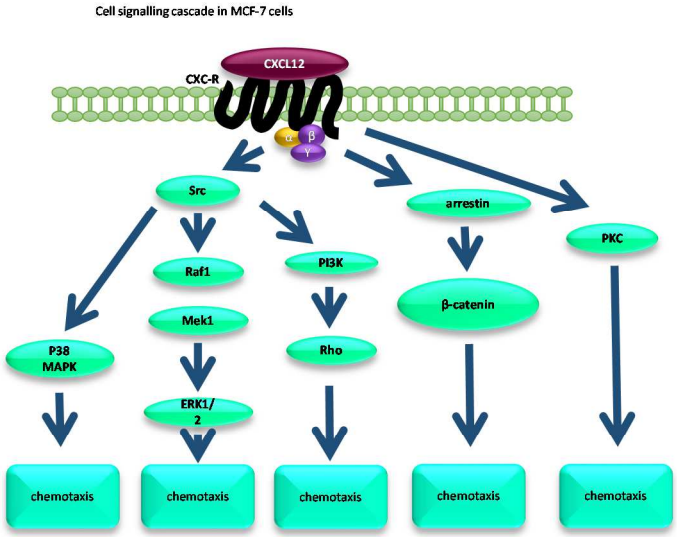

Cell signalling cascade in MCF-7 cells
CXC-R
CXCL12
Src
Raf1
Mek1
ERK1/ 2
PI3K
Rho
arrestin
β-catenin
PKC
P38 MAPK
chemotaxis
chemotaxis
chemotaxis
chemotaxis
chemotaxis

Figure 7