Roles of Slit-Robo Signaling in Pathogenesis of Multiple Human Diseases: HIV-1 Infection, Vascular Endothelial Inflammation and Breast Cancer

DISSERTATION

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By

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Abstract

The signal molecule, Slit, is a family of secreted glycoprotein, which contains 3 isoforms, Slit1-3. The cellular surface receptor for Slit is Robo (Roundabout), which contains 4 isoforms, Robo1-4. It is now clear that, Slit and Robo are expressed and functional in a variety of tissues besides the neuronal system, including but not limited to leukocytes, endothelial cells and epithelial cells. And Slit-Robo signaling also plays important roles in regulating cell functions that are not directly related to cell migration, such as cell attachment, survival and proliferation. Thus, Slit-Robo signaling is proposed to regulate the post-development pathogenesis of multiple human diseases.

**HIV-1 infection:** Slit2 is a ~ 200 kDa isoform of Slit, and it has been shown to regulate immune functions. However, not much is known about its role in HIV-1 (Human Immunodeficiency Virus type 1) pathogenesis. In our study, we show that the N-terminal fragment of Slit2 (Slit2-N) (~120 kDa) inhibits replication of HIV-1 virus in T-cell lines and peripheral blood T cells. Furthermore, we demonstrate inhibition of HIV-1 infection in resting CD4^+ T cells. In addition, we show that Slit2-N blocks cell-cell transmission of HIV-1. We further show that Slit2-N inhibits HIV-1 infection by blocking viral entry into T cells. We also rule out the possibility of Slit2-N-mediated inhibition of various other steps in the life cycle including binding, integration and viral transcription. Elucidation of the molecular mechanism reveals that Slit2-N mediates its functional effects by binding
to Robo1 receptor. Furthermore, we find that Slit2-N inhibits gp120-induced Robo1-F-actin association suggesting that Slit2-N may inhibit cytoskeletal rearrangements facilitating HIV-1 entry. Studies into the mechanism of inhibition of HIV-1 reveal that Slit2-N abrogates HIV-1 envelope-induced actin cytoskeletal dynamics in both T cell lines and primary T cells. We further show that Slit2-N specifically attenuates the HIV-1 envelope-induced signaling pathway consisting of Rac1, LIMK and cofilin that regulates actin polymerization. Taken together, our results show that Slit2-N inhibits HIV-1 replication through novel mechanisms involving modulation of cytoskeletal dynamics. Our study, thus, provides insights into the role of Slit2-N in HIV-1 infection and underscores its potential in limiting viral replication in T cells.

Vascular endothelial inflammation: Slit2 and its receptors Robo1 and Robo4 are considered to regulate mobility and permeability of endothelial cells and other cell types. However, the roles of Slit2 and its two receptors in endothelial inflammatory responses remain to be clarified. In our study, we show that, in primary HUVECs, Slit2 represses LPS-induced secretion of certain inflammatory cytokines/chemokines, cell adhesion molecule ICAM-1 upregulation, and monocyte adhesion. Slit2’s anti-inflammatory effect is mediated by its dominant endothelial-specific receptor Robo4. However, the minor receptor Robo1 has pro-inflammatory properties and is downregulated by Slit2 via targeting of miR-218. Elucidation of molecular mechanism reveals that Slit2 represses inflammatory responses by inhibiting the Pyk2–NF-kB pathway downstream of LPS–TLR4. Further studies reveal that LPS enhances endothelial inflammation by downregulating the anti-inflammatory Slit2 and Robo4 in HUVECs in vitro, as well as in
arterial endothelial cells and liver in vivo during endotoxemia. These results suggest that Slit2–Robo4 signaling is important in regulating LPS-induced endothelial inflammation, and LPS, in turn, enhances inflammation by interfering with the expression of the anti-inflammatory Slit2–Robo4 during the disease state. This implies that Slit2–Robo4 is a key regulator of endothelial inflammation, and its dysregulation during endotoxemia is a novel mechanism for LPS-induced vascular pathogenesis.

**Breast cancer I:** Targeting tumor angiogenesis is a promising alternative strategy for improvement of breast cancer therapy. Slit2-Robo4 signaling has been shown to protect endothelial integrity during sepsis shock and arthritis, and inhibit Vascular Endothelial Growth Factor (VEGF) signaling during pathological angiogenesis of retinopathy, which indicate that Robo4 might be a potential target for angiogenesis in breast cancer. In our study, we used immune competent Robo4 knockout mouse model to show that endothelial Robo4 is important for suppressing breast cancer growth and metastasis. And this effect does not involve the function of Robo4 on hematopoietic stem cells. Robo4 inhibits breast cancer growth and metastasis by regulating tumor angiogenesis, endothelial leakage and tight junction protein ZO-1 downregulation. Treatment of SecinH3, a small molecule drug which deactivates ARF6 downstream of Robo4, can enhance Robo4 signaling and thus inhibit breast cancer growth and metastasis. SecinH3 functioned by reducing tumor angiogenesis rather than directly affecting cancer cell proliferation. In conclusion, endothelial Robo4 signaling is important for suppressing breast cancer growth and metastasis, and it can be targeted (enhanced) by administrating a small molecular drug instead of recombinant Slit2 protein.
Breast cancer II: When overexpressing Slit2 in breast cancer cells (such as MCF7) which typically lack anti-cancer Slit2 expression, one of the most significantly downregulated proteins is S100A7 (Psoriasin). S100A7 is an inflammatory protein known to be broadly upregulated in breast cancer. However, the role of S100A7 in breast cancer has been elusive, since both pro- and anti-proliferative roles have been reported in different types of breast cancer cells and animal models. To date, the mechanism by which S100A7 differentially regulates breast cancer cell proliferation is still not clear. We used Gene Functional Enrichment Analysis to search for the determining factor of S100A7 differential regulation. We confirmed the factor and elaborated its regulating mechanism using in vitro cell culture. We further verified the findings using xenografts of human breast cancer cells in nude mice. In our study, we show that S100A7 significantly downregulates the expression of miR-29b in Estrogen Receptor (ER)-positive breast cancer cells (represented by MCF7), and significantly upregulates miR-29b in ER-negative cells (represented by MDA-MB-231). The differential regulation of miR-29b by S100A7 in ER-positive and ER-negative breast cancer is supported by the gene expression analysis of TCGA invasive breast cancer dataset. miR-29b transcription is inhibited by NF-κB, and NF-κB activation is differentially regulated by S100A7 in ER-positive and ER-negative breast cancer cells. This further leads to differential regulation of PI3K p85α and CDC42 expression, p53 activation and p53-associated anti-proliferative pathways. Reversing the S100A7-caused changes of miR-29b expression by transfecting exogenous miR-29b or miR-29b-Decoy can inhibit the effects of S100A7 on in vitro cell proliferation and tumor growth in nude mice. In conclusion, the distinct
modulations of the NF-κB – miR-29b – p53 pathway make S100A7 an oncogene in ER-negative and a cancer-suppressing gene in ER-positive breast cancer cells, with miR-29b being the determining regulatory factor.

All in all, Slit2 acts on different Robo receptors on various cell types, which modulates the cell behaviors during post-development pathological events. Upon comparing these regulatory effects of Slit-Robo in different diseases, there appears a common feature: “slowing down the cell activities as brakes slowing down a vehicle”. Slit-Robo slows down the T cell cytoskeletal dynamics during HIV-1 infection; Slit-Robo slows down the endothelial inflammatory responses to bacterial infection; Slit-Robo slows down the tumor blood vessel generation in breast cancer. Hence, it seems promising to target the Slit-Robo signaling for novel therapeutic methodologies of multiple human diseases.

*Target Slit-Robo signaling to hit the brake pedal for multiple human diseases:*

*HIV-1 infection, vascular endothelial inflammation and breast cancer*
Dedication

This PhD dissertation is dedicated to my parents and my folks.

My parents deeply and yet unnoticeably influenced me.
Father taught me to think deeply and to analyze comprehensively, which gave me the thirst for knowledge and the curiosity to the unknown.

Mother taught me to stay kind and to care for people, which gave me the interest in medicine and the concerns about human health.

Undoubtedly, my parents, to a great extent, helped make this dissertation possible.
The expectation and encouragement from my maternal grandfather also kept driving me forward.

谨以此哲学博士论文文献给我的父母和家人。
父母对我的影响论肌뎁인데而“润物细无声”。
父亲教我深刻的思维和全面分析的能力，他给了我对真知的渴望和对未知的好奇。
母亲教我保持内心的善良和对人的关爱，她给了我对医学的兴趣和对健康的关心。
父母无疑很大程度上决定了我生物医学科学博士的学习和研究。
外祖父的期许和鼓励，也给了我前进路上的很大的力量。

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Chapter 1: The role of Slit2-Robo1 signaling in regulating HIV-1 infection in T cells

1.1 Introduction

1.1.1 Acquired Immune Deficiency Syndrome (AIDS) and HIV-1

AIDS is a disease of insufficient T cell immunity caused by Human Immunodeficiency Virus (HIV) infection. When immune system is severely impaired, secondary infections are often lethal, which poses a great threat to human health. In the year 2013 (most recent data available), about 35 million people are living with HIV infection worldwide, over 2.1 million individuals were newly infected with HIV, and more than 1.5 million people died from AIDS (UNAIDS 2015).

Route of HIV-1 infection

The main pathogenic and virulent subtype of HIV is HIV-1. HIV-1 is transmitted through body fluids (such as blood, semen and vaginal fluid) from an infected person. HIV-1 viruses disseminated in the body fluids of infected patient can exist as free viral particles and as cell associated viruses. Upon contact with mucosal surface and skin, HIV-1 virus can pass the primary barriers at positions of impaired barrier integrity, or it can have contact with susceptible cells through transcytosis and subepithelial dendritic cell protrusion (Hladik and McElrath, 2008). The envelope protein of HIV-1, gp120, has binding affinity to various cellular receptors, including CD4, CXCR4, CCR5 and DC-SIGN. Thus, HIV-1 can infect or reside in cells that express these receptors. The first line
target cells of HIV-1 infection are dendritic cells and macrophages residing in the skin and mucus, which express DC-SIGN and CCR5. Then dendritic cells and macrophages carrying HIV-1 can transmit virus to T cells expressing CD4, CXCR4 and CCR5 in tissues or draining lymph node. Infected T cells can further disseminate HIV-1 throughout the whole body. Due to its susceptibility and abundance, CD4$^+$ T cells are the main target of HIV-1 infection and the main host for HIV-1 replication.

**Loss of T cells and cause of AIDS**

Massive loss of functional T cells is a hallmark of HIV-1 pathogenesis and AIDS. The causes of T cell death in HIV-1-positive patients are multifold on multiple levels (Khaitan and Unutmaz, 2011). In terms of cell types, there are (1) death of CD4$^+$ T helper cells and (2) death of CD8$^+$ cytotoxic T lymphocytes (CTLs). In terms of infection status, there are (i) death of HIV-1 infected T cells and (ii) death of non-infected bystander T cells. In terms of stimuli of cell death, there are (a) extracellular HIV-1 soluble proteins, (b) extracellular HIV-1 virions, (c) intracellular HIV-1 proteins, (d) cytolitic HIV-1 replication and (e) host derived cytokines and anti-viral proteins. In terms of the types of cell death, there are (α) apoptosis and pyroptosis, (β) necrosis, (γ) cell senescence and anergy and (δ) physical membrane insult. Regardless of the causes of T cell death, insufficient T cell functions render the adaptive immune system paralyzed, which undermines the ability to defend against different types of non-self existence (pathogens and cancers). Hence, lack of T cells *per se* is not lethal, but the consequences of that are often life-threatening. The opportunistic infections contribute to the majority of AIDS related death.
Disease progression from HIV-1 infection to AIDS

The disease progression of AIDS is often primed by a rapid raise of HIV-1 viral load and a sudden drop of CD4\(^+\) T cell count within a few weeks of infection. After that, HIV-1 viral load starts to lower down and CD4\(^+\) T cell count starts to elevate with fluctuations. Around week 4, CD8\(^+\) CTLs get fully activated and CD8\(^+\) CTL count soars up to a peak, which is followed by a quick drop back to a minimal level. During the chronic phase of HIV-1 infection, HIV-1 viral load gradually reduces, CD4\(^+\) T cell count continues to decrease with fluctuations and CD8\(^+\) CTL count remains low. At the late stage of HIV-1 infection, which is normally more than 3 years after infection, CD4\(^+\) T cell count drops to minimal level and CD8\(^+\) CTL count remains low. AIDS is thus formed (Goulder and Watkins, 2004).

HIV-1 life cycle

HIV-1 is a type of retrovirus. It carries two single-stand RNAs as its genome. RNAs are associated with accessory proteins, encapsulated by capsid and matrix proteins and wrapped in membrane. The typical life cycle of HIV-1 in T cells is composed of: virus binding to cell membrane, entry into cells, virus de-capsulation, reverse transcription of virus RNA genome, nuclear import of pre-integration complex, integration of HIV-1 genome into cell genome, production of virus proteins and genome RNAs, assembly of virus, budding and release of virus out of the cell. Among the steps of HIV-1 life cycle, theoretically almost every step can be targeted for anti-HIV-1 therapy.

Treatment for HIV-1 infection
Currently, the treatment for HIV-1 infection has been effective but not curative, which means that AIDS can be relived but HIV-1 infection cannot be completely eliminated. The combination of drugs that target multiple different viral proteins/activities, also known as Highly Active Anti-Retroviral Therapy (HAART), is the standard treatment for HIV-1 infection. With continuous HAART treatment, viral load remains undetectable and patients live a functionally normal life. Most of the patients under HAART treatment can expect an un-shortened life span. However, the side effects and the continuous drug intake significantly lower the life quality of HIV-1 patients. There has been a single case of complete elimination of HIV-1 infection in a HIV-1 positive leukemia patient, who received chemotherapy and bone marrow transplant from a CCR5-Δ32 donor. This type of treatment seems promising, but remains very risky and rarely accessible. Meanwhile, a few therapies under clinical trials seem attractive, including stem cell gene editing and reinfusion.

**Challenges of treatment**

One of the biggest headaches of HIV-1 clinicians and researchers is that current drug therapy cannot completely remove HIV-1 from the patients. The reasons why drug treatment cannot eliminate HIV-1 are, again, multifold and still remain partially unclear. Three main theories are proposed to be related with HIV-1 persistence with drug treatment, which are HIV-1 hypermutation(Boutwell et al., 2010), HIV-1 latency(Finzi et al., 1999) and HIV-1 reservoir(Ramratnam et al., 2000; Ramratnam et al., 2004). HIV-1 hypermutation is caused by the low fidelity of reverse transcriptase, low stability of RNA genome and defensive interventions by host cellular factors. Rapid and various mutations
of HIV-1 genome generate drug resistance thus facilitate viral evolution and adaptation. HIV-1 latency is the inactivity of HIV-1 genome after integration in resting or memory T cells. It can get active again when the integrated T cell is activated. HIV-1 reservoir is a controversial theory, which claims that there are “reservoirs” of T cells in the human body where HIV-1 actively replicates and transmits while staying out of the reach of drugs. Among these theories, HIV-1 hypermutation is the most well-studied and well-accepted cause of viral drug resistance. It is quite straight-forward for one to argue that viral hypermutation related drug resistance is inevitable when the drug is designed to target viral proteins. However, the drugs currently available are all designed to target viral proteins. On the contrary, host cell replicates its genome with high fidelity and barely allows mutations. Thus targeting host proteins which are necessary for HIV-1 infection would be a superior approach to develop novel drugs which can minimize the HIV-1 resistance.

1.1.2 Slit and its receptor Robo

Slit belongs to a group of large secretory glycoproteins that were initially described as regulating axonal guidance during the development of the central nervous system(Kidd et al., 1999; Yuan et al., 1999). Slit consists of a family of three genes: Slit1, Slit2 and Slit3 that are highly homologous to each other and encode ligands for the Robo receptors(Hohenester, 2008; Howitt et al., 2004). Slits and Robo receptors were originally discovered as chemorepellents that prevent developing commissural neurons from mistakenly re-crossing the midline(Brose et al., 1999). It is now clear that Slit and Robo genes are expressed in a panel of different tissues in addition to the brain, where
Slit-Robo signaling also has critical regulatory functions (Chédotal, 2007; Greenberg et al., 2004; Medioni et al., 2010; Piper et al., 2000). However, information on the effects of Slit-Robo in pathologies of human diseases of non-neuronal systems is less studied, with recent studies indicating that Slit2-Robo1 complex regulates inflammation and cancer (Grieshammer et al., 2004; Legg et al., 2008; London et al., 2010; Marlow et al., 2008; Prasad et al., 2004; Prasad et al., 2008; Wang et al., 2003; Wu et al., 2001).

1.1.3 Slit-Robo and regulation of HIV-1 infection

With regard to the immune system, Slit2 has been shown to inhibit migration of hematopoietic cells, monocytes, neutrophils, dendritic cells, and T lymphocytes, toward chemoattractant signals (Chen et al., 2004a; Guan et al., 2003; Tole et al., 2009; Wu et al., 2001). Specifically, we and others have shown Slit2 blocks CXCL12/CXCR4-mediated chemotaxis of T cells (Prasad et al., 2007; Wu et al., 2001). In addition, we recently showed that full-length Slit2 inhibited both X4 and R5-tropic HIV-1 replication in T cells (Anand et al., 2011). Recently, Slit2-N was also shown to regulate HIV-1-gp120-induced endothelial permeability (Zhang et al., 2012). A prototypical Slit2 protein contains an N-terminal signal peptide, four leucine-rich repeats (LRRs), seven (in Drosophila Slit) or nine (in vertebrate Slits) EGF repeats, and a C-terminal cysteine knot (Morlot et al., 2007b). Studies have shown that full-length Slit2 is cleaved between the fifth and sixth EGF-like repeat into a 120–140 kDa N-terminal and a 50–60 kDa C-terminal fragment (Nguyen Ba-Charvet et al., 2001b). Recent evidence suggests that the N-terminal region of Slit2 (Slit2-N) is responsible for the biological functions of Slit2 (Chédotal, 2007; London et al., 2010). The intracellular signal transduction
mechanism for Slit2-Robo1 signaling is not well-studied. However, several lines of recent evidence have demonstrated that Slit2 regulates actin polymerization after binding to Robo receptor (Ghose and Van Vactor, 2002; Liu et al., 2006; Prasad et al., 2007; Stella et al., 2009). Robo is a transmembrane receptor that consists of a fibronectin type III and immunoglobulin (Ig)-like domains and an intracellular cytoplasmatic domain. The intracellular domain of Robo has been shown to interact with proteins that regulate the Rho family of small guanosine triphosphates (GTPases), which play well-defined roles in cell migration, cytoskeletal organization and remodeling by eliciting changes in actin cytoskeleton (Schmitz et al., 2000). Furthermore, HIV has the capacity to bind to its receptors, CD4 and/or co-receptors (CXCR4 or CCR5) and induce signal transduction pathways that trigger actin cytoskeletal rearrangements facilitating viral entry (Harmon and Ratner, 2008; Jiménez-Baranda et al., 2007; Spear et al., 2012; Vorster et al., 2011; Yoder et al., 2008). In the present study, we analyzed the effect of N-terminal domain of Slit2 (Slit2-N) in HIV-1 infection and shown that it inhibits replication of both X4 and R5-tropic HIV-1. Furthermore, our mechanistic studies in T cell lines and primary T cells have revealed that Slit2 inhibits the HIV-1 viral entry through a novel mechanism involving modulation of actin cytoskeletal dynamics.

1.2 Materials and Methods

**Viral isolates**

The lab-adapted X4-tropic virus, HIV-1 IIIB and the R5-tropic virus, HIV-1 BaL, used in this study were obtained from the NIH AIDS Research and Reference Reagent Program.

**Anti-HIV-1 assays**
MT4 cells, a human T cell line bearing human T cell leukemia virus type 1 (NIH AIDS Research and Reference Reagent program) were grown in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, Carlsbad, CA) and antibiotics. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood collected from healthy HIV-1-seronegative donors (American Red cross, Columbus, OH) by Ficoll-Paque density gradient centrifugation (GE Biosciences, Piscataway, NJ). The study was done in compliance with the Helsinki declaration on rights of human subjects with approval from the Ohio State University Institutional Review Board (2007H0281). Monocytes were depleted by two rounds of adherence to plastic. Non-adherent cells were either rested overnight in complete RPMI and used for infection or stimulated with phytohemagglutinin (PHA) (5 μg/mL) for 3 days. CD4⁺ T cells were isolated according to the manufacturer’s protocol by immunomagnetic selection (EasySep Kit, Stem Cell Technologies, Canada).

MT4 cells pre-treated with various concentrations of Slit2-N (0.6-60 nM) were infected with HIV-1 at 10 ng p24/10⁶ cells. The cells were washed extensively after two hours of infection to remove the unbound virus and incubated for 48 hours. PHA-stimulated PBMCs were pre-treated with Slit2-N and infected similarly with HIV-1. The culture supernatant were assessed with the HIV-1 p24 ELISA (Advanced Bioscience Laboratories, Kensington, MD).

For infection of resting CD4⁺ T cells, HIV-1 IIIB (10 ng p24) was used to infect 10⁶ cells. For these studies, T cells were pretreated with Slit2-N (6nM), incubated with the
virus for 2 hours at 37°C and washed twice with medium to remove the unbound virus. Infected cells were resuspended into fresh complete RPMI containing Slit2-N (6nM) at a density of 10^6/mL and incubated for 5 days without stimulation. Cells were activated at day 5 with anti-CD3/CD28 magnetic beads (Dynal) at 2–4 beads per cell to initiate viral replication. Infected cells were pelleted at various time points and supernatants used for p24 ELISA.

**Quantification of cell-cell spread by flow cytometry**

To measure cell-cell spread by flow cytometry, an adaptation of the assay of Sourisseau et al. (Sourisseau et al., 2007) was used. Briefly, equal number of HIV-1-infected donor MT4 cells were mixed with CellTrace Far Red-labeled target T cells (2 μM; Invitrogen) in the presence of various concentrations of Slit2-N (0–60 nM) and incubated for 4 h at 37°C. In addition, infected cells were treated with trypsin-EDTA prior to fixing to further diminish doublet formation and to remove surface-absorbed p24. Cells were then fixed in 4% formaldehyde, washed, and permeabilized in BD Perm/Wash buffer (BD Biosciences), and HIV-1 p24 was detected with the FITC-conjugated HIV p24 antibody (Pierce) and data acquired using a FACSCalibur (BD Biosciences). The percentage of HIV-p24^+ cells among the CellTrace FarRed-labeled target cells was quantified and data analysis performed using CellQuest 5.0.

**Proteins antibodies and plasmids**

Slit2-N protein was obtained either from AbCam (Cambridge, MA) or Creative Biomart (Shirley, NY); LIMK1, Cofilin and GAPDH antibody was obtained from Cell signaling (Danvers, MA), while Robo1 antibodies were obtained from AbCam. Slit2-ΔD2 was
prepared by transfecting the Slit2-ΔD2 plasmid in 293T cells. The protein was purified from the supernatants as described before (Prasad et al., 2007). The Slit2-ΔD2 plasmid was cloned in pTT28 by deleting the second LRR domain of truncated Slit2 (Slit2-N) using restriction enzymes BsrGI and NheI. After deleting 210 amino acids (Q235-W444) of Slit2-N, a short synthesized linker corresponding to amino acids YTAGGSAGGSAGGSAGKL was inserted into BsrGI and NheI restriction sites (Patel et al., 2012).

**Preparation and infection of HIV-1 pseudotyped viruses**

HIV-1 viruses pseudotyped with vesicular stomatitis virus envelope protein (VSV-G) were prepared as previously described (Bartz and Vodicka, 1997; Canki et al., 2001). Briefly, 293T cells (27 × 10⁶ per 10 cm plate) were transfected with 20 μg of pNL4-3.Luc.R'E- (HIV-Luc) plasmid and 4 μg of pVSV-G (obtained from the NIH AIDS Research and Reference reagent program) by lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Cell culture supernatants were collected 48 hours later, filtered and saved as virus stocks. For infection, pseudotyped viruses corresponding to 10 ng p24 were used to infect target cells. Following 2 hour infection, the cells were washed extensively to remove unbound viruses. The cells were incubated for 48 hours and harvested for luciferase activity as previously described (Canki et al., 2001).

**Immunostaining and flow cytometry**

FITC- or PE-labeled monoclonal antibodies against human CD4, CCR5 or CXCR4 were purchased from BD Biosciences. Half a million cells were incubated with isotype control
or the labeled antibodies on ice in PBS-0.1% BSA for 30 minutes. Cells were washed with cold PBS-0.5% BSA, and then analyzed on a FACSCalibur (BD Biosciences). To determine intracellular p24, HIV-1 infected T cells were fixed and permeabilized using Fix/perm solution (BD Biosciences, San Jose, CA) and stained with KC57 monoclonal antibody (Coulter, Brea, CA) followed by flow cytometry analysis. The respective isotype control was also included. The flow cytometry data was analyzed by CellQuest software (BD Biosciences).

**gp120 binding assay**

MT4 cells were incubated for 1 hour at 37°C with differing concentrations of Slit2-N. After the incubation, cells were washed with PBS and incubated with 1 μg/mL FITC-conjugated recombinant gp120 (Immunodiagnostics, Woburn, MA) for 30 minutes at room temperature. The fluorescence intensity of gp120-FITC bound to the surface of lymphocytes was measured with the FACS Calibur.

**Virus binding assay**

The procedure used to detect the binding of HIV-1 particles into target cells was described previously (Finnegan and Blumenthal, 2006). Briefly, MT4 cells (1.0 × 10⁶ cells/mL) were exposed to HIV-1 (40 ng p24) in the absence or presence of the Slit2-N (3 and 6 nM) in 100 μL of PBS. After incubation at 4°C for 1 h, unbound virus particles were removed by washing the cells three times in PBS. Cell lysates were prepared by resuspending the pellet in lysis buffer (PBS containing 1% Triton X). Viral binding was monitored by measuring the amount of p24 in the cell lysates by ELISA. Total cell
protein was calculated using Bradford assay and all samples were normalized for protein content.

**Viral entry assays**

In the first method, MT4 cells \((1.0 \times 10^6 \text{ cells/mL})\) were exposed to HIV-1 (40 ng p24) in the absence or presence of the Slit2-N (3nM and 6nM) in 100 μL of PBS. After incubation at 37°C for 4 hours, unbound virus particles were removed by washing the cells three times in PBS. The cells were treated with 0.05% trypsin for 10 minutes to remove surface-bound viral particles. Cell lysates were prepared by resuspending the pellet in lysis buffer (PBS containing 1% Triton X). Viral entry was monitored by measuring the amount of p24 in the cell lysates by ELISA. Total cell protein was calculated using Bradford assay and all samples were normalized for protein content.

In the second approach, viral entry was measured by the virion-based fusion assay (Cavrois et al., 2002). Briefly, a total of \(1 \times 10^6\) MT4 cells were incubated 3 h with equivalent viral inputs of BlaM-Vpr-containing virions (500 ng of p24) in 500 μL RPMI 1640 medium. Cells were then extensively washed to remove free virions and incubated (1 h, room temperature) with CCF2-AM loading mix, as recommended by the manufacturer (GeneBLAzer detection kit; Invitrogen). Next, excess dye was washed off and cells were incubated for 16 h at room temperature before fixation with 4% paraformaldehyde. Then, \(8 \times 10^5\) cells were placed in a 96-well plate per each experimental condition. The associated emission light to cleaved CCF2 (blue; 447 nm) and intact CCF2 probe (green; 520 nm) was measured. The entry of BlaM-Vpr containing virions was measured as the ratio of the maximal fluorescence intensity between cleaved
and intact CCF2. Thereby, an increase in this ratio indicates more fused viruses with target cells. The percentage of infection was determined by measuring the fluorescence intensities of intact and cleaved CCF2 probe in control infected cells (scrambled or pCDNA.3 transfected cells) and subtracting the background blue and green fluorescence ratio determined in non-infected cells (without β-lactamase activity), (GeneBLAzer detection kit; Invitrogen).

**Virus integration**

Host cell nuclear integration of HIV-1 in MT4 cells was analyzed by PCR as described previously (Mbisa et al., 2009). DNA was prepared by using a DNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. DNA samples were used for the amplification of integrated DNA with Alu-LTR–specific primers (Nagaraja et al., 2012) using the following cycling conditions: 95°C for 10 minutes, then 40 cycles at 95°C for 15 s, 60°C for 1 minute, and 72°C for 1 minute. GAPDH was used as an internal control.

**Determining proviral transcription of full-length (FL) and multiple-spliced (MS) HIV-1 mRNA**

RNA was prepared from untreated or Slit2-N treated HIV-1 IIIB-infected cells using the QIAamp RNAeasy Mini Kit protocol (Qiagen). FL and MS HIV-1 mRNA transcript levels were determined by a modification of a protocol described (Sundstrom et al., 2007). qRT-PCR was performed using primers (200nM) specific for FL and for MS using 400 ng of sample RNA in a SYBR Green assay system using a Realplex Cycler (Eppendorf, Westbury, NY).
Transfections

siRNA-mediated knockdown of Robo1 was performed using specific ON-TARGETplus SMARTpool siRNA (Dharmacon, Lafayette, CO). Briefly, MT4 cells were nucleofected with 200 nM siRNA (Amaza Biosystems). The respective, non-targeted (NT) siRNA was used as a control. Robo1 siRNA-mediated knockdown was estimated by detecting Robo1 expression 48 hours after the initial transfection by Western blotting and/or flow cytometry.

Rac1 activation assay

Rac1 activation was determined by using the Rac/Cdc42 activation assay kit (Millipore). In brief, cell lysates were pre-cleared with sepharose beads were incubated with 20 μg/mL p21-activated kinase (PAK)-1 agarose for 60 minutes at 4°C, according to the manufacturer’s instructions. Agarose beads were collected by centrifugation, followed by denaturation, boiling of the samples, and SDS-PAGE analysis. Proteins were transferred to nitrocellulose membranes, and Western blotting was performed by using murine anti-human Rac1 antibody. Blotting of equal amounts of lysates with total Rac1 represented the loading controls.

FITC-Phalloidin staining of F-actin

One to two million cells were stimulated with HIV-1 gp120 IIIB and incubated at 37°C with gentle agitation (600 to 1200 rpm) to prevent cell settling at the bottom. F-actin staining using FITC-labeled phalloidin (Sigma, St. Louis, MO) was carried out according to the manufacturer’s recommendation with minor modifications. Briefly, each staining was carried out using 1–2 ×10^6 cells. Cells were pelleted, fixed and permeabilized with
CytoPerm/Cytofix buffer (BD Biosciences) for 20 minutes at room temperature, washed with cold Perm/Wash buffer (BD Biosciences) twice, followed by staining with 5 μL of 0.3 mM FITC-labeled phalloidin for 30 minutes on ice in dark. After washing twice with cold Perm/Wash buffer, cells were resuspended in 1% paraformaldehyde and analyzed on a FACSCalibur (BD Biosciences). The cells were also observed under a fluorescence microscope (Zeiss Axiophot). Cells were scored as positive if increased F-actin staining was observed.

**Western blotting**

Equivalent amounts of protein extracts were run on a 4% to 12% gradient acrylamide gel (NuPAGE Bis-Tris gel; Invitrogen) and transferred onto nitrocellulose membranes. Immunodetection involved specific primary antibodies, appropriate secondary antibodies conjugated to horseradish peroxidase, and the enhanced chemiluminescence Western blotting detection system (GE Lifesciences).

**Statistical analysis**

Reported data are the means ± S.E.M. of at least three independent experiments performed in duplicate or triplicate. The statistical significance was determined by the Student’s t test.

1.3 Results

1.3.1 N-terminal fragment of Slit2 mediates anti-HIV-1 activity

Slit2 contains the N-terminal region consisting of four leucine-rich repeats (LRRs), nine epidermal growth factor (EGF) repeats, a laminin G domain, and a C-terminal cysteine-
rich region (Figure 1A) (Morlot et al., 2007b). Studies have shown that full-length Slit2 is cleaved between the fifth and sixth EGF-like repeat into a 120–140 kDa Nterminal and a 50–60 kDa C-terminal fragment, and the biological effects of Slit2 are mediated by the N-terminal fragment (Nguyen Ba-Charvet et al., 2001b). In the present study, we first analyzed the anti-HIV-1 activity of a purified Slit2-N fragment.

Firstly, the purity of Slit2-N was determined by silver staining (Figure 1B). To confirm that Slit2-N does not affect the viability and proliferation of T cells, we performed the MTT assay (Roche, Indianapolis, IN) as per the manufacturer’s recommendations, using various concentrations of Slit2-N. We found that Slit2 did not affect cell viability or cell proliferation (data not shown). We also demonstrated that Slit2 did not affect T cell activation (data not shown). Next, we evaluated the anti-HIV-1 activity of Slit2-N against cell-free virus. We used a range of concentrations of Slit2-N (0.6-60 nM), and Slit2-N clearly showed a dose-dependent inhibition of HIV-1 replication in MT4 cells (Figure 1C). We obtained a maximum inhibition of 82.6% when Slit2-N was used at a concentration of 60 nM.

To check the specificity of activity of Slit2-N against HIV-1, we pre-incubated MT4 cells with or without Slit2-N and then infected these cells with HIV-Luc pseudotyped with the VSV-G envelope protein. This single-life cycle virus does not require HIV receptors to enter the target cells (Aiken, 1997). Incubation of MT4 cells with Slit2-N had no effect on infection with HIV-Luc pseudotyped with VSV-G (Figure 1D).
Figure 1. The N-terminal Slit2 fragment possesses anti-HIV-1 activity.
(A) Domain organization of Slit2 showing LRR, leucine-rich repeat; EGF, epidermal growth factor-like; LG, laminin G-like; CT, C-terminal cystine regions. (B) Silver staining of the Slit2-N protein. (C) MT4 cells pre-treated with various concentrations of Slit2-N were infected with HIV-1 IIIB (10 ng p24). We used heat-inactivated (H.I) Slit2-N as a control. After 48 hours, supernatants were collected for estimation of HIV-1 p24 antigen levels by ELISA. Virus production in the positive control (control HIV-1 infected MT4 cells): 4.8 ng/mL p24 Ag. Heat-inactivated Slit2-N at a concentration of 60nM was used as a control. (D) MT4 cells were treated with Slit2-N (6 nM) for 30 minutes and then infected with HIV-Luc viruses pseudotyped with VSV-G envelope (VSV-G) for 2 hours. Forty-eight hours post-infection, cells were harvested for the Luciferase assay. (E) MT4 cells were treated with equivalent concentrations of Slit2-N and Slit2-ΔD2 (≈6nM) and infected with HIV-1 IIIB (10 ng p24) for 48 hours. Virus production in the supernatants was estimated by HIV-1 p24 ELISA. (F) MT4 cells infected with HIV-1 IIIB were co-cultured with dye-labeled target Jurkat T-cells in the presence of various concentrations of Slit2-N for 4 hours, and the percentage of Gag+ target cells was measured by flow cytometry. The results are represented in a bar graph. Values are the mean percentages of Gag+ dye-labeled target cells with the SEMs. Infected cells were used when >70-80% of the donor cells were routinely Gag+ by flow cytometry. Data are representative of three independent experiments. *p < 0.05 versus untreated cells.
Figure 1. The N-terminal Slit2 fragment possesses anti-HIV-1 activity.
Several recent studies have indicated that the functional effects of Slit2 are concentrated in the D2 domain of Slit2 (Howitt et al., 2004; Piper et al., 2006). To verify whether the D2 region is responsible for the anti-HIV activity of Slit2, we obtained a mutant Slit2 protein that lacks the second leucine-rich region that is necessary for binding to Robo1 (Slit2-ΔD2) (Patel et al., 2012). MT4 cells were infected with HIV-1 IIIB in the presence of equivalent concentrations of Slit2-N and Slit2-ΔD2 (≈6nM). We found that Slit2-ΔD2 showed no inhibition of HIV-1 replication in comparison to Slit2-N (Figure 1E), indicating that the anti-HIV activity of Slit2 is restricted to the D2 domain of Slit2.

In addition to infection with cell-free virions, the importance of cell-associated spread across connecting membrane bridges and close cell–cell contacts is increasingly recognized and thought to constitute the predominant mechanism of HIV-1 propagation in vivo (Jolly et al., 2004; Ruggiero et al., 2008). We, therefore, examined whether Slit2-N has an effect on cell-cell transmission of HIV-1. Briefly, MT4 cells were first productively infected with HIV-1 IIIB. After 24 hours, 70-80% of the cells were HIV-1 p24 positive by intracellular p24 staining and flow cytometry (data not shown). These cells were used as donors and co-cultivated with Far-red Cell trace dye-labeled Jurkat T cells in the presence of various concentrations of Slit2-N, as described in the Methods. As shown in Figure 1F, we clearly observed a dose-dependent inhibition of HIV-1 infection in the target cells with maximum inhibition of 62.5% when Slit2-N was used at 60nM. These studies indicate that Slit2-N also inhibits cell to cell spread of HIV in T cells.
We further evaluated the effect of Slit2-N on HIV-1 IIIB (X4) and BaL (R5) replication in PHA-stimulated primary CD4+ T cells. We observed an approximately 40% reduction in HIV-1 replication with both X4-tropic virus and R5-tropic virus (Figure 2A, left and right panel), indicating that Slit2-N inhibits HIV-1 replication irrespective of the co-receptor used by the virus. We also tested the effect of Slit2-N on infection of resting CD4+ T cells. Cells pre-treated with Slit2-N and infected with HIV-1 IIIB were incubated for 5 days, after which they were activated with anti-CD3/CD28 beads to initiate viral replication (Figure 2B, upper panel). Supernatant collected at various time points after activation was examined for HIV-1 p24 levels by ELISA. We observed a significant reduction in HIV-1 infection (approximately 65%) in resting CD4+ T cells (Figure 2B, lower panel).
Figure 2. Slit2-N inhibits HIV-1 infection of activated and resting CD4\(^+\) T cells.

(A) PHA-stimulated CD4\(^+\) T cells were infected with HIV-1 IIIB (left panel) or HIV-1 BaL (right panel) in the presence of Slit2-N (3nM). The cells were incubated in IL-2 containing medium, and the cells were collected after 48 hours and analyzed for HIV-1 p24 expression by flow cytometry. *p < 0.05 versus untreated cells infected with HIV-1.

(B) Resting CD4\(^+\) T cells were infected with HIV-1 IIIB in the presence or absence of Slit2-N (3nM). Following infection, the cells were washed to remove unbound virus and cultured in the presence of Slit2-N for 5 days. Cells were activated at day 5 with anti-CD3/CD28 magnetic beads, and viral replication was measured by p24 release. Results are representative of 3 separate experiments.
1.3.2 Slit2 attenuates viral entry

In order to determine how Slit2-N-mediated inhibition works, we first determined the step in the HIV-1 life cycle that was inhibited by Slit2-N. Time of addition assays pointed to the inhibition of early pre-integrative steps in the viral life cycle (data not shown). Our next objective was to further determine the exact pre-integrative step in the viral life cycle that is inhibited by Slit2-N. The first step in the viral life cycle is the binding of the virus onto the receptors on the T cells. Therefore, we first determined whether HIV-1 receptor expression was affected by Slit2-N. Treatment of cells with Slit2-N did not affect membrane expression of CD4, CXCR4 or CCR5, which are required for HIV-1 binding and entry into cells (Figure 3A). To investigate whether Slit2-N is capable of inhibiting the binding of HIV-1 gp120 to its receptors on T cells, we analyzed the binding ability of FITC-conjugated recombinant gp120 to the Slit2-N-treated and untreated MT4 cells. As shown in Figure 3B, Slit2-N did not affect the binding of gp120 to T cells. We also determined the binding of HIV-1 virus to MT4 cells using the virus binding assay. As shown in Figure 3C, though there was no difference in the binding of HIV-1 onto the T cells. Together, these results indicated that the binding of virus to the receptors on T cells was not affected by Slit2-N. Next, we further specifically tested the ability of Slit2-N to block cellular entry of HIV-1 in MT4 cells using a HIV-1 virus entry assay. We observed a significant reduction (~40%) in the amount of virus that enters the cell in Slit2-N-treated cells as compared to the control (Figure 3D). To further analyze the role of Slit2-N in inhibiting HIV-1 infection, we performed viral fusion and entry experiments by using X4-tropic HIV-1 viral particles containing the BlaM-Vpr chimera(Cavrois et al.,
2002). These chimera virions have been designed to specifically study the first steps of viral infection, since β-lactamase activity directly correlates with viral entry. Untreated and Slit2-N-treated MT4 cells were incubated (3 h) with equivalent viral inputs of X4-tropic virions containing BlaM-Vpr fusion protein. Untreated cells were susceptible to viral entry, whereas in cells treated with Slit2-N, viral entry was strongly reduced (~50%) (Figure 3E). Taken together, these data indicate that Slit2-N inhibits HIV-1 viral fusion and entry.
Figure 3. Slit2-N blocks HIV-1 entry, but does not affect HIV-1 receptor expression, envelope binding or HIV-1 virus binding to T cells.

(A) To analyze the effect of Slit2-N on HIV-1 receptor expression, MT4 cells or PBMCs were treated with Slit2-N for various time periods. MT4 cells were analyzed for CD4 and CXCR4 expression while PBMCs were analyzed for CCR5 expression, respectively, by flow cytometry. (B) MT4 cells were treated with Slit2-N and analyzed for gp120 binding by flow cytometry using FITC-tagged gp120. (C) For virus binding, untreated and Slit2-N (3 nM and 6 nM) treated MT4 cells were infected with HIV-1 IIIB (40 ng/10^6 cells) and incubated for one hour at 4°C, and the cell lysates were analyzed for HIV-1 p24 levels. (D) MT4 cells infected with HIV-1 IIIB were incubated for 3 hours at 37°C in the presence or absence of Slit2-N (3nM and 6nM). The cells were treated with trypsin, washed, lysed and analyzed for intracellular p24 antigen levels by ELISA. *p < 0.05 versus the control preparation. (E) Untreated and Slit2N-treated MT4 cells were incubated with BlaM-Vpr-containing virions (500 ng of p24), after which the cells were washed and incubated with CCF2-AM loading mix (GeneBLAzer detection kit; Invitrogen). The excess dye was washed off and cells were incubated for 16 h at room temperature before fixation with 4% paraformaldehyde. The entry of BlaM-Vpr containing virions was measured as the ratio of the maximum fluorescence intensity between cleaved and intact CCF2. *p < 0.05 versus the control infected cells. Results are representative of 3 separate experiments.
Figure 3. Slit2-N blocks HIV-1 entry, but does not affect HIV-1 receptor expression, envelope binding or HIV-1 virus binding to T cells.
We further evaluated the effect of Slit2-N on integration and post-integrative steps including transcription. In one set of samples, we pre-treated MT4 cells with Slit2-N prior to infection with HIV-1 IIIB. In another set of samples, we treated the infected cells with Slit2-N, 3 hours after infection to bypass the entry step. After twenty-four hours, proviral HIV-1 DNA was measured with qRT-PCR assays toward LTR-Alu sequences (LTR-Alu) as previously described (Nagaraja et al., 2012), to measure virus integration. We also quantitated the number of full-length and spliced viral transcripts in the infected cells by qRT-PCR. We did not detect a significant difference in virus integration (Figure 4A) or full-length (Figure 4B) and spliced mRNA levels (Figure 4C) between HIV-1 infected untreated MT4 cells and Slit2 -treated MT4 cells, when Slit2-N was added after HIV-1 entry step. However, when Slit2-N was added before infection of the cells, Slit2-N-treated cells showed a significant difference in the both viral integration and transcription. These results suggest that Slit2-N does not regulate HIV-1 integration or transcription in T cells, but inhibits steps prior to integration.
Figure 4. Slit2-N does not affect HIV-1 integration or transcription.

(A) MT4 cells were treated with Slit2-N prior to HIV-1 infection or three hours after infection (to bypass the entry step). Twenty-four hours after infection, DNA was extracted from the respective samples, and the integrated HIV-1 DNA was analyzed by qPCR for Alu-LTR. (B) The full length and spliced (C) RNA transcription was evaluated by qRT-PCR. Uninfected and HIV-1 infected MT4 cells were used as controls. Results are representative of 3 separate experiments.
1.3.3 Anti-HIV-1 activity of Slit2-N is mediated through Robo1

Our next aim was to decipher the molecular mechanism by which Slit2-N mediates HIV-1 inhibition. Robo1 is a protein with a single transmembrane domain, serving as a Slit2 receptor in various cells and mediates a majority of Slit2-N-mediated effects. We, therefore, assessed the role of Robo1 in Slit2-N-mediated inhibition of HIV-1 infection. MT4 cells transfected with Robo1 siRNA showed a significant knockdown of Robo1 expression as compared to cells transfected with non-targeting siRNA (Figure 5A). The transfected cells were infected with HIV-1 IIIB and observed for HIV-1 p24 levels after 48 hours of incubation. As shown in Figure 5B, Robo1 downregulation abrogated Slit2-mediated inhibitory effects compared to control, suggesting that the anti-HIV-1 activity of Slit2-N is predominantly transduced by the Robo1 receptor.

1.3.4 Slit2 inhibits the HIV-1-envelope induced Robo1-actin association

Since studies have shown that Robo associates with several actin-related proteins to mediate its functions (Bashaw et al., 2000; Wong et al., 2001; Ye et al., 2010), we evaluated whether HIV-1 gp120 regulates the association of Robo1 with actin. Interestingly, we found that treatment with HIV-1 gp120 enhanced the association of the Robo1 receptor with actin. However, pre-treatment with Slit2-N attenuated the gp120-induced Robo1-actin association at 5, 15 and 30 minute intervals (Figure 5C), indicating
that Slit2-N upon binding to Robo1 inhibits Robo1/actin association and may thus modulate HIV-1-induced cytoskeletal changes facilitating viral entry.

Figure 5. Slit2-N mediates HIV-1 inhibition by binding to the Robo1 receptor.

(A) MT4 cells transfected with non-targeting (NT) or Robo1-siRNA by nucleofection (Amaza biosystems) were analyzed for Robo1 expression by Western blotting. GAPDH was used as a loading control. (B) The NT and Robo1 siRNA-transfected cells were pre-treated with Slit2-N (3nM) or control, and left uninfected or infected with HIV-1 IIIB for 48 hours. The supernatants were analyzed for HIV-1 p24 levels by ELISA. *p < 0.05 versus the mock-infected cells transfected with NT-siRNA. (C) MT4 cells were stimulated with HIV-1 gp120 for various periods of time in the presence and absence of Slit2-N. The lysates were immunoprecipitated (IP) with anti-Robo1 antibody. The immune complexes were then immunoblotted with β-actin antibody. The same blot was re-probed with Robo1 antibody. TCL- total cell lysate. IgG- antibody control. Results are representative of 3 separate experiments.
1.3.5 Robo1 interacts with F-actin via LSP1 and LSP1 is important for HIV-1 infection

To our knowledge, the structure of Robo1 molecule does not allow its direct association with F-actin. Thus, we intended to find the binding partner of Robo1 in human T cells that mediates the indirect association between Robo1 and F-actin. In order to do so, we first used Robo1 polyclonal antibody (or its non-specific IgG as negative control) to pull down Robo1 and its associated proteins from lysates of MT4 cells (human adult T-cell leukemia cell line). The associated proteins were determined by MS-MS peptide segment alignment analysis, and IgG sample was used as the negative/background control. In the list of Robo1 associated proteins, Leukocyte Specific Protein 1 (LSP1) was the F-actin binding protein with the highest abundance. Thus LSP1 was chosen to be the candidate for further verification. We detected the binding of LSP1 to Robo1 using GST-Robo1-cyt (fusion protein of GST and cytoplasmic domain of Robo1) pull-down assay (Figure 6A).

We then verified the F-actin binding function of LSP1 by analyzing the filapodia formation after transfecting LSP1 in the HEK293 cell which naturally does not express LSP1 (Figure 6B).

In order to investigate the importance of LSP1 in HIV-1 infection, we generated LSP1-knock-down MT4 cell subclone using shRNAs targeting LSP1 mRNA. HIV-1 replication was significantly inhibited in LSP1-knockdown cells comparing to the control cells transfected with non-targeting shRNA, using p24 ELISA assay (data not shown).
Figure 6. Robo1 associates with LSP1, and LSP1 is important for HIV-1 infection.

(A) Robo1 binding proteins in MT4 cell lysates were pulled down by glutathione beads bound to GST or GST-Robo1-cyt, and analyzed by WB. (B) HEK293 cells were transfected by LSP1 construct containing GFP reporter gene. Control and transfected cells were shown as light-field and green fluorescence images.

In conclusion, LSP1 mediates the association between Robo1 and F-actin, and it is important for HIV-1 infection in T cells.

1.3.6 Slit2-N attenuates HIV-1-envelope-induced actin polymerization in T cells

We further investigated the signaling mechanisms of Slit2-N-mediated inhibition of viral entry. In HIV-1 infection, binding of the virus to CD4+ T cells initiates an early actin polymerization in T cells (Balabanian et al., 2004; Vorster et al., 2011) followed by depolymerization (Vorster et al., 2011), a process mimicking the chemotactic response initiated from chemokine receptors. The actin polymerization has been suggested to promote viral entry (Spear et al., 2012; Vorster et al., 2011; Yoder et al., 2008). To
determine whether Slit2-N affects HIV-1-induced cytoskeletal changes, MT4 cells were untreated or treated with Slit2-N and stimulated with HIV-1 gp120, fixed and stained for F-actin. As expected, gp120 induced actin polymerization at early time points (15 and 30 minutes). However, in the presence of Slit2-N, gp120-induced actin polymerization was significantly inhibited, as demonstrated by flow cytometry (Figure 7A). We also observed a significant inhibition of gp120-induced actin polymerization by Slit2-N by fluorescence microscopy (15 minutes) (Figure 7B).
Figure 7. Slit2-N inhibits HIV-1-envelope-mediated actin polymerization.

(A) MT4 cells were pre-treated with Slit2-N (3nM) and treated with gp120 for various time points, as indicated. Cells were taken at various time points and stained with phalloidin-FITC and quantified by flow cytometry. The graph represents the percentage increase in F-actin positive cells over the control. (B) The cells stained with phalloidin-FITC were also observed under a fluorescence microscope. The images shown are cells stimulated with gp120 for 5 minutes. (C) Resting T cells were pretreated with Slit2-N (3nM) and treated with gp120 for various time points, as indicated. Cells were taken at various time points and stained with phalloidin-FITC and quantified by flow cytometry. The graph represents the percentage increase in F-actin positive cells over the control. (D) The cells stained with phalloidin-FITC were also observed under a fluorescence microscope. The images shown are cells stimulated with gp120 for 5 minutes.
To determine whether Slit2-N affects HIV-mediated actin dynamics leading to virus entry in primary cells, we examined the effect of Slit2-N on HIV-1 envelope mediated actin dynamics in resting CD4$^+$ T cells. Actin polymerization was demonstrated by changes in phalloidin-FITC binding to F-actin over time. We found that HIV-1 gp120 transiently triggered low actin polymerization at early time points in primary T cells. Pretreatment with Slit2-N inhibited early actin polymerization in these cells (Figure 7C and D), indicating that Slit2 may inhibit HIV-1 entry by modulating the cytoskeletal changes induced by HIV-1.

1.3.7 Slit2 inhibits signaling mechanisms that regulate gp120-induced actin cytoskeletal dynamics

We further evaluated the mechanism by which Slit2-N inhibits HIV-1 gp120-induced actin polymerization. The mechanism that triggers early actin polymerization through HIV-1 envelope-mediated transient activation of the Rho GTPase, Rac1 and the LIM domain kinase (LIMK), has recently been described (Costantino et al., 2012; Harmon and Ratner, 2008; Vorster et al., 2011; Wu et al., 2008; Yoder et al., 2008). LIMK1 is a protein that phosphorylates and inactivates cofilin that in turn enhances actin polymerization thus facilitating HIV-1 entry. We therefore hypothesized that Slit2-N may decrease early actin polymerization by preventing HIV-1-envelope-induced activation of the Rac1/LIMK1/cofilin pathway.
We first tested the effects of Slit2-N on HIV-1-envelope-induced Rac1 activation in T cells. We used agarose beads conjugated to the PBD of PAK (PAK-PBD) to detect the activated, GTP-bound species of Rac1. Unstimulated T cells had low basal levels of activated Rac1. Exposure to gp120 increased levels of activated Rac1. Slit2-N did not affect basal levels of activated Rac1 (data not shown), but inhibited gp120-induced activation of Rac1 significantly (Figure 8A). Furthermore, we found that Slit2-N inhibited gp120-induced phosphorylation of both LIMK1 (Figure 8B) as well as phosphorylation of cofilin (Figure 8C). We also showed that treatment of resting CD4+ T cells with Slit2-N also interfered with HIV-envelope induced phosphorylation of LIMK1 and cofilin. (Figure 8D-E). Together, these results suggest that Slit2-N inhibits gp120-induced Rac1/LIMK1/cofilin pathway that mediates actin polymerization, thereby blocking HIV-1 entry.
Figure 8. Slit2-N attenuates HIV-1 envelope-induced signaling pathway that mediates actin cytoskeletal reorganization.

(A) Lysates from untreated, full-length Slit2 and Slit2-N (3nM)-treated MT4 cells were stimulated with X4-tropic HIV-1 gp120 (10nM) for 10 minutes. The lysates were incubated with 20 μg/mL p21-activated kinase (PAK)-1 agarose for 60 minutes at 4°C. Agarose beads were analyzed for Rac1-binding activity by WB with anti-Rac1 antibody (upper panel). Total Rac1 (lower panel) represents loading controls. (B) MT4 cells, pretreated with Slit2-N, were treated with HIV-1 gp120 for various time points, blotted for phospho-LIMK1 and total LIMK1. (C) The same lysates were blotted with phospho-cofilin, and total cofilin. (D) Resting primary T cells were treated as indicated. Lysates were blotted for phospho-LIMK1 and total LIMK1. (E) The same lysates were also blotted with phospho-cofilin and total cofilin.
1.4 Discussion

A prototypical Slit2 protein contains an N-terminal signal peptide, four leucine-rich repeats (LRRs), seven in Drosophila Slit) or nine (in vertebrate Slits) EGF repeats, and a C-terminal cysteine knot (Morlot et al., 2007a). Structural-functional analysis of Slit2 has revealed that the anti-HIV-1 activity of Slit2 was present in the N-terminal domain of Slit2. In the present study, we also showed that the N-terminal region of Slit2 was sufficient to mediate anti-HIV-1 activity in T cells. A recent study has also evaluated the administration of Slit2-N in a murine model of H5N1 influenza (London et al., 2010). Furthermore, Slit2-N also attenuated HIV-1 gp120-induced endothelial permeability (Zhang et al., 2012). Our findings are thus in agreement with recent studies that indicate that many of the biological functions of Slit2 are mediated by the N-terminal fragment (Chédotal, 2007; London et al., 2010).

In the present study, we showed that Slit2 not only inhibited infection of T cells with cell-free virus, but also inhibited cell-cell transmission of HIV-1 using co-culture of HIV-1–infected and non-infected cells. Cell-cell transmission of HIV-1, through the formation of a virological synapse, is the predominant mode of HIV-1 transfer in vivo and probably occurs in secondary lymphoid organs, where most lymphocytes are present and cells are in close contact with each other and with antigen presenting cells (Jolly et al., 2004; Ruggiero et al., 2008).
The initial steps in the HIV-1 viral life cycle include the binding of the viral envelope glycoproteins to the primary receptor, CD4 and co-receptors, CXCR4/CCR5 followed by membrane fusion and viral entry. Recently, Slit2 was shown to downregulate CXCR4 expression in breast cancer cells (Marlow et al., 2008). However, in contrast to breast cancer cells, Slit2-N did not have any effect on the downregulation of either CXCR4/CCR5 or CD4 expression, ruling out down-modulation of HIV-1 receptors/coreceptors as a mechanism of action for Slit2. In addition, virus binding assays indicated that Slit2-N did not inhibit HIV-1 binding onto target cells. However, specific entry assays demonstrated that Slit2 inhibited viral entry into the target cell.

In our mechanistic studies, we showed the importance of Robo1 in Slit2-mediated inhibition of HIV-1 replication using siRNA-mediated Robo1 knockdown experiments. This is in agreement with various genetic and biochemical experiments that have shown that Slit2 binds to Robo1 receptor to mediate signaling and many of its functional effects (Howitt et al., 2004; Kidd et al., 1999; Liu et al., 2006; Morlot et al., 2007b; Prasad et al., 2007; Wu et al., 2001). Our next aim was to investigate the exact mechanism of inhibition of virus entry. The early interaction between HIV-1 and T cells initiates intracellular signaling cascades that are important for the early steps of the HIV-1 life cycle (Naghavi et al., 2007; Yoder et al., 2008).

The cortical actin is a common structure that is targeted by most viruses for entry and intracellular transport (Naghavi and Goff, 2007; Ploubidou and Way, 2001). In HIV-1 infection, the direct involvement of the cortical actin in early stages of viral infection has been suggested in HIV-mediated CD4-CXCR4 receptor clustering (Barrero-Villar et al., 2008).
2009; Jiménez-Baranda et al., 2007; Naghavi and Goff, 2007), and intracellular migration (Yoder et al., 2008). Actin polymerization has been shown to promote viral entry in both the simple model of infection by free virus and the more physiologically relevant route of infection through the virological synapse (Jiménez-Baranda et al., 2007; Naghavi et al., 2007; Pontow et al., 2004; Spear et al., 2012; Vorster et al., 2011; Yoder et al., 2008). In this study, we showed that Slit2-N inhibits gp120-induced actin polymerization and Robo-actin association at early time points. This is in agreement with the common theme emerging from a number of studies that Slit2 mediates cellular guidance through regulation of actin polymerization as well as data from neuronal cells linking Robo to proteins associated with the actin cytoskeleton including Ena and srGAP1 (Ghose and Van Vactor, 2002; Kanellis et al., 2004; Liu et al., 2006; Prasad et al., 2008; Tole et al., 2009).

The data implicating that Slit2-N inhibits gp120-induced actin polymerization led us to evaluate the intracellular molecules that transduce the signal to regulate the actin polymerization. Several studies indicate that the regulatory effect of Slit2 involve modulation of Rho family of small GTPases, Rac1 and CDC42. Rho-GTPases play a definitive role in cell migration, cytoskeletal organization and actin remodeling. A recent study has also shown that the binding of HIV-1 envelope glycoprotein with the primary receptor CD4 and one of the co-receptors, CXCR4 or CCR5 activates a signaling cascade resulting in activation of Rho GTPases, specifically Rac1 and actin cytoskeletal remodeling that facilitate HIV-1-induced membrane fusion and virus entry (Harmon and Ratner, 2008). Rac1 has been shown to be activated by gp120 to trigger actin
polymerization to mediate both cell-cell (Pontow et al., 2004) and virus-cell fusion (Harmon and Ratner, 2008). Furthermore, another recent study has described a pathogenic mechanism for triggering early actin polymerization through HIV-1 envelope-mediated transient activation of Rac1-LIMK1-cofilin pathway (Vorster et al., 2011).

The LIM domain kinase, LIMK, is a protein that phosphorylates cofilin and LIMK1-mediated actin polymerization was shown to directly facilitate early CD4 CXCR4 clustering and viral entry. These studies led us to test the effects of Slit2-N on the HIV-1-envelope-induced activation of LIMK1 and cofilin. We found that gp120-induced phosphorylation of LIMK1 and cofilin was inhibited by Slit2-N. Previously, it has been shown that early LIMK1 activation in response to HIV envelope occurs in both unstimulated and stimulated T cells (Vorster et al., 2011). Furthermore, the authors also showed LIMK activation upon infection of human primary macrophages with the CCR5-utilizing viruses (Vorster et al., 2011). These findings indicate that the Rac1/LIMK1/cofilin pathway leading to early actin polymerization is rather a general mechanism occurring early in both X4 and R5-tropic HIV infection of both primary resting/active CD4 T cells and macrophages. Based on our results, we hypothesize that Slit2-N blocks HIV-1 induced activation of Rac1-LIMK1-cofilin pathway that leads to actin polymerization, thus blocking HIV-1 entry (Figure 9).
In summary, our studies reveal that N-terminal region of Slit2 possesses anti-HIV-1 activity against both X4 and R5-tropic viruses. Furthermore, elucidation of the signaling mechanisms revealed that Slit2-N regulates HIV-1 infection by inhibiting HIV entry into target cells through modulation of cytoskeletal dynamics. We have shown that Slit2-N inhibits gp120-induced Robo1-actin association. In addition, our studies revealed that Slit2-Robo1 modulates cytoskeleton by attenuating Rac1-LIMK1-cofilin pathway. Our findings thus elucidate the role of Slit2-N in HIV-1 infection and could provide insights into novel approaches to limit HIV-1 infection.
Chapter 2: The role of Slit2-Robo4 signaling in regulating vascular endothelial inflammation

2.1 Introduction

2.1.1 Vascular endothelial inflammation and its relevant diseases

Inflammation is a type of innate immune response to pathogens and self pathological alterations. Some would also argue that inflammation is caused by both innate and adaptive immunity. Based on the current concept, inflammation is initiated by activation of immune cells, and such immune cells include almost all cell types in both liquid and solid tissues, ranging from leukocytes to endothelial cells, fibroblasts, smooth muscle cells, epithelial cells and even adipocytes. The typical symptoms of topical inflammation on appearance are: redness, swelling, heat, pain and malfunctioning. Among them, redness and swelling are mainly caused by inflammation involving vascular endothelial cells.

Inflammatory responses

In general, the typical inflammatory response is composed of 5 steps: (1) appearance of pathogen or host derived inflammatory stimuli in the tissue, and the sensing of such stimuli by a variety of immune cells residing in the tissue; (2) a series of signaling events which lead to local secretion of inflammatory cytokines and chemokines; (3) activation of endothelial cells in adjacent blood vessels, including secretion of inflammatory cytokines
and chemokines, upregulation of cell adhesion molecules and increased endothelial permeability; (4) recruitment of responsive leukocytes from blood stream to local endothelium and then across endothelial barrier into the tissue; (5) effector leukocyte immune reaction in the local tissue. The inflammation of endothelial cells is a necessary and important link in the above chain of human inflammatory responses to multiple pathological challenges.

**Sepsis and endothelial inflammation**

Sepsis is the uncontrollable multi-bacterial infection. Sepsis is characterized by cytokine storm caused by bacteria-derived pathogenic stimuli, among which lipopolysaccharide (LPS) from Gram-negative bacteria is a major inflammatory activation factor. The excessive amount of cytokines (cytokine storm) in blood stream can lead to severe endothelial inflammation (Aird, 2003). Such endothelial inflammation and the consequent endothelial barrier leakage are considered the direct cause of sepsis-related symptoms, including hypotension, shock, ischemia and organ failure (London et al., 2010; Nathan, 2002). The current treatments for acute sepsis shock are far from satisfying. One of the standard treatments is anti-microbial drugs. However, the time needed for microbial identification and pathogen clearance by drug is often too long to improve the survival of patients (Morens and Fauci, 2008; Morse et al., 2006). And the secondary injuries caused by cytokines cannot be eliminated. Another common therapeutic strategy is to attenuate the inflammatory cytokine signaling, which is believed to be able to directly block the cytokine-induced inflammation. However, clinical outcomes of this treatment have been
quite disappointing (Abraham et al., 2001; Cronin et al., 1995; Crum et al., 2005). Thus, a novel approach for sepsis treatment is in urgent need.

**Atherosclerosis and endothelial inflammation**

Atherosclerosis is the formation of lipid plaques on aortic/arterial wall and narrowing of aorta/artery, which is the cause of coronary artery disease (heart attack) and stroke. Atherosclerosis is a common chronic disease in people with hyperlipidemia. Insult to aortic endothelial barrier and endothelial inflammation are considered the initiation of atherosclerosis development. After that, macrophages are recruited to sub-endothelial tissues mainly by chemokine CCL2. With the extensive accumulation of low density lipoprotein (LDL) under endothelia in hyperlipidemic people, macrophages internalize LDL to form foam cells and enhance local inflammation, which generates more chemokines (mainly CCL2 and CXCL12) and forms a positive feedback loop. The continuous inflammation beneath endothelial cells causes the accumulation of lipid, immune cells and necrotic tissues, which narrows the artery and renders the plaques fragile. Finally, the rupture of such lipid plaques can clog the small blood vessels in the brain and heart, which leads to the ischemia in such vital organs. Although macrophage has been considered to play the major role in cause atherosclerosis, endothelial inflammation also initiates and enhances the process. As a chronic disease, atherosclerosis is generally not treated until heart attack or stroke happens. Thus, a novel method of suppressing endothelial inflammation could potentially be useful in preventing atherosclerotic development and in treating secondary atherogenesis after surgery.
2.1.2 Slit2-Robo4 signaling and endothelial inflammation

Endothelial inflammation plays a critical role in the pathogenesis of septic shock–induced organ injury and atherosclerosis (Riedemann et al., 2003; Ross, 1999). LPS of Gram-negative bacteria is one of the main inflammatory pathogens in septic shock and atherogenesis (Alexander and Rietschel, 2001; Cohen, 2002; Hack and Zeerleder, 2001). LPS induces inflammation by directly activating the vascular endothelium and monocyte/macrophage system and eliciting a series of specific cell responses, including an increase in cell adhesion molecule and pro-inflammatory cytokine/chemokine expression in endothelial cells (Bierhaus et al., 2000). This leads to hyperpermeability of endothelium and recruitment of leukocytes (especially monocytes/macrophages) to enhance inflammation (Cohen, 2002). Both the enhanced vascular permeability and the increased monocyte adhesion on endothelial cells are thought to play important roles in the pathogenesis of septic shock and atherosclerosis (Cohen, 2002).

Slit and Robo are evolutionarily conserved proteins that are widely expressed in different tissues (Chedotal, 2007; Fernandis and Ganju, 2001; Nguyen Ba-Charvet et al., 2001a). The secretory protein Slit has 3 isoforms, Slit1–3, and it has four membrane receptors, Robo1–4 (Chedotal, 2007; Fernandis and Ganju, 2001; Greenberg et al., 2004). Robo1–3 are expressed in a broad spectrum of tissues, but Robo4 is specifically expressed in endothelial cells (Jones et al., 2008; Niclou et al., 2000; Sheldon et al., 2009; Zhang et al., 2012). Slit2 is present in blood and is also expressed in endothelial cells (Fish et al., 2011; Patel et al.; Zhang et al., 2012). Slit2-induced signaling has different roles in different cell types, such as regulating axon guidance in neuronal cells, regulating
chemotaxis and HIV infection in leukocytes, regulating metastasis and proliferation in carcinoma cells, and regulating angiogenesis in endothelial cells (Anand et al., 2013; Dallol et al., 2002a; Guan et al., 2003; Jones et al., 2008; Jones et al., 2009; Liu et al., 2006; Ye et al., 2010; Zhang et al., 2012). In endothelial cells, Slit2 functions by binding to its receptors Robo1 and Robo4 (Jones et al., 2008; Sheldon et al., 2009; Wang et al., 2008b), subsequently inducing a series of intracellular signaling events (London et al., 2010; Zhang et al., 2012). Slit2 was shown to regulate angiogenesis (Jones et al., 2008; Wang et al., 2003; Wang et al., 2008b) and protect endothelial integrity during sepsis and when exposed to HIV (London et al., 2010; Zhang et al., 2012). However, not much is known about the role of Slit2 in regulating endothelial inflammatory responses other than the increase in permeability.

With its critical regulating functions, Slit2 signaling is often dysregulated or deficient in pathological status. Slit2 and Robo1 are commonly silenced by DNA methylation in several human cancers (Dallol et al., 2002a; Dallol et al., 2002b; Dong et al., 2012; Narayan et al., 2006), and the expression of Slit2 can also be regulated by cytokines (Zhang et al., 2012) (Ning et al., 2011). However, there is no report showing the regulation of Slit-Robo expression during inflammation. Thus, it is important to understand the role of LPS in regulating Slit and Robo expression and disease progression using in vitro and in vivo models.

MicroRNAs are short noncoding RNAs that regulate the translation and/or degradation of target messenger RNAs (Ambros, 2004). They were shown to regulate the pathogenesis of numerous diseases (Calin and Croce, 2006; Tili et al., 2008). miR-218 is a microRNA
that is broadly expressed in different tissues, including endothelial cells (Alajez et al., 2011; Fish et al., 2011; Small et al., 2010). One of the precursors of miR-218, mir-218-1, is encoded in the intron of slit2 genes, and it is expressed along with Slit2 protein (Alajez et al., 2011; Fish et al., 2011). Robo1 is one of the main targets of miR-218, and miR-218 represses Robo1 expression by inhibiting its translation (Alajez et al., 2011; Fish et al., 2011; Small et al., 2010). Therefore, it is possible that miR-218 also plays a role in regulating Slit2 signaling during endothelial inflammation.

In the current study, we characterized the role of Slit2 signaling in regulating LPS-induced endothelial inflammatory responses. Based on in vitro and in vivo studies, we proposed a novel pathogenic model of endotoxemia involving LPS-induced endothelial inflammation and liver injury through modulation of Slit2 signaling.

2.2 Materials and Methods

Reagents and cells

LPS from *Escherichia coli* O111:B4 (Sigma-Aldrich; 600,000 EU/mg and <0.80% protein contamination, as shown by the manufacturer’s certificate of analysis) was dissolved in PBS. N-terminal human Slit2 (Slit2-N) protein and Oct1 Ab were obtained from Abcam (Cambridge, MA). ICAM-1, VCAM-1, and GAPDH Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Slit2, Robo1, and Robo4 polyclonal Abs were obtained from Abcam. p-Pyk2 (Y402) and Pyk2 Ab were obtained from Cell Signaling Technology (Danvers, MA). TLR4 Ab (neutralizing) was obtained from R&D Systems (Minneapolis, MN). Pyk2 inhibitor Tyrphostin A9 was obtained from Calbiochem (San Diego, CA), and PF431396 was obtained from Pfizer (New York, NY).
HUVECs were cultured in complete ECM medium (both from ScienCell Research Laboratories, Carlsbad, CA). Human microvascular endothelial cells (HMVECs; adult dermis; Clonetics, San Diego, CA) were maintained in EGM-2MV growth medium containing growth factors, antimicrobials, cytokines, and 5% FBS. HUVECs and HMVECs were grown to confluence in tissue culture plates before treatment with LPS and/or Slit2-N. THP-1 (National Institutes of Health AIDS Research and Reference Reagent program), a human monocytic cell line, was grown in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% heat-inactivated FCS (Invitrogen) and penicillin/streptomycin antibiotics. A human multiple tissue cDNA panel was obtained from Clontech Laboratories (Mountain View, CA).

**Quantitative RT-PCR (qRT-PCR)**

Total RNA was extracted from cells using TRIzol reagent (Life Technologies) and purified with an RNeasy kit (QIAGEN). Total RNA was then reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Real-time PCR was then performed on an Eppendorf Mastercycler realplex using Power SYBR Green Master Mix (Life Technologies). Data analysis was performed using the standard “ΔΔCt method.”

**Western blotting**

Please refer to Chapter 1.

**Cytokine secretion–quantification assay**

Cumulative cytokine secretion in the supernatant of HUVEC culture under different conditions was detected with a Human Cytokine Array Kit, Panel A (R&D Systems),
according to the manufacturer’s manual. Confluent HUVECs were pretreated with Slit2-N (30 nmol/l) or PBS as a control for 30–60 min. Cells were then stimulated with LPS (100 ng/mL) or PBS as a negative control for 12 h before the supernatant of each group was collected. Assays were performed in duplicates and quantified by densitometry with the ChemiDoc XRS+ System (Bio-Rad, Hercules, CA).

**Cell adhesion assay**

THP-1 cell adhesion assay was modified from the method reported previously (Prasad et al., 2007). Briefly, HUVECs were grown to confluence in 96-well plates and were stimulated with LPS (100 ng/mL) for 6 h, with or without Slit2-N (30 nmol/l) pretreatment, before washing with warm complete RPMI 1640. THP-1 cells were washed and stained with 1 mmol/L CFSE (Life Technologies) in PBS for 5 min. 10^6 THP-1 cells (5 × 10^6 cells/mL) were added to treated HUVECs for 60 min. Cells were then washed with warm medium, and fluorescence intensity was detected using a Synergy 2 Microplate Reader (BioTek, Winooski, VT).

**Small interfering RNA knockdown**

Small interfering RNA (siRNA)-mediated knockdown of Robo1 and Robo4 was performed using Robo1- and Robo4-specific ON-TARGETplus SMARTpool siRNA (Dharmacon, Lafayette, CO). Briefly, confluent HUVECs were transfected with 200 pmol siRNA/well in 6-well plates using TransPass HUVEC Transfection Reagent (New England BioLabs), according to the manufacturer’s protocol. Nontargeting small RNA was used as control.

**Flow cytometry**
Please refer to Chapter 1.

**NF-κB activity assay and MCP-1 ELISA assay**

HUVECs, with or without Slit2-N (30 nmol/l) pretreatment, were stimulated with LPS (100 ng/mL) for 4 h before harvest. Nuclear and cytoplasmic extractions of cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific), per the product manual. Activated NF-κB levels of both nuclear and cytoplasmic extracts were measured using a TransAM NF-κB p65 Transcription Factor ELISA Kit (Active Motif, Carlsbad, CA), per the product manual. MCP-1 concentration in the HMVEC culture supernatants was detected using an MCP-1 ELISA Kit (Invitrogen), per the product manual.

**in vivo endotoxemia study**

Male C57BL/6 mice, at 12 wk age, were randomly separated into two groups, with five mice per group. Mice were injected i.p. with 2.5 mg/kg LPS (E. coli O111:B4; Sigma-Aldrich; 1 mg/mL in PBS) or an equal amount of PBS (saline) as control. Twenty-four hours after injection, mice were euthanized with CO₂. Immediately, aorta and main arteries connecting to the heart were isolated, and liver was removed. Aortic endothelial cells were isolated by the method adapted from Chen et al. (Chen et al., 2004b). Blood was emptied from arteries, and lumens were washed with PBS. Then, ~50 mL 37°C enzyme solution (0.25% trypsin and 225 U/mL collagenase type II in RPMI 1640 with 25 mmol/l HEPES) was injected into the lumen of arteries with one end tied. After digestion for 1 min, enzyme solution was collected. This was repeated five times, and endothelial cells were isolated by centrifugation. The purity of isolated endothelial cells was detected
by flow cytometry with murine CD31 Ab (Santa Cruz Biotechnology). To detect specific
gene expression, liver tissue and aortic endothelial cells were homogenized, and RNA
was extracted for qRT-PCR analysis, as stated above. All mice were kept in the animal
facility of Ohio State University in compliance with the guidelines and protocols
approved by the Institutional Animal Care and Use Committee.

**Immunohistochemistry (IHC) staining**

Samples from mouse livers were dissected, fixed in formalin, and embedded in paraffin.
Standard IHC techniques were used, according to the manufacturer’s recommendations
(Vector Laboratories), using Abs against CD31 (Santa Cruz Biotechnology; 1:100) and
Robo4 (Abcam; 1:200). VECTASTAIN Elite ABC reagents (Vector Laboratories),
coupled with avidin-DH:biotinylated-HRP-H complex with 3,3'-diaminobenzidine
(Polysciences) and Mayer’s hematoxylin (Fisher Scientific), were used for detection of
the bound Abs.

**Statistical analysis**

Reported data for cell line studies are the mean ± SEM of at least three independent
experiments performed in duplicate or triplicate. The animal study was done with n = 5
mice/group. The statistical significance was determined by the Student t test. Linear
regression analysis was used to determine dependence/correlation between Slit2 and
Robo1 expression levels.
2.3 Results

2.3.1 Slit2 inhibits LPS-induced cytokine expression

Studies showed that Slit2 can be cleaved into a 120–140 kDa N-terminal and a C-terminal fragment, and the biological effects of Slit2 are mediated by the N-terminal fragment, which interacts with its receptor Robo (Anand et al., 2013; Chedotal, 2007; London et al., 2010). In this study, we used Slit2-N to elaborate its effect.

In the pathogenesis of septic shock–induced organ injury and atherosclerosis, LPS-stimulated endothelial cells can initiate and enhance topical and systematic inflammation by secreting pro-inflammatory cytokines and chemokines, which increase the permeability of endothelium and recruit and activate leukocytes to clear the infection. To examine the role of Slit2 in regulating LPS-induced endothelial inflammation, we first analyzed its role in pro-inflammatory cytokine/chemokine expression. Slit2-N pretreatment significantly inhibited LPS-stimulated MCP-1 (CCL2) and GM-CSF expression at the mRNA level, as assessed by qRT-PCR, in HUVECs in a dose-dependent manner (Fig. 10A). In accordance with the mRNA level, Slit2-N pretreatment also significantly inhibited cumulative MCP-1 and GM-CSF secretion at the protein level after 12 h of stimulation with LPS. In addition, LPS-induced secretion of CXCL1 (GROa) and macrophage migration inhibitory factor (MIF) were significantly inhibited by Slit2-N treatment (Fig. 10B). Moreover, Slit2-N also inhibited LPS-induced MCP-1 secretion in HMVECs (Fig. 10E). However, Slit2-N did not significantly affect the LPS-induced secretion of other common inflammatory cytokines, including IL-6 and IL-1b (data not shown). Meanwhile, Slit2-N (30 nmol/l) treatment 30 min after LPS stimulation
showed much less effect on cytokine expression (Fig. 10A), which suggests that Slit2 may regulate the LPS-induced cellular signaling. These data indicate that Slit2 can repress the LPS-induced endothelial inflammatory response by inhibiting secretion of certain cytokines/chemokines. These affected cytokines/chemokines are well-known for their functions in inducing endothelial tight junction disruption, leukocyte recruitment, and activation.
FIGURE 10. Slit2-N inhibited LPS-induced cytokine and cell adhesion molecule ICAM-1 expression in HUVECs, as well as monocyte adhesion on HUVECs.

HUVECs were treated with 100 ng/mL LPS for 2 h (A) or 12 h (B), with or without Slit2-N. (A) Cells were stimulated by LPS with pretreatment of a gradient of 3, 30, and 60 nmol/L Slit2-N or post-treatment with 30 nmol/L Slit2-N (post–Slit2-N). Total RNA was isolated, and qRT-PCR was performed to detect mRNA expression level of MCP-1 and GM-CSF. Values are normalized to control group and are not comparable between cytokines. (B) Cell culture supernatants were collected after treatment with Slit2-N (30 nmol/l) plus LPS. Cytokines secreted in the supernatants were detected with an R&D Cytokine Array Kit. Values are net optical signal intensity. (C) HUVECs were treated with 100 ng/mL LPS for 12 h, with or without Slit2-N pretreatment (30 nmol/l). Cells were lysed, and WB was performed using ICAM-1, VCAM-1, or GAPDH Ab. (D) HUVECs were stimulated with LPS (100 ng/mL) for 6 h, with or without Slit2-N (30 nmol/l). CFSE-labeled THP-1 monocytic cells were added to treated HUVECs. After washing, attached THP-1 cells were quantified by mean fluorescence intensity at 528 nm. (E) HMVECs were stimulated with 100 ng/mL LPS in the presence or absence of 30 nmol/L Slit2-N for 12 h. MCP-1 secretion in the supernatant was analyzed by ELISA. (F) ICAM-1 expression was detected by qRT-PCR in HMVECs stimulated with stimulation in the absence or presence of Slit2-N. *p , 0.05, **p , 0.01.
Figure 10. Slit2-N inhibited LPS-induced cytokine and cell adhesion molecule ICAM-1 expression in HUVECs, as well as monocyte adhesion on HUVECs.
2.3.2 Slit2 inhibits LPS-induced cell adhesion molecule ICAM-1 expression and monocyte adhesion on endothelial cells

During LPS-induced endothelial inflammation, endothelial cells upregulate the expression of cell adhesion molecules, including ICAM-1 and VCAM-1, to increase leukocyte (including monocyte) attachment. Recruited monocytes are critical in atherogenesis and enhancement of inflammation. We observed that Slit2-N treatment potently inhibited LPS-induced ICAM-1 protein expression without significantly affecting VCAM-1 (Fig. 1C). Moreover, Slit2-N also inhibited ICAM-1 upregulation by LPS in HMVECs (Fig. 1F). To check whether this inhibition of ICAM-1 expression influences monocyte adhesion on endothelial cells, we performed a cell adhesion assay with the monocytic cell line THP-1 and HUVECs. LPS treatment enhanced THP-1 adhesion on HUVECs, and Slit2-N treatment of HUVECs significantly decreased THP-1 adhesion upon stimulation with LPS (Fig. 1D). These results suggest that Slit2 can decrease LPS-induced monocyte adhesion on endothelial cells by inhibiting ICAM-1 expression.

2.3.3 Anti-inflammatory effect of Slit2 is mediated through Robo4 rather than Robo1

Slit2 is a secreted signaling molecule that functions by binding to its receptor Robo and, subsequently, induces a series of intracellular signaling events. Given that Slit2 inhibits LPS-induced cytokine/chemokine expression, we wanted to verify whether the effect of
Slit2 was mediated through Slit2–Robo interaction. Moreover, of the two endothelial Slit2 receptors, Robo4 is expressed much more abundantly in endothelial cells than is Robo1 (Fig. 11A). Thus, we set out to identify which Robo receptor is responsible for the effect of Slit2 on LPS-induced inflammation in endothelial cells. GM-CSF mRNA expression was used as the indicator of endothelial inflammation, which was inhibited by Slit2 (Fig. 10A, 10B). Robo1 was knocked down in HUVECs using Robo1-siRNA (Fig. 11B), and GM-CSF expression was measured by qRT-PCR at the mRNA level after 2 h of LPS stimulation. Slit2 inhibited LPS-induced GM-CSF expression in non-targeting siRNA-transfected cells, as well as in the Robo1-siRNA group (Fig. 11C), which shows that the anti-inflammatory effect of Slit2 is not dependent on the Robo1 receptor. In contrast, Slit2 enhanced LPS-induced GM-CSF expression when Robo4 was knocked down in HUVECs (Fig. 11B, 11D), which showed that the anti-inflammatory effect of Slit2 was mediated through the Robo4 receptor. However, Robo1 could be pro-inflammatory, considering the fact that the amount of LPS-induced GM-CSF is less after Robo1 knockdown and it is more after Robo4 knockdown. These results suggested that Slit2 mediates its anti-inflammatory effects in endothelial cells through Robo4 rather than Robo1. Because Robo4 is dominantly expressed (Fig. 11A), the overall effect of Slit2 treatment is anti-inflammatory in endothelial cells.
Figure 11. The anti-inflammatory effect of Slit2 was dependent on Robo4 rather than Robo1.

(A) Relative mRNA expression levels of Robo1 and Robo4 in resting HUVECs were quantified by qRT-PCR; PCR product is shown on Agarose Gel. (B) Forty-eight hours after transfection with Robo1-siRNA or Robo4-siRNA, HUVECs were lysed, and WB was used to detect the protein levels of Robo1 and Robo4. GAPDH was used as loading control. Forty-eight hours after Robo1 (C) or Robo4 (D) knockdown, GM-CSF mRNA expression in HUVECs was analyzed by qRT-PCR 2 h after LPS (100 ng/mL) treatment, with or without Slit2-N (30 nmol/l). Non-targeting siRNA was used as control for Robo1-siRNA or Robo4-siRNA. *p , 0.05, **p , 0.01.
2.3.4 Slit2 represses LPS-induced endothelial inflammation by inhibiting the Pyk2–NF-κB pathway

Next, we analyzed the molecular mechanism by which Slit2 inhibits LPS-induced endothelial inflammation. We observed that Slit2 did not change the surface level of TLR4 on HUVECs (Fig. 12A, 12B). This indicates that Slit2 affects LPS-triggered intracellular signaling. It was reported that, upon binding to TLR4, LPS activates Pyk2 through phosphorylation, which then activates NF-κB and initiates NF-κB translocation into the nucleus to start the transcription of inflammatory cytokines/chemokines. It was reported that Pyk2 is important for LPS-induced chemokine IL-8 and MCP-1 expression in endothelial cells (Anand et al., 2009; Anand et al., 2008). By inhibiting Pyk2 activation using a Pyk2 inhibitor Tyrphostin A9 (Anand et al., 2008), we also showed that Pyk2 is important for LPS-induced MCP-1, CXCL1, MIF, and GM-CSF expression, which were repressed by Slit2 (Fig. 12C). Then we confirmed that Slit2-N treatment significantly inhibited LPS-induced phosphorylation of Pyk2 (Fig. 12D). In addition, a high concentration of TLR4-neutralizing Ab and a potent and highly specific Pyk2 inhibitor PF431396 prevented LPS-induced cytokine expression at the transcriptional level (Fig. 12E). This confirmed that LPS-induced cytokine gene transcription is mediated by TLR4-Pyk2 signaling. Next, downstream of Pyk2, we analyzed NF-κB activation and its nuclear translocation in the presence and absence of Slit2 in HUVECs using an ELISA-based NF-κB activity assay. We extracted the cytoplasmic and nuclear portions of HUVEC cell lysates, the purity of which was indicated by the levels of nuclear protein Oct1 and GAPDH (mainly present in the cytoplasm) (Fig. 12F). Slit2-N
treatment decreased the LPS-induced activation and nuclear translocation of NF-κB (Fig. 12F). This indicates that Slit2 treatment inhibited LPS-induced Pyk2 activation and further decreased the downstream activation and nuclear translocation of NF-κB in HUVECs.
Figure 12. Slit2 inhibits LPS-induced Pyk2 activation and NF-kB activation and translocation.

(A) Surface levels of TLR4 on HUVECs treated with Slit2-N (30 nmol/L) for different times (0, 0.5, 1, and 3 h) were analyzed by flow cytometry. IgG isotype control is represented by the filled red graph. TLR4 levels at the different time points are shown as colored lines. Black, 0 h; red, 0.5 h; green, 1 h; and purple, 3 h. (B) Flow cytometry data were quantified as mean fluorescence intensity. Differences over time are not statistically significant. (C) HUVECs were treated with 100 ng/mL LPS for 12 h, with or without pretreatment with Pyk2 inhibitor Tyrphostin A9 (Tyr A9; 10 mmol/L). Cell culture supernatants were collected after treatment. Cytokines secreted in the supernatants were detected using an R&D Cytokine Array Kit. Values are relative net optical signal intensity and are not comparable between cytokines. (D) HUVECs were treated with 100 ng/mL LPS for 2 h, with or without Slit2-N pretreatment (30 nmol/L). Phosphorylated Pyk2 was detected by WB using p-Pyk2 (Y402) or Pyk2 Ab. GAPDH was used as loading control. (E) HUVECs were pretreated with Pyk2 inhibitor PF431396 (PF; 10 mmol/L), the indicated concentration of TLR4-neutralizing Ab (TLR4.Neu.Ab), or their controls for 1 h and then stimulated with LPS for 2 h. Protein expression was analyzed by qRT-PCR. (F) Representative cytoplasmic (CY) and nuclear (NU) portions of HUVEC cell lysates were probed for nuclear marker Oct1 (exclusively in nuclear portion) and cytoplasmic marker GAPDH (mostly in cytoplasmic portion). HUVECs were treated with 100 ng/mL LPS for 4 h, with or without Slit2-N pretreatment (30 nmol/L). Cells were lysed, and nuclear and cytoplasmic portions were separated. Active NF-kB was detected using an ELISA-based assay. *p , 0.05.
Figure 12. Slit2 inhibits LPS-induced Pyk2 activation and NF-κB activation and translocation.
2.3.5 LPS enhances endothelial inflammation by downregulating Slit2 and Robo4

We showed above that Slit2 and Robo4 are anti-inflammatory in endothelial cells. Thus, we asked whether LPS has any effect on the expression of Slit2 and Robo4, as well as whether this effect may contribute to endothelial inflammation. By qRT-PCR and WB analysis, we observed that LPS stimulation significantly downregulated Slit2 and Robo4 in HUVECs, without significantly changing the Robo1 level (Fig. 13A–C). This showed that LPS may enhance endothelial inflammation by downregulating the anti-inflammatory molecules Slit2 and Robo4.

2.3.6 Slit2 downregulates Robo1 protein level through upregulation of miR-218

Slit2-N treatment not only inhibited LPS-induced inflammation, it also upregulated Slit2 and downregulated Robo1 expression (Fig. 13A, 13C). It was reported that primary microRNA, mir-218-1, located in the intron of the slit2 gene, and mature miR-218 are expressed along with Slit2 because they share the same transcript. Thus, we hypothesized that downregulation of Robo1 could be mediated through miR-218, which targets the 39-untranslated region of Robo1 and inhibits its protein translation. We observed that the cellular miR-218 level changed in a manner consistent with the Slit2 mRNA level (Fig. 13A, 13D). In addition, Slit2-N treatment per se significantly increased the levels of both Slit2 and miR-218, the latter of which knocked down Robo1, leaving Robo4 unchanged (Fig. 13E, 13F). This indicates that there is a positive feedback regulation of Slit2 expression, and Slit2 treatment can regulate Robo1 receptor expression via intronic miR-
Similar to our findings in Fig. 4, this repressing effect of Slit2 toward Robo1 expression appears to be universal in different human tissues. By analyzing the Slit2 and Robo1 expression levels in a human tissue panel, we observed a strong negative correlation between Slit2 and Robo1 (Fig. 13G). This negative correlation could be mediated, at least in part, by miR-218.
Figure 13. The modulation of Slit2, Robo1, and Robo4 expression and the role of miR-218.

(A and B) HUVECs were treated with 100 ng/mL LPS for 2 h, with or without Slit2-N pretreatment (30 nmol/L). qRT-PCR was used to detect mRNA expression of Slit2 and Robo4. The changes in protein levels of Robo4 in HUVECs treated for 12 h were similar to those in the mRNA levels. (C) Protein levels of Robo1 in the above-treated HUVECs or Robo1 in HUVECs treated only with Slit2-N for 12 h were assessed by WB. (D) The level of miR-218 was detected using miR-218–specific qRT-PCR primers in HUVECs treated with LPS in the presence or absence of Slit2-N (30 nmol/L), as well as in HUVECs treated with Slit2-N or PBS as control for 2 h. (E) Slit2-N treatment induced Slit2 and miR-218 upregulation, as assessed by qRT-PCR. (F) Slit2-N treatment reduced Robo1 expression, without affecting Robo4. (G) Slit2 and Robo1 expression levels in 10 human tissues were detected using qRT-PCR with a human multiple tissue cDNA panel (ovary, prostate, kidney, colon, heart, small intestine, liver, pancreas, lung, and skeletal muscle). Values were normalized to human normal tissue control. Each symbol represents one human tissue, with Slit2 and Robo1 expression levels on separate axes. *p < 0.05, **p < 0.01.
Figure 13. The modulation of Slit2, Robo1, and Robo4 expression and the role of miR-218.
2.3.7 LPS downregulates Slit2 and Robo4 expression in arterial endothelial cells and in liver during endotoxemia *in vivo*

With the observation that LPS regulated Slit2 and Robo4 expression in HUVECs *in vitro*, we wanted to verify whether LPS also regulates their expression during endotoxemia (sepsis) *in vivo* using a mouse model. During endotoxemia/septic shock, multiple organ injury (including liver) is one of the main life-threatening events caused by endothelial inflammation. Additionally, inflammation of arterial endothelial cells caused by LPS is important for atherosclerosis development. Thus, we planned to analyze the changes in expression in mouse arterial endothelial cells and whole liver. Male C57BL/6 mice, at 12-wk age, were injected i.p. with 2.5 mg/kg LPS or saline. Twenty-four hours after injection, mice were sacrificed, and the liver and aorta were removed. We separated aortic endothelial cells from the aorta by enzyme digestion, and 96% of the cells were CD31⁺, as detected by flow cytometry (Fig. 14A). In mouse aortic endothelial cells, LPS significantly downregulated Slit2 and Robo4. Similarly, LPS significantly downregulated the expression of Slit2 and Robo4 in mouse liver (Fig. 14B). Because Robo4 is specifically expressed in endothelial cells, its expression in whole liver mainly represents the Robo4 level of liver endothelial cells, whereas Slit2 expression in the liver represents its overall level in the tissue environment. Both of these observations were in agreement with the changes in HUVECs in vitro.
Figure 14. Slit2, Robo1, and Robo4 in arterial endothelial cells and liver are regulated by LPS during endotoxemia in vivo.

(A) Endothelial cells were isolated from mouse arteries by enzyme digestion. Cells were stained for CD31 or IgG control, and the purity of endothelial cells was analyzed by flow cytometry. Percentage of CD31+ cells is shown (region R1). IgG isotype control Ab (left panel). Murine CD31 Ab (right panel). (B) C57BL/6 mice were injected i.p. with LPS or saline. Twenty-four hours later, mice were sacrificed, and aortic endothelial cells and liver were isolated. qRT-PCR was performed to detect murine Slit2 and Robo4 expression after RNA extraction. Symbols representing saline group mice are on the left of each plot, whereas those representing LPS group mice are on the right of each plot. Black lines indicate the differences in the averages between groups. (C) Liver lysates of mice injected with PBS or LPS were used to detect Slit2 secretion by WB. Quantification of Slit2 secretion from five mice/group. (D) Liver samples from LPS- or PBS-injected mice were fixed and embedded in paraffin. IHC was performed to stain Robo4 and CD31 on overlying sections. Main blood vessel endothelial cells are indicated by black arrows, and sinusoidal endothelial cells are indicated by red arrows. CD31 was used as the endothelial marker. *p < 0.05.
Figure 14. Slit2, Robo1, and Robo4 in arterial endothelial cells and liver are regulated by LPS during endotoxemia in vivo.
Additionally, we analyzed data from two other microarrays in the National Center for Biotechnology Information Gene Expression Omnibus DataSets database. They showed similar changes in Slit2 and Robo4 expression upon LPS or proinflammatory cytokine stimulation (Edgar et al., 2002) (Table 1). We also observed dramatic downregulation of Slit2 in mouse liver with non-LPS–induced inflammation, including vascular injury and blood leakage (data not shown). Furthermore, we analyzed the Slit2 protein expression by WB and endothelial Robo4 protein level by IHC with mouse liver tissue from the LPS or saline group. Liver lysates from mice injected with LPS have less Slit2 expression compared with those from the saline group (Fig. 14C). Moreover, after LPS injection, liver main blood vessel endothelial cells and liver sinusoidal endothelial cells showed significantly less Robo4 expression compared with those of the saline group (Fig. 14D). LPS-stimulated upregulation of endothelial cell marker CD31 in mouse liver endothelial cells during endotoxemia is shown as a positive control (Fig. 14D).
Table 1. LPS and proinflammatory cytokines regulate Slit2 and Robo4 expression

Based on National Center for Biotechnology Information Gene Expression Omnibus DataSets, GSE23070 assessed the treatment of HUVECs with a mixture of pro-inflammatory cytokines (TNF-α, IL-1β, IL-8), and GSE9567 assessed the effects of LPS i.p. injection on murine heart. Similar to our findings, microarray data from both of these studies indicate that LPS or pro-inflammatory cytokines can regulate Slit2-Robo1/4 signaling.

The t values and p values are from a t test between control and treatment groups. A positive t value indicates increased expression with treatment, whereas a negative t value indicates decreased expression.

These data showed that LPS downregulated anti-inflammatory Slit2–Robo4 in vivo, which may be responsible for enhancing endothelial inflammation and liver injury. A proposed mechanism for the role of Slit2-Robo1/4 is summarized in Figure 15.
Figure 15. Proposed mechanism of Slit2 regulation of LPS-induced.

The proinflammatory TLR4–Pyk2–NF-κB and Slit2–Robo1 pathways are represented by red shapes, whereas the anti-inflammatory Slit2–Robo4 pathway is represented by green shapes. Robo4 is dominantly expressed in endothelial cells, compared with Robo1.

2.4 Discussion

LPS-induced endothelial inflammation is a critical pathological event in numerous diseases, especially acute endotoxemia/sepsis. We discovered that the secretory protein Slit2 can repress LPS-induced endothelial inflammatory responses, including secretion of...
inflammatory cytokines/chemokines, upregulation of cell adhesion molecule ICAM-1, and monocyte adhesion. The two endothelial receptors Robo1 and Robo4 were shown to play differential roles in endothelial cells, and Slit2–Robo4 interaction is responsible for the anti-inflammatory effects. Slit2 can downregulate the minor receptor Robo1 via miR-218. In addition, LPS was shown to downregulate Slit2–Robo4 to enhance endothelial inflammation in vitro and in vivo.

In the current study, to our knowledge, we showed for the first time that Slit2 represses the expression of certain LPS-induced inflammatory cytokines/chemokines in HUVECs, including MCP-1, MIF, CXCL1, and GM-CSF. This is in agreement with a study (London et al., 2010) of cecal ligation and puncture–induced multi-bacterial sepsis (including Gram-negative) in a mouse model, which showed that there is a trend, although not significant, toward deceased inflammatory cytokine levels in the serum after Slit2 administration. The lack of significant differences could be due to mixed and complicated cytokine/chemokine sources in vivo and large detection errors, given that differentiated leukocytes do not express Robo4. In addition, it was reported that Slit2 can protect LPS-and HIV-1 gp120–induced endothelial hyperpermeability by preventing the tight junction disruption (London et al., 2010; Zhang et al., 2012). Although unlikely, it is possible that Slit2 also inhibits the increase in accessible membrane TLR4 to LPS during LPS-induced endothelial tight junction breakdown, and this could contribute, in part, to the anti-inflammatory effect of Slit2. Our work suggests that the protection of endothelial integrity by Slit2 might be mediated, at least in part, through its repression of inflammatory cytokine–induced indirect tight junction disruption. Along with these pro-
inflammatory cytokines, some LPS-induced anti-inflammatory cytokines (including soluble ICAM-1 and IL-1Ra) were repressed by Slit2 (data not shown). However, these anti-inflammatory cytokines are a part of the self-protective responses of endothelial cells, and their expression levels are relatively low.

LPS-induced expression of ICAM-1 in HUVECs was also inhibited by Slit2. Consequently, LPS-induced THP-1 monocytic cell adhesion was also reduced by Slit2. This role of Slit2 in the regulation of inflammation has not been reported before. However, similarly, we (Prasad et al., 2007) and other investigators (Patel et al.) showed that Slit2 can inhibit T cell and platelet adhesion onto endothelial cells or extracellular matrix proteins by acting on T cells and platelets.

In the current study, we showed that the dominant endothelial receptor Robo4 is responsible for the anti-inflammatory effect of Slit2, which supports the findings of another study showing that Slit2–Robo4 can reduce inflammation-induced organ damage and death by protecting endothelial integrity during sepsis. In addition, our data indicate that Robo1 could be pro-inflammatory in endothelial cells. This is a new discovery illustrating the differential roles of Robo1 and Robo4 receptors in endothelial inflammation. However, several studies indicated that Robo1 and Robo4 may have opposite functions in the regulation of angiogenesis and endothelial cell migration (Guo et al., 2012; Jones et al., 2009; London et al., 2010; Wang et al., 2003; Wang et al., 2008b; Zhang et al., 2012). Furthermore, in agreement with other studies, we showed that Robo4 is expressed 14 times more abundantly than Robo1, which renders Robo4 the dominant anti-inflammatory endothelial receptor for Slit2.
The proline-rich kinase 2, Pyk2, also known as RAFTK, is a cytoplasmic tyrosine kinase related to focal adhesion kinase. The Pyk2–NF-κB pathway was shown to be important for LPS-triggered MCP-1 and IL-8 expression in endothelial cells (Anand et al., 2009; Anand et al., 2008). We also showed that Pyk2 is important for LPS-induced expression of MIF, CXCL1, and GM-CSF. Given the importance of Pyk2–NF-κB in LPS-induced cytokine/chemokine expression, our molecular signaling study revealed that Slit2 represses LPS-induced endothelial inflammation through the inhibition of LPS-triggered Pyk2 activation, as well as NF-κB activation and nuclear translocation. Upstream of Pyk2, LPS-induced inflammatory cytokine expression was also strongly blocked by the higher concentration of TLR4-neutralizing Ab, although it did not decrease to the control baseline level. This could either be due to incomplete blocking of TLR4 by Ab or the effect of trace amounts of contaminants (including TLR2 ligand) in the LPS product. miR-218 was shown to inhibit Robo1 expression in endothelial and other cells (Fish et al., 2011; Tie et al., 2010). In this study, we showed that Slit2 treatment increases Slit2 and miR-218 expression, and the increased miR-218, in turn, inhibits Robo1 protein translation.

To our knowledge, our in vitro and in vivo data indicate for the first time that LPS stimulation or endotoxemia can downregulate Slit2 and Robo4 in endothelial cells and in liver. Thus, we hypothesize that LPS enhances endothelial and organ inflammation by impairing anti-inflammatory Slit2–Robo4 signaling. In addition to liver, we observed similar effects in mouse heart (data not shown). Multiple organ injury (including liver and heart) due to endothelial inflammation is one of the primary life-threatening events
during sepsis. LPS-induced arterial endothelial cell inflammation is also important for atherogenesis. Therefore, our findings may provide a novel mechanistic explanation of the pathogenesis of sepsis and atherosclerosis. Other studies also suggested that Slit2 expression can be modulated by PDGF and HIV gp120 (Zhang et al., 2012), (Ning et al., 2011). Furthermore, as shown in Table 1, our analysis of the microarray data (Edgar et al., 2002) revealed that i.p. injection of LPS induced significant downregulation of Slit2 and Robo4 in the whole heart of mice, and that pro-inflammatory cytokine (TNF-a, IL-1b, IL-8) treatment induced significant downregulation of Slit2 and Robo4 in HUVECs. Taken together, our studies imply that Slit2 is an important inflammation regulator, and it may be widely regulated by inflammatory stimuli and cytokines.

In conclusion, our study showed that Slit2–Robo4 represses LPS-induced endothelial inflammation. LPS and endotoxemia may enhance inflammation by downregulating Slit2 and Robo4 in endothelial cells. The anti-inflammatory effect of Slit2 is mediated through inhibition of cytokine/chemokine expression and monocyte adhesion. Furthermore, we showed that Slit2–Robo4 suppresses LPS-induced signaling by inhibiting the activation of the Pyk2–NF-κB pathway. These suggest that Slit2–Robo4 signaling plays an important role in modulating endothelial inflammation and endotoxemia-induced organ injury. Therefore, Slit2 appears to be a promising candidate for developing novel therapies against sepsis-induced organ injury and atherosclerosis. In addition, serum Slit2 level could be used as an indicator of vascular wall inflammation.
Chapter 3: The role of Robo4 signaling in controlling breast cancer growth and metastasis

3.1 Introduction

3.1.1 Breast cancer

Breast cancer is a type of cancer originated from the breast tissue. The most common type of breast cancer is breast carcinoma which initiated from the mammary gland epithelial cells. Breast cancer is the most common type of cancer in women, and is a leading cause of death in women with cancer. Thanks to the improvement of diagnosis and therapies, the prognosis of breast cancer patients has been prolonged and the death rate has been reduced. However, the poorly controlled metastasis and severe side effects of non-selective drugs still urge scientists to develop better therapeutic methodologies for breast cancer.

Epidemiology of breast cancer

For many decades, breast cancer has been the most commonly diagnosed cancer and the leading cause of cancer-related death among females both worldwide and in developed countries like the US. Recently, in the year of 2012, in women of developed countries, death caused by lung cancer (209,990) surpassed that caused by breast cancer (197,600) for the first time (Torre et al., 2015). Once was a growing major threat to women’s health, breast cancer incidence rate seem to plateau or even drop in developed countries, like the
US, due to reduced menopausal hormone therapy, increased women participating in mammogram screening. Thanks to early detection and improved treatment, breast cancer caused death in women of developed countries has been reducing since the 1990s (Torre et al., 2015). On the other hand, breast cancer incidence and death rates have been and still are increasing in developing countries. Epidemiologically, risk factors for breast cancer include reproductive and hormonal factors (such as a long menstrual history), use of contraceptives and never having children (Colditz et al., 2006). It has been shown that giving birth to children and breast feeding reduce the chance of developing breast cancer (Colditz et al., 2006). Besides, other risk factors potentially modifiable are adulthood weight gain, postmenopausal obesity, physical inactivity and drinking alcohol (Chlebowski et al., 2013; Colditz et al., 2006).

**Progression of breast cancer**

The majority of breast cancers are carcinomas derived from mammary gland epithelial cells. When the cancerous tissue initiates from mammary gland ducts, it is classified as ductal carcinoma (DC). When initiating from mammary gland lobules, it is classified as lobular carcinoma (LC). At the early stage of atypical breast epithelial hyperplasia, cancer tissues are growing and yet well locally restricted in the lumen of mammary glands, which are called ductal/lobular carcinoma in situ (DCIS/LCIS). After this stage, the growth of cancerous tissue starts to break through the basement membrane and invade into the adjacent tissue, which is called invasive ductal/lobular carcinoma (IDC/ILC) and indicates malignancy. At late stage, IDC/ILC can invade into blood vessels and
lymphatics, which leads to distal metastasis of breast cancer through circulation. The metastatic foci can reside in separated organs such as bone, lung, liver and brain.

**Subtypes of breast cancer**

Breast cancer can be grouped into different subtypes based on their tissue origin and receptor status. Pathology analysis is used to identify the tissue origin of carcinoma cells. Based on the relative location and closeness to the mammary gland lumen, breast cancer can be grouped into luminal-cell carcinoma and basal-like-cell carcinoma. Histochemical and molecular analyses are used to separate breast cancer into different groups based on the presence/absence of cellular expression of three receptors, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). ER and PR are hormonal receptors which are considered enhancing factors of breast cancer development. And hormonal receptors, especially ER, can be targeted by pharmaceutical inhibitors. HER2, also known as Erbb2 and Neu, is a receptor tyrosine kinase type of oncogene. It can be targeted by receptor neutralizing antibody and pharmaceutical inhibitors. For breast cancers that are positive for ER/PR or HER2, they can be specifically treated with the above mentioned countermeasures to suppress the pro-cancer functions of ER/PR/HER2. However, for breast cancers that are negative for all three receptors (triple negative breast cancer, TNBC), targeted treatments are relatively limited. Moreover, TNBC is considered more aggressive comparing to the other subtypes. In fact, there are also overlaps between the tissue origin subtypes and receptor status subtypes. Luminal-cell carcinomas are generally ER-positive (ER⁺), and basal-like-cell carcinomas are often triple negative (ER⁻/PR⁻/HER2⁻).
3.1.2 Tumor angiogenesis

Angiogenesis, or neovascularization, is the generation of blood vessels. Angiogenesis occurs during development, physiological vascular remodeling and pathological events (Carmeliet and Jain, 2011). Blood vessels are vitally important for supporting the normal functional of tissues. Thus, angiogenesis is a necessary component of development, wound healing, pregnancy and even cancer development. In breast cancer, tumor angiogenesis directly and indirectly supports tumor growth and metastasis (Folkman, 2002), which is why the extent of angiogenesis often serves as an indicator of the aggressiveness of breast cancer.

Ways of tumor angiogenesis

There have been two major theories proposed to be the mechanism of tumor angiogenesis, and both of which have abundant supporting evidence.

The classical way is called sprouting angiogenesis, which involves the pre-existing endothelial cells on the blood vessel. In this theory, endothelial cells disassociate from adjacent cells after tight junction breakdown, sprout toward source of pro-angiogenic cytokines, proliferate and connect to form tubes, recruit perivascular cells to provide stability and maturation, and remodel to form a functional vascular network (Carmeliet and Jain, 2011). This theory has long been accepted and well-studied. A well-accepted model of sprouting tumor angiogenesis starts from the hypoxia of the tumor tissue. Hypoxia causes a stress response in the tumor cells, which leads to the secretion of pro-angiogenic cytokines, inflammatory cytokines and matrix metalloproteinases (MMPs). These pro-angiogenic cytokines may include vascular endothelial growth factor (VEGF),
platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF). The inflammatory cytokines may include interleukin-1-beta (IL-1β), interleukin-6 (IL-6) and tumor necrotic factor-alpha (TNF-α). The common MMPs are MMP2 and MMP9 (Weis and Cheresh, 2011). Pro-angiogenic and inflammatory cytokines have been shown to interact with stromal cells and immune cells in the tumor environment and cause endothelial tight junction breakdown, lead to endothelium leakage and induce vascular endothelial cell migration and tube formation. MMPs can break down the extracellular matrix (ECM) and facilitate new vessel formation (Weis and Cheresh, 2011).

The other major way of angiogenesis is supported by bone marrow derived endothelial progenitor cells (EPCs). This theory suggests that other than the classical way, circulating EPCs and stem cells derived from bone marrow can be recruited to the site in need and integrate into the forming or newly formed vessels (Butler et al., 2010; Kobayashi et al., 2010; Lyden et al., 2001).

Besides, there are also some less common ways of angiogenesis-like events reported by a few groups. It has been shown that cancer stem cells are potentially capable of differentiating into vascular cell types (Alvero et al., 2009; Ricci-Vitiani et al., 2010; Wang et al., 2010), including in breast cancer (Bussolati et al., 2009). In certain types of cancer, the vascular mimicry behavior of cancer cells creates intra-tumor canals providing additional blood supply to the tumor (Maniotis et al., 1999; Sefior et al., 2012).

**Angiogenesis and metastasis**

In the classical sprouting angiogenesis model, the induction of endothelial barrier leakage is the major initiating event of vascular sprouting. As stated above, the tumor tissue
(including cancer cells, stromal cells and immune cells) secrets multiple cytokines (such as VEGF, TNF-α, IL-1β and IL-6) which can act on endothelial cells and lead to breakdown of endothelial tight junctions. And the MMPs secreted from tumor tissue also cleave ECM fibers to reduce to the blockade of cell migration. Consequently, it is easier for the cancer cells to invade across the endothelial layer through trans-endothelial migration and into the blood flow and lymphatics. The vascular/lymphatic invasion is considered the initial step of cancer metastasis, and is also a key characteristic of cancerous malignant conversion. In other words, enhanced angiogenesis is often coupled by increased metastasis (Folkman, 2002; Weis and Cheresh, 2011).

**Targeting tumor angiogenesis**

It is not at all irrational to compare cancer remission in oncology to species extinction in paleontology, since they share numerous similarities from an evolutionary point of view (Walther et al., 2015). Treatment of cancer is and should be comparable to the selection pressure of species extinction. Just like most cancer treatments are not capable of eliminating cancer permanently, most selection pressures are not capable of causing species extinction. Both cancer and species have ways of resisting or circumventing the detrimental pressure on them and survive the evolution. However, the lessons from hundreds’ millions’ years of “successful” species extinction should provide the oncologists with ways of improving cancer treatment. In order to successfully remove cancer, treatment should mimic all of the aspects of the causes of extinctions, which includes maintaining multiple and diverse selective pressures for many generations (prolonged chemotherapies of diverse targets), targeting escape phenotypes (actively
target cancer chemoresistance), destroying habitat (target cancer stroma and supporting tissues) and reducing evolvability (preventing cancer hypermutation) (Walther et al., 2015). And targeting tumor angiogenesis is a type of “destroying habitat” approach. Despite what some oncologists think (Walther et al., 2015), anti-angiogenesis therapy has been largely explored. To suppress tumor angiogenesis, three main approaches can be targeted: blood vessels, pro-angiogenic cytokines/mediators and tumor microenvironment.

Targeting blood vessels can include damaging of intra-tumor endothelial cells, inhibiting endothelial cell migration and proliferation, preventing EPC generation and recruitment, and targeting supportive cells of blood vessels. As the security of non-tumor blood vessels is critically important for the cancer patient, tumor specificity of the approach of damaging intra-tumor endothelial cells is the key factor to consider during therapy development. A simple way is to deliver endothelial damaging drugs directly into the tumor, which obviously has its restrictions. Thus, plenty of work has been devoted to the search for tumor endothelial specific markers for targeted therapy (Nanda and St Croix, 2004). A few cell surface molecules have been proposed to be specific for newly formed tumor endothelium, with an interesting instance being Robo4 (Yoshikawa et al., 2013). Interestingly, a group also claims that certain oncolytic viruses are capable of infecting only tumor endothelial cells and causing tumor vascular collapse (Jeng et al., 2013).

Targeting pro-angiogenic cytokines/mediators has been a research focus that generated several clinically available drugs. Theoretically, pro-angiogenic cytokines/mediators can be blocked by synthesis/secretion inhibitors, neutralizing antibodies, receptor blocking
antibodies and receptor signaling inhibitors. Among these targets, VEGF neutralizing antibody has been utilized as anti-angiogenic drug in the treatment of multiple cancers (Ebos et al., 2009).

Targeting tumor microenvironment may include regulating MMPs, modulating ECM and basement membrane, and suppress stromal inflammation. However, there are very limited “drugable” targets to investigate in this approach.

3.1.3 Robo4 and breast cancer angiogenesis

In current breast cancer treatment, frequent somatic mutation and heterogeneity of cancer cells have often rendered aggressive tumors resistant to chemotherapy (Gerlinger et al., 2012). Given the important supportive function and the rarity of mutation of tumor blood vessel endothelium, inhibiting tumor angiogenesis by targeting tumor endothelial cells has been considered an alternative strategy to overcome drug resistance. Tumor angiogenesis is necessary for tumor growth and it also promotes metastasis (Weidner et al., 1991). Angiogenesis can be initiated by chemoattractive and proliferative cytokines in the tumor environment, such as Vascular Endothelial Growth Factor (VEGF), which induces endothelial cell migration and proliferation (Ferrara, 2002). Meanwhile, tumor derived inflammatory cytokines, such as IL-1β, causes leakage of endothelium by disrupting the tight junctions, and in turn promotes endothelial sprouting during angiogenesis (Voronov et al., 2003). Moreover, undermined endothelial integrity promotes breast cancer metastasis, especially when tight junction protein ZO-1 is degraded in tumor endothelial cells (Martin and Jiang, 2009).
Robo4 is one of the cell surface receptors for the secreted signaling protein, Slit2 (Park et al., 2003). Its expression is restricted to endothelial cells and their progenitor, hematopoietic stem cells (HSCs) (Huminiecki et al., 2002; Smith-Berdan et al., 2011). By binding to Slit2 ligand, Robo4 signaling counteracts with VEGF signaling and IL-1β signaling in endothelial cells to inhibit angiogenesis during retinopathy and protect endothelial integrity during sepsis shock (Jones et al., 2008; London et al., 2010). It has also been shown that Robo4 regulates HSC homing to bone marrow during HSC transplant (Smith-Berdan et al., 2011), and is important for vascularization during mammary gland development (Marlow et al., 2010).

Recently, it was shown that the main signaling event of Robo4 pathway upon Slit2 stimulation is the deactivation of a GTPase, ARF6 (Jones et al., 2009). And deactivation of ARF6 could be achieved by pharmacologically inhibiting ARF6 activating proteins, guanine nucleotide exchange factors, with the small molecule drug SecinH3 (Zhu et al., 2012). This drug has been shown to be effective in enhancing Robo4 function in treating arthritis and endotoxemia (Davis et al., 2014; Zhu et al., 2012). Although targeting Robo4 has been shown to be very promising in treatment of sepsis shock (London et al., 2010), to date, the quest for Robo4 targeting strategy has been limited to injection of large doses of recombinant Slit2 protein (London et al., 2010). However, given the high molecular weight (120 – 170 kDa) and low stability of Slit2 protein, it is far from a good drug candidate. Hence, targeting ARF6 downstream of Robo4 with SecinH3 seems a promising alternative strategy.
In the present study, we elucidated the role of endothelial Robo4 in breast cancer growth and metastasis by knocking out endothelial Robo4 and by enhancing Robo4 downstream signaling, and explored the possibility of using a novel small molecule drug to target Robo4 in treating breast cancer.

3.2 Materials and Methods

Cells
PyMT cells were cultured in DMEM:F12 medium supplemented with 10% FBS and 1% dual antibiotics. E0771 and their subclones, E0.1 and E0.2, were cultured in complete RPMI 1640 or DMEM medium as indicated. Human microvascular endothelial cells (HMVEC) were cultured in ECM medium (ScienCell, San Diego, CA). MDA-MB-231 was cultured in DMEM medium. Cancer cell supernatant was collected from subconfluent cultures after 48 h of serum starvation.

Animal study
Robo4+/− C57BL/6 mice (generated by Dr. Dean Li, University of Utah) were bred for Robo4+/+ and Robo4−/− genotyped female mice. At 8-week age, 2 × 10⁶ PyMT cells, or 0.2 × 10⁶ E0771, E0.1, E0.2 cells in 50% Matrigel (Corning, MA) were injected into the right mammary fat pad of mice of all groups. For drug treatment experiments, mice were injected daily i.p. with 1mg/kg SecinH3 in PBS with 5% DMSO, or vehicle control, from the 2nd day after tumor injection. Tumor size was assessed once a week and tumor volume was calculated according to the formula: volume = length × (width)² / 2. Mice were sacrificed at the end of study or per the request of the veterinarian. All mice were
kept in OSU animal facility in compliance with the guidelines and protocols approved by the IACUC.

**Cell proliferation assay**

Cell proliferation assay was performed using Cell Proliferation Kit I (MTT) from Roche (Indianapolis, IN) per the kit manual. Proliferation assays were done in triplicates. O.D. values were measured using Model 680 microplate reader from BIO-RAD (Hercules, CA).

**IHC, Flow cytometry, WB and qRT-PCR**

Please refer to Chapter 1 and 2.

**Statistical analysis**

Reported data for cell line studies are the means ± S.E.M. of three independent biological samples. Animal studies use n=5~8 mice for each group. The statistical significance was determined by the Student’s t test or as specified.

3.3 Results

3.3.1 Endothelial Robo4 suppresses tumor growth and metastasis

Robo4 is strictly expressed only on endothelial cells and HSCs. Both endothelial cells and HSC-derived leukocytes can affect tumor development. By comparing the peripheral blood leukocyte profiles between wildtype (Robo4+/−) and Robo4 knockout (Robo4−/−) mice, we showed that Robo4 knockout did not affect the HSCs differentiation or the relative composition of peripheral blood leukocytes (Figure 16-17). Thus, we
hypothesized that Robo4 deficiency in endothelial cells, rather than HSCs, affects breast cancer development.
Figure 16. Peripheral blood leukocyte profiles of Robo4\(^{+/+}\) and Robo4\(^{-/-}\) mice.

Peripheral blood of adult female Robo4\(^{+/+}\) and Robo4\(^{-/-}\) mice was collected into heparin solution, and red blood cells were lysed. White blood cells were then stained with conjugated antibodies against cell surface markers (Biolegend, CA) and analyzed by flow cytometry. There was not any significant difference between the peripheral blood leukocyte profiles of Robo4\(^{+/+}\) and Robo4\(^{-/-}\) mice. (gating strategy is described in Figure 17)
Figure 17. Gating strategy for analyzing peripheral blood leukocyte profiles by flow cytometry.

After orthotopically injecting E0.2 cells (a subclone of C57BL/6 derived breast cancer cell line E0771) into female C57BL/6 Robo4^{+/+} and Robo4^{−/−} mice, tumors developed significantly faster in Robo4^{−/−} mice (Figure 18A, B). The numbers of lung metastasis were also significantly greater in Robo4^{−/−} mice comparing to Robo4^{+/+} mice (Figure 18C). Importantly, the probability of distal metastasis is known to be positively correlated with the primary tumor size(Koscielny et al., 1984). Thus, to eliminate the factor of tumor size from the influences on the number of lung metastatic foci, we analyzed the tumors on the Metastasis-Log_{10} Volume plot, in which the slope of linear regression indicates the aggressiveness of the breast tumors(Koscielny et al., 1984). On the Metastasis-Log_{10} Volume plot, the tumors in Robo4^{−/−} mice are more aggressive in generating distal lung metastasis (Figure 18D). This is reflected by the significantly prolonged relapse free survival (RFS) of breast cancer patients with high Robo4 expression in a large combined cohort(Györffy et al., 2010) (Figure 18E). Taken together, we showed that Robo4 expression on endothelial cells inhibits breast cancer growth and metastasis.
Figure 18. Endothelial Robo4 knockout enhanced breast cancer growth and metastasis.

(A) Progression of tumor volumes of E0.2 tumors. (B) Left: Representative tumors of Robo4\textsuperscript{+/+} and Robo4\textsuperscript{-/-} mice. Right: Tumor weight of Robo4\textsuperscript{+/+} and Robo4\textsuperscript{-/-} groups. (C) Number of lung metastasis in two groups. (D) Metastasis-$\log_{10}$Volume plot of tumor and metastasis in two groups. Formula of linear regressions (in the form of: $y = ax + b$) were labelled by the lines. Slope is the value of a. (E) Kaplan-Meier plot of RFS probability of breast cancers in a combined cohort of 3458 patients. Patients were separated by mean of Robo4 level. (*p<0.05)
3.3.2 Endothelial Robo4 suppresses tumor angiogenesis and protects vascular integrity

Vascular staining showed that there was more angiogenesis in the tumors of Robo4⁻/⁻ mice, comparing to Robo4⁺/⁺ mice (Figure 19A). The increased vascular staining was coupled by a similar increase in the percentage of CD31⁻CD45⁻ tumor endothelial cells (Figure 19B). Disrupted tumor endothelial integrity not only initiates neovascularization, but also directly promotes cancer cell vascular invasion and metastasis. When knocking out Robo4 in endothelial cells, in addition to increased tumor blood vessel quantity, the blood vessels also showed significantly increased percentage of branching phenotype (Figure 20A). As a sign of angiogenesis and compromised integrity, the increased branching in Robo4⁻/⁻ tumor blood vessels was accompanied by the reduced level of tight junction protein, ZO-1, in tumor endothelial cells (Figure 20B). These data showed that endothelial Robo4 suppresses tumor growth and metastasis by protecting endothelial integrity and angiogenesis.
Figure 19. Endothelial Robo4 knockout led to increased tumor.

(A) Left: H&E staining and IHC staining of tumor blood vessels (CD31). Right: Quantitative analysis of the CD31 staining areas. (B) Flow cytometry analysis of relative numbers of tumor endothelial cells. Statistical summary is shown in the bar graph of upper right panel.
Figure 20. Endothelial Robo4 knockout led to undermined vascular integrity.

(A) Left: H&E staining and IHC staining of tumor blood vessels (CD31). Right: Quantitative analysis of the CD31 staining areas. (B) Flow cytometry analysis of relative numbers of tumor endothelial cells. Statistical summary is shown in the bar graph of upper right panel. (C) Upper: Representative vessel staining patterns of tumors in both groups. Patterns were isolated through adjusting color threshold of CD31 IHC images. Lower-left: Statistical summary of numbers of tumor blood vessels per field on IHC slides at 100x magnification. Lower-right: Percentage of branching tumor vessels of both groups. Branching is defined as the staining pattern with obvious lateral protrusions. (D) Flow cytometry analysis of tumor endothelial cell ZO-1 levels. Mean fluorescent intensity (MFI) is statistically summarized in the bar graph of lower left panel. (*p<0.05)
Figure 20. Endothelial Robo4 knockout led to undermined vascular integrity.
3.3.3 Pharmacological enhancement of Robo4 signaling downstream of Robo4 inhibits tumor angiogenesis, tumor growth and metastasis

The small molecule drug, SecinH3, can enhance Robo4 signaling through deactivating the ARF6 activating proteins downstream of Robo4. SecinH3 treatment did not inhibit the in vitro proliferation of a non-aggressive murine breast cancer cell line, PyMT (Figure 21).

Figure 21. SecinH3 treatment does not inhibit cell proliferation or affect mouse body condition.

(A) MTT proliferation assay of PyMT cells in 96-well plates. (B) Body weight of mice bearing PyMT tumors, with or without treatment of SecinH3. (C) MTT proliferation assay of E0.1 cells in 96-well plates. Extra high concentration 25 μM is more than 10 times higher than in vivo dosage. (*p<0.05, N.S.: non-significant)
However, SecinH3 significantly reduced tumor growth of PyMT-cell orthotopic tumors *in vivo* (Figure 22A-B), without affecting the body condition of the mice (Figure 21B). This showed that SecinH3, with low toxicity, reduced tumor growth by influencing the host, rather than tumor cells. To further analyze the effect of Robo4 enhancement by SecinH3 on breast cancer metastasis, we used the highly aggressive murine breast cancer cell line, E0.1 (an E0771 subclone, Figure 23). Again, SecinH3 significantly suppressed tumor growth of E0.1 cells (Figure 22C). More importantly, SecinH3 largely inhibited lung metastasis of E0.1 tumors (Figure 22D). Judging by its Metastasis-Log10Volume plot, SecinH3 reduced the aggressiveness (slope of linear regression) of E0.1 tumors (Figure 22D). Moreover, SecinH3 treatment rescued breast cancer cell supernatant induced loss of ZO-1 protein and mRNA in endothelial cells (Figure 24 A-B). And accordingly, in both PyMT tumors and E0.1 tumors, SecinH3 treatment suppressed tumor angiogenesis and the branching phenotype (Figure 24C-D). Taken together, these data showed that, in accordance with the functions of Robo4, enhancing Robo4 signaling using small molecule drug (SecinH3) can inhibit breast tumor growth and metastasis through reducing tumor angiogenesis.
Figure 22. SecinH3 inhibited tumor growth and metastasis.

(A) Tumor volume of PyMT tumor in C57BL/6 mice with or without SecinH3 treatment. (B) Left: Representative PyMT tumors of vehicle and SecinH3 treatment groups. Right: PyMT tumor weight at end of study. (C) Left: Tumor volumes of E0.1 tumors in C57BL/6 mice with or without SecinH3 treatment. Right: E0.1 tumor weight at end of study. (D) Left: Representative lungs of mice bearing E0.1 tumors. Lungs were fixed with Bouin’s solution so that metastatic foci appeared as white nodules (such as the ones pointed by arrows). Middle: Numbers of lung metastatic nodules of both groups. Right: Metastasis-$\log_{10}$Volume plot of tumor and metastasis in two groups. (*p<0.05)
Figure 22. SecinH3 inhibited tumor growth and metastasis.
Figure 23. Generation and characterization of E0771 subclones, E0.1 and E0.2.

Generation of E0.1 and E0.2 is described in the flow chart in the upper panel. After 10 passages of in vitro culture, E0.1 and E0.2 showed significantly different phenotypes. E0.1 proliferated faster, but had less attachment to the plate, comparing to E0.2. When injecting same amount of E0771, E0.1 and E0.2 (0.2 x 10^6) cells into mouse mammary fat pads, E0.2 grew slower and E0.1 generated more lung metastasis than the other two (shown in lower panels, 3 weeks post-injection). So E0.1 cell is considered highly aggressive, and E0.2 cell is considered less aggressive.
Figure 24. SecinH3 inhibited angiogenesis in PyMT and E0.1 tumors.

(A and B) HMVEC cells were treated with supernatant of breast cancer cell MDA-MB-231 (BC sup.), with or without pre-treatment of 30μM SecinH3. ZO-1 protein (A) and mRNA (B) levels were analyzed by WB and qRT-PCR respectively. (C and D) Left: H&E and CD31 IHC staining of PyMT and E0.1 tumors. Middle: Quantification of CD31 staining area on slides of PyMT and E0.1 tumors. Right: Percentage of branching phenotype in tumor blood vessel staining. (*p<0.05)
Figure 24. SecinH3 inhibited angiogenesis in PyMT and E0.1 tumors.
3.4 Discussion

In the present study, we used immune competent Robo4 knockout mouse model to show that Robo4 is important for suppressing breast cancer growth and metastasis. Moreover, knocking out Robo4 in HSCs did not affect the HSC differentiation or the leukocyte profile of the mice. Thus, endothelial Robo4 should be responsible for the anti-cancer effects.

Robo4 knockout led to enhanced tumor angiogenesis. This is in agreement with previous works showing that Robo4 counteracts with VEGF signaling in endothelial cells (Jones et al., 2008; Koch et al., 2011; Marlow et al., 2010). Importantly, more blood vessels showed the branching phenotype in Robo4\(^{-/-}\) tumors. The sprouting induced branching of tumor blood vessels is caused by decrease of tight junctions and increase of endothelium leakage (Carmeliet and Jain, 2011). And reduced tight junction protein ZO-1 expression and increased leakage of endothelium have been shown to promote tumor angiogenesis and metastasis (Zhou et al., 2014). Indeed, we also observed a decrease of ZO-1 in Robo4\(^{-/-}\) tumor endothelium, which should at least partly contribute to the enhanced angiogenesis and metastasis.

In this study, we used two subclones of C57BL/6 derived breast cancer cell line E0771, E0.1 and E0.2. E0.1 and E0.2 are generated in our lab by culturing isolated E0771 tumor cells in RPMI 1640 and DMEM media, respectively. It has been suggested that lower environmental pH, increased glucose metabolism and less cell attachment favor
cancerous progression of tumor cells. Comparing to DMEM, RPMI 1640 has less pH buffering capacity, less glucose and less calcium concentration. Thus, it is possible that RPMI 1640 is more likely to promote in vitro cancerous progression (evolusion) of tumor cells than DMEM. In fact, E0.1 has less cell-to-plastic attachment, and generates bigger tumors and more lung metastasis in mice than E0.2 cell. Thus, we chose to use E0.2 to study tumor growth and E0.1 to study lung metastasis for in vivo experiments.

To confirm the role of Robo4, we investigated the effect of enhancing Robo4 signaling with the small molecule drug (SecinH3) which targets downstream of Robo4. Similar to previous studies of sepsis and arthritis(Davis et al., 2014; Zhu et al., 2012), SecinH3 showed robust effects on enhancing Robo4 signaling. It rescued tight junction protein ZO-1 expression and inhibited breast tumor angiogenesis, tumor growth and metastasis.

For the non-aggressive cell line PyMT, SecinH3 not only did not inhibit but also surprisingly enhanced its in vitro proliferation, especially at lower concentrations. This confirmed that SecinH3 acts on the host not the cancer cells in vivo, although the reason behind the SecinH3-induced increase in cell proliferation still needs further study. Similarly, for the aggressive cell line E0.1, SecinH3 increased its proliferation at low concentration. Whereas, it inhibited proliferation at excessively high concentration (25 µM, 10 times higher than the 2.2 µM dosage of in vivo administration). Such a difference between the responses of PyMT and E0.1 cells to SecinH3 may result from their different stages of cancerous progression and their distinct cell types. In addition, by using the analysis (Metastasis-Log10Volume plot) of cancer aggressiveness in lung metastasis
study, we eliminated the factors of cell proliferation and tumor size in determining influences of metastatic potential.

In conclusion, endothelial Robo4 is important for controlling tumor angiogenesis and endothelial leakage, which suppresses the growth and metastasis of breast cancer. The small molecule drug, SecinH3, can inhibit breast cancer growth and metastasis by enhancing Robo4 function.
Chapter 4: miR-29b determines the pro-/anti-proliferative effects of S100A7 in breast cancer

4.1 Introduction
The inflammatory protein S100A7 (Psoriasin) was discovered as a marker of human psoriasis lesion (Eckert et al., 2004; Jinquan et al., 1996). S100A7 is mainly expressed in epithelial cells (Eckert et al., 2004), and can be induced by pathogens and inflammatory cytokines (Glaser et al., 2005; West and Watson, 2010). It has been noticed that S100A7 is upregulated in breast cancer cells (Al-Haddad et al., 1999; Carlsson et al., 2005; Enerback et al., 2002). However, the role of S100A7 in breast cancer progression has been elusive, since both pro- and anti-proliferative roles have been reported in different types of breast cancer cells and animal models (Deol et al., 2011; Emberley et al., 2003b; Krop et al., 2005; Nasser et al., 2012). It has been shown that S100A7 promotes cancer growth and metastasis in basal-like (ER–) breast cancer cells (Emberley et al., 2003a; Nasser et al., 2012). Accordingly, S100A7 upregulation correlates with poor prognosis for patients with basal subtype breast carcinoma (Emberley et al., 2003b). Conversely, it inhibits cancer growth and cell migration in luminal (ER+) breast cancer cells (Deol et al., 2011). To date, the mechanism by which S100A7 differentially regulates ER– and ER+ breast cancer cell proliferation is unknown.
microRNAs bind to untranslated regions (UTRs) of mRNAs, which inhibits protein translation and/or degrades mRNAs (Ambros, 2004). miR-29b is considered to be a tumor suppressor in multiple types of cancers (Garzon et al., 2009; Xiong et al., 2010), including breast cancer (Chou et al., 2013; Wu et al., 2013). The anti-cancer effect of miR-29b has been shown to be related to its targeting of the 3’ UTRs of multiple key cancer regulators, thus suppressing the growth and metastasis of breast cancer. miR-29b is encoded by two genes, mir-29b-1 on chromosome 7 and mir-29b-2 on chromosome 1. It has been shown that NF-κB binds to the promoter of mir-29b-1 and inhibits its transcription (Mott et al., 2010). In addition, NF-κB has been shown to transactivate YY1, which then binds to the promoter of mir-29b-2 and inhibits its transcription (Wang et al., 2008a). Since NF-κB governs numerous survival genes and apoptotic genes, its functions in cancer development has long been noted. The activity of NF-κB can be either upregulated or downregulated in cancer cells, depending on whether it is inducing survival signaling or apoptotic signaling (Baud and Karin, 2009). The differential activation statuses of NF-κB in different breast cancer subtypes has led to the discovery of reciprocal regulation of NF-κB by ER (Sas et al., 2012).

Among the targets of miR-29b, PI3K p85α and CDC42 have been shown to regulate of p53 activation (Park et al., 2009). Activated p53 translocates into the nucleus and activates multiple anti-proliferative pathways, including the ones of DNA repair and mitosis check point (Vousden and Lu, 2002). It was shown that miR-29b targets p85α and CDC42 and consequently inhibits p53 activation and cell proliferation (Park et al., 2009).
In the present study, we described a novel role of the NF-κB – miR-29b – p53 pathway, which defines the distinct effects of S100A7 on regulating cell proliferation and tumor growth of ER− and ER+ breast cancer.

4.2 Materials and Methods

Reagents and cells

NF-κB p65, p53, PI3K p85α, CDC42, Oct1, β-actin and GAPDH antibodies and NF-κB inhibitor QNZ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). miR-29b real-time PCR assay kit and miR-29b inhibitor were purchased from Life Technologies (Carlsbad, CA). Luciferase substrate was purchased from Promega (Madison, WI). p-p53 (S15), p-ATR, p-Chk-1, p-Chk-2 antibodies were purchased from Cell Signaling Technology (Danvers, MA). S100A7 antibody was purchased from Novus Biologicals (Littleton, CO). MDA-MB-231 cell and its derivatives were cultured in DMEM medium supplemented with 10% FBS and 1% dual antibiotics. MCF7 cell and their derivatives were cultured in MEMα medium supplemented with 10% FBS and 1% dual antibiotics. p53-conditional-knockdown-MCF7 cell line was provided by Dr. Jill Bargonetti (The City University of New York). 231-S-miR-29b cell was generated by stably transfecting pcDNA3-miR29b plasmid shared by Dr. Joshua Mendell (Johns Hopkins University) (Hwang et al., 2007). MCF7-S-miR-29b-Decoy cell was generated by stably transfecting AB.pCCL.sin.cPPT.U6.miR-29b-Decoy.hPGK.GFP.WPRE plasmid shared by Dr. Brian Brown (Mount Sinai School of Medicine) (Mullokandov et al., 2012).

Animal study
Adult female nude mice (NU/NU), obtained from Charles River Laboratories (Wilmington, MA), were housed under specific pathogen-free conditions. MDA-MB-231, MCF7 or their derivatives (3x10^6 cells/200 μL PBS) were injected into the right mammary fat pad of each mouse. Mice with MCF7 cell or derivatives were also injected subcutaneously with 2.5 μg of β-estradiol 17-valerate in 50 μL of Sesame oil once a week. Tumor size was assessed once a week and tumor volume was calculated using digital calipers according to the formula: volume = length × (width)^2 / 2. Mice were sacrificed at the end of study or per the request of the veterinarian. All mice were kept in OSU’s animal facility in compliance with the guidelines and protocols approved by the IACUC.

**Gene functional enrichment analysis**

Gene functional enrichment (GFE) analysis is performed using the bioinformatics engine, ToppGene (Chen et al., 2009), developed by the Division of Biomedical Informatics, Cincinnati Children's Hospital Medical Center (Cincinnati, OH). Training gene list was determined by gating the significantly up-/down-regulated genes from microarray data as specified. Enrichment analysis was corrected by Bonferroni method with a p-Value cutoff of 0.05.

**qRT-PCR**

For mRNA detection, total RNA was then reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies) and quantified using Power SYBR Green Master Mix (Life Technologies). p53 pathway PCR array was performed using p53 Signaling Pathway RT2 Profiler PCR Array from Qiagen. For
miRNA detection, total RNA was reverse transcribed and miRNAs quantified using miRNA specific real-time PCR kits from Life Technologies.

**NF-κB activity assays**

For the κB-site-DNA-bait ELISA assay, nuclear and cytoplasmic extractions of cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) per the product manual. Activated NF-κB p65 levels of both nuclear and cytoplasmic extracts were measured using TransAM NF-κB p65 Transcription Factor ELISA Kit (Active Motif, Carlsbad, CA) per the product manual. For the luciferase reporter assay, cells were transfected with equal amount of NF-κB-Luc reporter plasmids by lipofectamine 2000 (Life Technologies) and harvested after 48 h. Cells were lysed and luciferase activity was measured with the luciferase assay kit from Promega (Madison, WI) and Synergy plate reader (BioTek, Winooski, VT) per the manufacturer’s manual.

**Confocal microscopy**

Cells grown on micro-chamber-slides were fixed and permeabilized using the Fix/Perm solution (BD Biosciences, San Jose, CA) for 20 min at 4°C. The cells were washed with BD Perm/Wash buffer (BD Biosciences), and stained with respective primary antibodies for 1 h at 4°C. After the incubation, cells were washed and stained with Alexa Fluor 488 or 568 secondary antibodies for 1 h at 4°C. The slides were mounted using Vectashield mounting medium with DAPI (Vector Laboratories) and then imaged with Olympus FV1000 confocal microscope. The pictures were processed using Olympus FV-10 ASW software.

**Chromatin immunoprecipitation (ChIP)**
Cells were fixed and cross-linked in 2% paraformaldehyde, and then cross-linking was quenched with 10% volume of 1.375 M glycine. After washing with ice cold PBS, cells were harvested and lysed with NP-40 ChIP buffer. Nuclei were isolated by centrifuge, and lysed with SDS nuclei lysis buffer. DNA was digested using Micrococcal Nuclease from New England Biolabs, and digestion was stopped by adding 50 mM EDTA. DNA samples were then immunoprecipitated overnight at 4°C with NF-κB p65 antibody and Protein G / Protein A Agarose Beads (Calbiochem). After washing, DNA was eluted using SDS elution buffer, and then reverse cross-linked by incubation overnight at 65°C. DNA was purified using TRIzol reagent (Life Technologies) and chloroform, and then subjected to PCR with specific primers.

**Cell proliferation assay and WB**

Please refer to Chapter 1 and 2.

**Statistical analysis**

Reported data for cell line studies are the means ± S.E.M. of at least three independent experiments performed in duplicates or triplicates. Animal studies use n=5~8 mice for each group. The statistical significance was determined by the Student’s t test or as specified.
4.3 Results

4.3.1 S100A7 differentially regulates proliferation of ER⁻ and ER⁺ breast cancer cells

To investigate the effect of S100A7 upregulation in human breast cancer, we generated a panel of S100A7 overexpressing breast cancer cell lines (Deol et al., 2011; Nasser et al., 2012). S100A7 overexpression significantly increased the proliferation of ER⁻ MDA-MB-231 cells and decreased that of ER⁺ MCF7 cells (Table 2). Furthermore, with S100A7 overexpression, MDA-MB-231 cells gained mesenchymal properties (fibroblast-like cell shape and absence of cell-cell adhesion) and increased tumor growth in nude mice; in contrast, with S100A7 overexpression, MCF7 cells gained epithelial properties (flat scale-like cell shape and increased cell-cell adhesion) and showed reduced tumor growth in nude mice. These observations are in accordance with previous publications from our group and others (Deol et al., 2011; Emberley et al., 2003a; Nasser et al., 2012). In this study, we used MDA-MB-231 and MCF7 as representative cellular models of ER⁻ and ER⁺ breast cancers to analyze the mechanism by which S100A7 may differentially regulate cell proliferation.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>MDA-MB-231</th>
<th>MCF7</th>
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<tbody>
<tr>
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<td>Luminal</td>
</tr>
<tr>
<td>ER status</td>
<td>ER negative</td>
<td>ER positive</td>
</tr>
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<td>Proliferation change with S100A7 overexpression</td>
<td>↑ 57%*</td>
<td>↓ 33%*</td>
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<tr>
<td>Morphology change with S100A7 overexpression</td>
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<td>Gained epithelial</td>
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<tr>
<td>Tumor growth with S100A7 overexpression</td>
<td>↑ 1089%*</td>
<td>↓ 79%*</td>
</tr>
</tbody>
</table>

Table 2. Differential effects of S100A7 overexpression in human breast cancer cell lines.

Changes of cell proliferation, morphology, tumor formation in nude mice caused by S100A7 overexpression in given cell lines are listed. Mesenchymal properties of cell morphology include fibroblast-like cell shape, absence of cell-cell adhesion; epithelial properties include flat and scale-like cell shape, increased cell-cell adhesion and clustering. (*p<0.05)

4.3.2 S100A7 overexpression induced differential miR-29b expression changes in MDA-MB-231 and MCF7 cells

In order to analyze the mechanism by which S100A7 differentially regulates cell proliferation, we characterized the gene expression signatures in the microarray data of S100A7 overexpressing MDA-MB-231 and MCF7 cells (231-S100A7 and MCF7-S100A7). We postulated that we would pinpoint the driving factor of proliferative regulation by comparing the differential imprints of S100A7 overexpression in the transcriptomes of MDA-MB-231 and MCF7 cells. To do this, we performed the Gene Functional Enrichment (GFE) analysis to the microarray data of 231-V/231-S100A7 and
MCF7-V/MCF7-S100A7 cells with the bioinformatic engine, ToppGene. We used the genes which were up-/down-regulated greater than 2 folds in 231-S100A7 (v.s. vector control) and a comparable number of genes which were up-/down-regulated greater than 2.5 folds in MCF7-S100A7 (v.s. vector control) as training sets for randomized separate GFE analysis. We then compared the GFE analysis outcomes of these four sets and discovered a common imprint between MDA-MB-231 and MCF7 that is associated with opposite changes of genes: the expression changes of miR-29b targets. As shown in Figure 25A, miR-29b target genes were significantly enriched in the genes upregulated in 231-S100A7 and also in those downregulated in MCF7-S100A7 cells. miR-29b has been considered a strong tumor suppressor in multiple cancers. Based on the fact that miR-29b target genes were upregulated in 231-S100A7 and downregulated in MCF7-S100A7 cells, we hypothesized that S100A7 differentially regulates miR-29b expression which subsequently affects cell proliferation in MDA-MB-231 and MCF7 cells through modulation of miR-29b target genes. In order to verify this, we first analyzed the expression levels of both mature miR-29b and its two primary microRNAs on chromosome 7 and 1. In agreement with our GFE analysis, we observed that S100A7 overexpression significantly downregulated miR-29b expression in MDA-MB-231 cells and upregulated miR-29b in MCF7 cells, in both mature (miR-29b) and primary (pri-miR-29b-1 and pri-miR-29b-2) forms (Figure 25B-C).
Figure 25. S100A7 differentially regulate miR-29b transcription and NF-κB activation in MDA-MB-231 and MCF7 cells.

(A) GFE analysis was performed on the top up/down-regulated genes of 231-S100A7 and MCF7-S100A7 cells. Genes up/down-regulated > 2 folds in 231-S100A7 and those up/down-regulated > 2.5 folds (similar amounts of genes) in MCF7-S100A7 were filtered from microarray data and used as training sets for separate analysis. Training sets from microarray data are indicated by heat maps, and percentage of miR-29b targets are indicated with pie charts with significance of enrichment analysis. (B) mature miR-29b was measured by qRT-PCR in the given cell lines, with RNU6B as internal control. Values are not comparable between MDA-MB-231 and MCF7 cells. (C) primary mir-29b-1 and mir-29b-2 transcript levels were detected by qRT-PCR using specific primers. 18S rRNA was used as internal control. (D) total cell lysates were subjected to κB-site-DNA-bait ELISA assay to detect total active NF-κB dimer level. Values are not comparable between MDA-MB-231 and MCF7 cells. (E) cells transfected with NF-κB reporter plasmids were harvested after 48h, luciferase activity was analyzed in cell lysates. (F) activated p-p65 subunit of NF-κB in different cells were observed with confocal microscopy. Nuclei were stained with DAPI. (G) nuclear and cytoplasmic portions of cells were extracted and activated p65 NF-κB were detected with κB-site-DNA-bait ELISA. Data show the relative percentage of active NF-κB in the two portions. (* p<0.05, ** p<0.01)
Figure 25. S100A7 differentially regulate miR-29b transcription and NF-κB activation in MDA-MB-231 and MCF7 cells.
Importantly, in agreement with our *in vitro* cell line data (Figure 25B,C), analysis of TCGA invasive breast cancer patient data (Network, 2012) showed that S100A7 overexpression in ER+ patients is more likely to correlate with miR-29b upregulation than ER- patients; and S100A7 overexpression in ER- patients is more likely to correlate with miR-29b downregulation than ER+ patients (Figure 26-28). These data showed that S100A7 differentially regulates miR-29b transcription in ER- and ER+ breast cancers.
Figure 26. Expression of S100A7 in different subtypes of invasive breast cancer patients of the TCGA database.

mRNA expression levels of S100A7 in different subtypes were analyzed among the 825 available patient samples in TCGA (Network, 2012) invasive breast cancer database. (Left Panel) Patients were grouped by cancer type. **p<0.01 comparing to normal breast tissue. (Right Panel) Patients were grouped by receptor status. **p<0.01 comparing to ungrouped (data not available/N.A.) and positive status. 75 percentile of S100A7 upregulation is labeled in each figure. Figures were generated through Oncomine (Rhodes et al., 2007).
mRNA/miRNA expression levels of S100A7, ESR1, MIR-29-1/29B and MIR-29B-2/29B were analyzed among the 825 available patient samples in TCGA (Network, 2012) invasive breast cancer database. In TCGA cohort, most patients have ER-positive tumors, with only 22.95% patients have ER-negative tumors. To match this, ER-negative is defined as ESR1 mRNA expression being less than 0.1SD above the mean value of the whole cohort, the rest being ER-positive. In invasive breast cancer patients, 26.32% of them have S100A7 overexpression (Al-Haddad et al., 1999). To match this, S100A7 overexpression is defined as S100A7 mRNA expression being greater than 0.1SD below the mean. miR-29b upregulation is defined as MIR-29B-1/29B and MIR-29B-2/29B expression being greater than the mean, and downregulation is being less than the mean. p values of Fisher’s Exact Test are shown in the table. Color coding indicates the odds ratio. S100A7 upregulation is enriched in ER-negative cancers, and is simultaneously associate with miR-29b downregulation.
Figure 28. Influence of S100A7 overexpression on miR-29b expression level in invasive breast cancer patients of the TCGA database.

mRNA/miRNA expression levels of S100A7, ESR1, MIR-29-1/29B and MIR-29B-2/29B were analyzed among the 825 available patient samples in TCGA(Network, 2012) invasive breast cancer database. Definitions of conditions are same as Figure 26. Number of patients and its percentage is shown in each cell of table. Data are summarized in bar graphs. S100A7 overexpression in ER$^+$ patients is more likely to correlate with miR-29b upregulation than ER$^-$ patients. Meanwhile, S100A7 overexpression in ER$^-$ patients is more likely to correlate with miR-29b downregulation than ER$^+$ patients.
Figure 28. Influence of S100A7 overexpression on miR-29b expression level in invasive breast cancer patients of the TCGA database.
4.3.3 S100A7 differentially modulates NF-κB activation in MDA-MB-231 and MCF7

Interestingly, we observed differential regulation of NF-κB activity by S100A7, which is similar to the differential regulation of miR-29b expression in MDA-MB-231 and MCF7 cells. Using both κB-site-DNA-bait ELISA and NF-κB-luciferase reporter assay, we observed an increase of NF-κB activity in MDA-MB-231 and a decrease of NF-κB activity in MCF7 after S100A7 overexpression (Figure 25D,E). κB-site-DNA-bait ELISA detected the overall activated p65 NF-κB level, and NF-κB-luciferase reporter assay detected the actual transcription driving NF-κB level. Similar to changes in NF-κB activation level, we also observed increased NF-κB nuclear translocation in MDA-MB-231 and decreased NF-κB nuclear translocation in MCF7 after S100A7 overexpression (Figure 25F,G).

4.3.4 miR-29b transcription is differentially regulated by S100A7 via NF-κB in MDA-MB-231 and MCF7

miR-29b expression has been shown to be inhibited by NF-κB in non-breast cancer cells (Ma et al., 2011; Mott et al., 2010; Wang et al., 2008a). We intended to find out whether S100A7 affected miR-29b transcription via regulating NF-κB activation in MDA-MB-231 and MCF7. We first verified that, in breast cancer cells, inhibiting NF-κB activation with its inhibitor, QNZ, significantly enhanced the transcription of pri-mir-29b-1 and pri-mir-29b-2 (Figure 29A). This showed that NF-κB activity was inversely correlated with miR-29b transcription in breast cancer cells. pri-mir-29b-1 promoter contains three NF-κB binding sites. The binding of NF-κB to the promoter directly suppresses pri-mir-29b-1 transcription (Figure 29B). In addition, NF-κB transactivates
the transcriptional factor, YY1, which in turn can bind to the promoter of pri-mir-29b-2 to suppress its transcription (Figure 29C). By ChIP assay and qRT-PCR, we observed that S100A7 overexpression differentially altered the binding of NF-κB to the promoter of pri-mir-29b-1 and/or the expression of pri-mir-29b-2 suppressor, YY1, in MDA-MB-231 and MCF7 cells (Figure 29D-F). Both direct and indirect transcriptional regulations by NF-κB led to the differential regulation of miR-29b levels by S100A7, in MDA-MB-231 and MCF7 cells.
Figure 29. S100A7 differentially regulates miR-29b transcription in MDA-MB-231 and MCF7 cells.

(A) MCF7 cells were treated with 5nM QNZ for 24h before they were harvested for qRT-PCR analysis. A well-known NF-κB driven gene GM-CSF served as the positive control for effect of QNZ inhibition. (B) κB binding sites at the proximal of mir-29b-1 gene transcriptional start site. Two sets of primers, κB-1 and κB-2, were designed to detect three κB binding sites in ChIP. (C) indirect inhibition of mir-29b-2 transcription by NF-κB and YY1. (D) ChIP assay of NF-κB p65 subunit binding to mir-29b-1 promoter region in 231-V and 231-S cells. (E) ChIP assay of NF-κB p65 subunit binding to mir-29b-1 promoter region in MCF7-V (M-V) and MCF7-S (M-S). (F) qRT-PCR quantification of YY1 level in different cells. (* p<0.05, ** p<0.01, N.S.: non-significant)
4.3.5 S100A7 downregulates PI3K p85α and CDC42 via targeting of miR-29b to activate and stabilize p53 in MCF7

It has been reported that miR-29b activates p53 through targeting the 3’ UTR of the oncogenes, PI3K p85α and CDC42 (Park et al., 2009) (Figure 30A). Activated (phosphorylated) p53 translocates into nucleus and is protected from degradation in the cytoplasm (stabilized). We showed that S100A7 induced upregulation of miR-29b in MCF7 cells was able to reduce the level of p85α and CDC42 proteins (Zhao et al., 2015). Transient knockdown of miR-29b with miR-29b inhibitor (antagomir) partially rescued the expression of p85α and CDC42 (Zhao et al., 2015). Prolonged knock down of miR-29b by miR-29b-Decoy transfection reduced p53 activation in MCF7-S100A7 (Figure 30B). Compared to MDA-MB-231, S100A7 caused a much more dramatic change of miR-29b in MCF7 cells (Figure 28B). This dramatic rise of miR-29b led to a significant increase of p53 phosphorylation, nuclear translocation and total p53 protein level (Figure 30C) in MCF7-S100A7 cells (Zhao et al., 2015).
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Figure 30. miR-29b targets p85α and CDC42, and determines the effects of S100A7 on p53 activation and stability in different cells.

(A) target sites of miR-29b on 3’ UTRs of CDC42 and p85α mRNAs. (B) prolonged knock down of miR-29b with miR-29b-Decoy reduced p53 phosphorylation and total p53 level in MCF7-S cells. (C) relative p53 levels in nuclear extract of cells. p53 levels were normalized to nuclear marker Oct1. (* p<0.05, ** p<0.01)

4.3.6 S100A7 enhances p53 related anti-proliferative pathways in MCF7

We further investigated the mechanism by which S100A7 inhibited MCF7 proliferation. p53 pathway qRT-PCR array data revealed that S100A7 overexpression in MCF7 cells led to the upregulation of p53 activating factors (such as ATM, ATR, CDKN1A/p21, CDKN2A and CHEK2), p53 targeted apoptotic factors (such as FAS and TNFRSF10B),
p53 targeted DNA repairing factors (such as PCNA and PTTG1) and p53 targeted anti-survival factors (such as PTEN) (Figure 31A). Consequently, DNA repair pathway in MCF7-S100A7 cells was also activated through p53. Phosphorylation of ATR, Chk-1 and Chk-2 was enhanced by S100A7, which was absent when p53 is knocked down in MCF7 cells (Brekman et al., 2011) (Figure 31B,C). S100A7 induced p53 upregulation appeared to be concurrently related with significantly enhanced anti-proliferative pathways in MCF7 cells.
Figure 31. S100A7 activated p53 related anti-proliferative pathways.

(A) Log2 fold change of mRNA expression of p53 pathway factors after S100A7 overexpression. (B) WB of p53 and S100A7 in MCF7-p53-CKD transfected with S100A7 or vector control. p53-shRNA transcription and p53 knockdown in MCF7-p53-CKD was induced by Doxycyclin (Dox). (C) S100A7 activated p53, ATR, chk-1, chk-2 in MCF7 cells. p53 knock down depleted the effect of S100A7.
4.3.7 S100A7 induced differential regulation of miR-29b is important for its
differential effects on cell proliferation of MDA-MB-231 and MCF7 in vitro and
their tumor growth in vivo

To verify the importance of miR-29b in determining the differential effects of S100A7,
we stably transfected 231-S100A7 and MCF7-S100A7 cells with exogenous miR-29b
and miR-29b-Decoy, respectively, to reverse the changes of miR-29b caused by S100A7
(Figure 32A,C). The S100A7 induced proliferation increase in MDA-MB-231 was
repressed by exogenous miR-29b (Figure 32B), and the decrease of proliferation in
MCF7 was also partially rescued by miR-29b-Decoy (Figure 32D, Figure 33). When we
orthotopically injected these cells into nude mice mammary fat pad, miR-29b
overexpression repressed S100A7 induced tumor growth of MDA-MB-231 cells (Figure
34A-C), whereas miR-29b knockdown rescued tumor growth from S100A7 suppression
in MCF7 cells (Figure 34D, E). Thus, we confirmed that miR-29b is the determining
factor of the differential effects of S100A7 in ER− and ER+ breast cancer cells.
Figure 32. Manipulating miR-29b could counteract the effects of S100A7 on cell proliferation in MDA-MB-231 and MCF7.

The proliferation of MDA-MB-231 and MCF7 cell lines were determined with MTT assay. (A,C) miR-29b levels of indicated cell lines. (B,D) MTT cell proliferation assays of indicated cell lines. (* p<0.05, ** p<0.01)
Figure 33. miR-29b-Decoy transfection reversed the phenotypic changes (epithelial transition) induced by S100A7 in MCF7 cells.

48h after miR-29b-Decoy transfection in MCF7-S100A7, its morphology was examined under microscope. EGFP expression indicates cells transfected with miR-29b-Decoy plasmids.
Figure 34. miR-29b determines the effects of S100A7 on tumor growth in MDA-MB-231 and MCF7 cells.

(A and D) representing tumors of each group at the end of study. Brackets mark the size of tumors on mice. All tumors are to the same scale. (B and E) tumor weight of each group. * p<0.05 as analyzed by ANOVA. 231-V and 231-S-miR-29b are not significantly different, neither are MCF7-V and MCF7-S-miR-29b-Decoy. (C) tumor volume of each group. * p<0.05 as analyzed by ANOVA. 231-V and 231-S-miR-29b are not significantly different.
Figure 34. miR-29b determines the effects of S100A7 on tumor growth in MDA-MB-231 and MCF7 cells.
4.4 Discussion

In the present article, we proposed a novel mechanism for the differential roles of the inflammatory protein S100A7 on the proliferation of ER\(^-\) and ER\(^+\) breast cancer cells. S100A7 can either promote or suppress breast cancer cell proliferation through distinct modulation of the NF-\(\kappa\)B – miR-29b – p53 pathway in ER\(^-\) MDA-MB-231 and ER\(^+\) MCF7 cells, respectively (summarized in Figure 35).

Figure 35. Proposed mechanism of S100A7 differentially regulating NF-\(\kappa\)B – miR-29b – p53 pathway in different types of breast cancer cells.

miR-29b governs numerous genes’ expression by targeting their 3’ UTRs, leading to both translational suppression and instability of mRNAs. Since large portions of these regulated genes are associated with cell proliferation, miR-29b has been considered as a tumor suppressor in various cancers(Balkhi et al., 2013; Garzon et al., 2009; Wang et al., 2008a; Xiong et al., 2010), including breast cancer(Chou et al., 2013; Wu et al., 2013).
Moreover, other than regulation of cell proliferation, miR-29b has also been shown to inhibit breast cancer metastasis (Chou et al., 2013). Through bioinformatic analysis and in vitro assays, we found that miR-29b expression was reduced by S100A7 in ER⁻ MDA-MB-231 and increased by S100A7 in ER⁺ MCF7 cells, which at least partly explained the different roles of S100A7 in regulating proliferation of different types of breast cancer cells. The clinical relevance of this was supported by patient data of the TCGA cohort, which showed that S100A7 upregulation is more likely to be associated with increased miR-29b expression in ER⁺ breast cancer patients than ER⁻ patients and vice versa. Interestingly, we noticed that, although S100A7 is overexpressed in both ER⁻ and ER⁺ breast cancer patients, there is a significant mutual-exclusivity of S100A7 and ER expression in human breast tumors (Figure S1-S2). In other words, S100A7 overexpression is more commonly seen in ER⁻ tumors. This is in agreement with previous works based on different cohorts of breast cancer patients (Al-Haddad et al., 1999).

Moreover, the differential regulation of miR-29b expression was associated with differential regulation of NF-κB activation by S100A7. NF-κB has been shown to either directly or indirectly inhibit the expression of miR-29b, which is transcribed from mir-29b-1 on chromosome 7 and mir-29b-2 on chromosome 1 (Mott et al., 2010; Wang et al., 2008a). Directly, NF-κB can bind to the promoter of mir-29b-1 to block its transcription. Indirectly, NF-κB can transactivate YY1 which then block the transcription of mir-29b-2 by binding to its promoter (Wang et al., 2008a). In the present study, we showed that S100A7 enhanced NF-κB activity in MDA-MB-231 and inhibited NF-κB activity in MCF7, which then directly and/or indirectly influences miR-29b transcription. It was
known that ER negatively regulates activity in breast cancer (Biswa, et al., 2000; Cvoro et al., 2006; Parker et al., 2009; Sas et al., 2012), and may even block the transcriptional function of NF-κB (Cvoro et al., 2006). Thus, the absence/presence of ER in breast cancer cells may lead to the differential regulations of NF-κB activation by S100A7 in MDA-MB-231 (ER−) and MCF7 (ER+) cells. In this work, ER status was used as the primary standard for dividing breast cancer cells into two groups with distinct responses to S100A7. To our knowledge, the distinct regulation of NF-κB - miR-29b by S100A7 is shown for the first time.

Among the targets of miR-29b, PI3K p85α and CDC42 have been shown to be closely related to cancer growth (Lang et al., 2010; Namløs et al., 2012; Park et al., 2009). And, as oncogenes, they inhibit the activation of tumor suppressor, p53 (Park et al., 2009). Activated p53 translocates into the nucleus and avoids degradation in the cytoplasm. Using MCF7, whose miR-29b was more significantly affected by S100A7, we showed that miR-29b targeted PI3K p85α and CDC42, which consequently increased p53 level by enhancing its activation and nuclear translocation.

Activated p53 can enhance multiple anti-proliferative pathways to inhibit cancer growth (Vousden and Lu, 2002). Here, we showed that increased p53 activation and total protein level increased the expression of multiple p53 downstream anti-proliferative factors, including mitosis checkpoint pathways and DNA repair pathways. These effects were considered to exert anti-proliferative influences to breast cancer cells.

In addition, we reversed the effects of S100A7 on cell proliferation and tumor growth by overexpressing miR-29b in 231-S100A7 cells and knocking down miR-29b in MCF7-
S100A7 cells, which reflected that miR-29b is not only sufficient but also necessary for determining the differential effects of S100A7 in breast cancer cells. This demonstrated that miR-29b functions downstream of S100A7 and is important in determining the differential effects of S100A7 in breast cancer cells.

In conclusion, we report a novel regulatory route that determines the pro-/anti-proliferative roles of S100A7 in ER− and ER+ breast cancer. In these different types of breast cancer cells, S100A7 differentially regulates NF-κB activation, which then differentially affects miR-29b expression and p53 functions. And reversing miR-29b changes can suppress the effects of S100A7 in these cells. Thus, it is suggested that miR-29b may be paired with S100A7 to serve as more accurate biomarkers for breast cancer diagnosis and prognosis. On the other hand, S100A7 does not have kinase activity, which makes it difficult to be targeted with drugs. Recently, many lipid-nanoparticle-based technologies have made it possible to target miRNAs in clinical trials of cancer treatment (Kanasty et al., 2013). Hence, with the discovery of S100A7 - miR-29b regulatory route, miR-29b may be potential to serve as an alternative target for S100A7 overexpression in breast cancer treatment.
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