# Modulation of Heparan Sulfate Proteoglycans During Herpes Simplex Virus Type-1 Infection

BY

GHADAH ABDULLAH KARASNEH

B.S., Jordan University of Science and Technology, 2004

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Microbiology and Immunology
in the Graduate College of the
University of Illinois at Chicago, 2012.

Chicago, Illinois

Defense Committee:

Deepak Shukla, Chair and Advisor

Alan McLachlan

Zheng Chen

Nancy Freitag

Tibor Valyi-nagy, Pathology

This thesis is dedicated to my loving parents Abdullah and Wafa, you are dearly missed but your encouragement as I began this journey will continue to echo throughout my life.

I also dedicate this thesis to my dear husband Faris for being by my side throughout this journey.

# ACKNOWLEDGMENTS

I would like to express my gratefulness to my advisor, Dr. Deepak Shukla for his supervision and support throughout my PhD study. Your support has nurtured my fond of science and helped shape my skills as a scientist. Furthermore, I would like to thank all my committee members, Dr. Alan McLachlan, Dr. Nancy Freitag, Dr. Tibor Valyi-Nagy, and Dr. Zheng Chen. Your valuable input and suggestions have solidified the cornerstones of this thesis.

I would also like to thank the Shukla Lab members past and present: Thessicar Antoine, Jim Trigilio, Rui Liu, Dr. Mohamed Ali, Min Jung Jarding, Tania Vadakara, Asim Farooq, Abdinassir Gababa, Asim Qureshi, Fatima Z. Rizvi, Dr. Christopher O'Donnell, Dr. Praveen Pandey, Sarolta Basca, and Myung-Jin Oh. My interaction with you has enriched my learning experience and helped me get through tough moments during my study. I am sure glad that I have met you.

I would like to acknowledge my colleagues in the Microbiology and Immunology department, and my friends in other departments.

Finally, I would like to thank my sisters and brother (Jumana, Sanna', Reema, Danah, and Ahmad) who have always found the time to support me and listen to me despite their busy life and the distance that have separated us.

## TABLE OF CONTENTS

CHAPTER PAGE

**TABLE OF CONTENTS** (continued)

CHAPTER PAGE

## LIST OF TABLES

## LIST OF FIGURES

FIGURE PAGE

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 3-OS HS | 3-O-Sulfated heparan sulfate |
| Ab | antibody |
| BSA | Bovine serum albumin |
| CFP | Cyan fluorescent protein |
| CHO | Chinese Hamster Ovary |
| CS | Chondroitin sulfate |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dpi | Day post infection |
| E | Early |
| ECM | Extracellular matrix |
| ER | Endoplasmic reticulum |
| FACS | Fluorescence-activated cell sorter |
| FBS | Fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| GAG | Glycosaminoglycans |
| gB-gN | Glycoprotein B-N |
| GFP | Green fluorescent protein |
| HCC | Hepatocellular carcinoma |
| HCE | Human corneal epithelial |
| HCV | Hepatitis C virus |
| HepI | Heparinase I |
| HepIII | Heparinase III |
| HIV | Human immunodeficiency virus |
| HPSE | Heparanase |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| HPV | Human papillomavirus (HPV) |
| HS | Heparan sulfate |
| HSPG | Heparan sulfate proteoglycans |
| HSV-1 | Herpes Simplex Virus Type-1 |
| HSV-2 | Herpes Simplex Virus Type-2 |
| HVEM | Herpesvirus entry mediator |
| ICAM | Intercellular adhesion molecule 1 |
| IgG | Immunoglobulin G |
| IE | Immediate-early |
| IgV | V-like immunoglobulin |
| L | Late |
| mAb | Monoclonal antibody |
| MAG | Myelin-associated glycoprotein |
| MEM | Minimum essential medium |
| MMP | Methalloproteinase |
| MOI | Multiplicity of infection |
| NES | Nuclear export signal |
| NLS | Nuclear localization signal |
| NMHC-IIA | Non-muscle myosin heavy chain IIA |
| ONPG | O-nitrophenyl-β-d-galactopyranoside |
| PBS | Phosphate buffered saline |
| PDZ | Postsynaptic density-95/ disc large protein/zonula occludens-1 |
| PG | Proteoglycans |
| PILRα | Paired immunoglobulin-like type 2 receptor-α |
| PKA | protein kinase A |
| pK | Lysine-rich |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| PMA | Phorbol 12-Myristate 13-Acetate |
| PRR | Proline-rich region |
| P/S | Penicillin and streptomycin |
| RNA | Ribonucleic acids |
| RFP | Red fluorescent protein |
| SDC1 | Human syndecan-1 |
| SD | Standard deviation |
| SDC-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| Ser | Serine |
| siRNA | Small interfering RNA |
| TBS | Tris buffered saline |
| TGFβ | Transforming growth factor β |
| TTBS | 0.1% Tween 20 in TBS |
| Tyr | Tyrosine |
| VCAM-1 | Vascular cell adhesion protein 1 |
| VEGF | Vascular endothelial growth factor |
| VP | Viral protein |
| wt | Wild type |

## SUMMARY

Herpes simplex virus type-1 (HSV-1) is an important human pathogen that relies heavily on cell-to-cell spread for establishing a lifelong latent infection. The result is a very successful prevalence of the virus in the human population infecting 40-80% of people worldwide. It causes oral and ocular lesions and more serious diseases, such as blindness, meningitis, and encephalitis. HSV-1 is a leading cause of viral corneal blindness and viral encephalitis in developed countries. HSV-1 entry into host cell is a multistep process that involves the interaction of the viral glycoproteins with various cell surface receptors. The role of heparan sulfate proteoglycans (HSPG) during HSV-1 infection has focused solely on the role of HS chains as an attachment receptor for the virus, while the core protein has been assumed to perform a passive role of only carrying the HS chains. Likewise, very little is known about the involvement of any specific HSPGs in HSV-1 lifecycle. The purpose of this study was to further analyze the contribution and modulation of HSPG during HSV-1 infection.

Our results have identified syndecan-1, a member of the syndecan family of HSPG, as an important contributor to HSV-1 infection. Syndecan-1 is predominantly expressed in epithelial cells which are prime targets for HSV-1 initial infection. Syndecan-1 downregulation in human corneal epithelial cells (HCE) resulted in reduced viral entry at a step prior to viral nuclear transport. Furthermore, our results suggest an important role of syndecan in HSV-1 mediated cell-to-cell fusion, as syndecan-1 induced expression enhanced cell fusion while syndecan-1 downregulation inhibited virus mediated cell fusion. Interestingly, these results were obtained in wild type Chinese hamster ovary cells (CHO-K1) as well as in HS-deficient mutant CHO cell (CHO-745). This suggests that syndecan-1 contribution to virus mediated cell-to-cell fusion is

HS independent. In addition, inducing the expression of syndecan-1 cells along with HSV-1 fusion glycoproteins (gB, gD, gH-gL) inhibited virus mediated cell-to-cell fusion in CHO-K1 and CHO-745 cells, suggesting a possible interaction between syndecan-1 core protein and at least one of the above mentioned HSV-1 fusion glycoproteins. Syndecan-1 also contributes in virus cell-to-cell spread as syndecan-1 downregulation and overexpression have quantifiable effects on HSV-1 plaque sizes. Further analysis of virus spread to HCE cells with reduced expression of syndecan-1 supported the significance of syndecan-1 in HSV-1 cell-to-cell spread.

The modulation of HSPG by Heparanase during HSV-1 infection was also studied. Although Heparanase induced expression did not show effect on HSV-1 entry into host cells, it enhanced virus release from infected cell to culture supernatant. Our result was supported by utilizing exogenous Heparinases that resulted in enhanced HSV-1 release from infected cell. This is a novel observation where the same receptor (HSPG) that the virus uses for its attachment, and efficient viral entry and spread, is used to facilitate viral release from infected cell through the action of Heparanase. Importantly, HSV-1 infection resulted in enhanced expression of active Heparanase, the form that degrades HS chains and is known to enhance syndecan-1 shedding. This strongly suggests that the virus has evolved to exploit Heparanase to modulate HSPG to regulate their contribution to the infection. Our results suggest that Heparanase induces virus release by not only degrading HS, but also by enhancing syndecan-1 shedding. This is supported by the fact that virus release can be induced by treatment with a syndecan-1 shedding agonist.

# 1. INTRODUCTION

## 1.1 Herpes Simplex Virus Type-1

Herpes simplex viruses (HSV) are part of the alphaherpesvirus subfamily of herpesviruses. There are two types of HSV: type-1 (HSV-1) and type-2 (HSV-2). These viruses are neurotropic capable of infecting the nervous system and causing neurological diseases. Moreover, HSV results in a lifelong infection by establishing latency in the host sensory neurons and replicating in epithelial cells during primary infection and reactivation (Heldwein et al., 2008). The virus is spread and transmitted among humans through physical contact and commonly causes localized mucocutaneous lesions (Akhtar et al., 2009). Oral and ocular lesions are primarily caused by HSV-1 and genital lesions by HSV-2. These viruses are also capable of causing more serious diseases, such as blindness, meningitis, and encephalitis (Connolly et al., 2011). HSV-1 is a leading cause of viral corneal blindness and viral encephalitis in developed countries (Herpetic Eye Disease Study Group 1998, Shoji et al., 2002). Current treatment of HSV-1 infection is Acyclovir or similar nucleoside analogs that target virus replication. However, there is no effective treatment to eradicate the virus from the body, or an effective vaccine against HSV-1. Moreover, the therapeutic effectiveness is frequently compromised by the emergence of drug-resistant virus isolates especially in immunocopromised individuals (Eizuru et al., 2003).

Unlike many herpesviruses, HSV has low species specificity and a wide host range. It has the unparalleled ability to infect human and nonhuman cells alike (Spear et al., 2003). The reason behind this successful story of infection is an accumulation of multiple supporting factors. These include:

- Involvement of several multifunctional HSV glycoproteins in entry.

- Existence of multiple alternative receptors. An array of HSV entry receptors for HSV glycoproteins already exists, and evidence suggests even more unidentified HSV receptors.
- Multiple entry modes. HSV has the ability to enter into host cells by direct fusion with the plasma membrane, or via endocytic pathways. The latter can be pH dependent or independent.
- Multiple spread strategies of HSV, including: HSV mediated cell-to-cell fusion, lateral cell-to-cell spread, and transmission of free virions.

## 1.2 HSV Structure

The mature infectious HSV consists of four components from the core outward: an opaque dense core that contains linear double stranded deoxyribonucleic acid (DNA) (approximately 152 kB), encoding at least 74 genes (McGeoch et al., 2006). HSV genome is encapsulated within an icosahedral capsid that consists of 162 capsomeres with six different viral proteins (VPs) present on the surface (Diefenbach et al., 2008). The capsid is surrounded by a protein layer called the tegument that contains 22 VPs. Finally, an outer envelope that contains 16 membrane proteins, including 12 different proteins that contain oligosaccharide chains (glycoproteins). These glycoproteins are of particular importance for virus entry since their interactions with the host cell surface proteins mediate HSV entry into the cell. These glycoproteins are: gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, and gN (Diefenbach et al., 2008, Mettenleiter et al., 2004, Campadelli-Fiume et al., 2000). Figure 1 shows a schematic of HSV-1 structure.

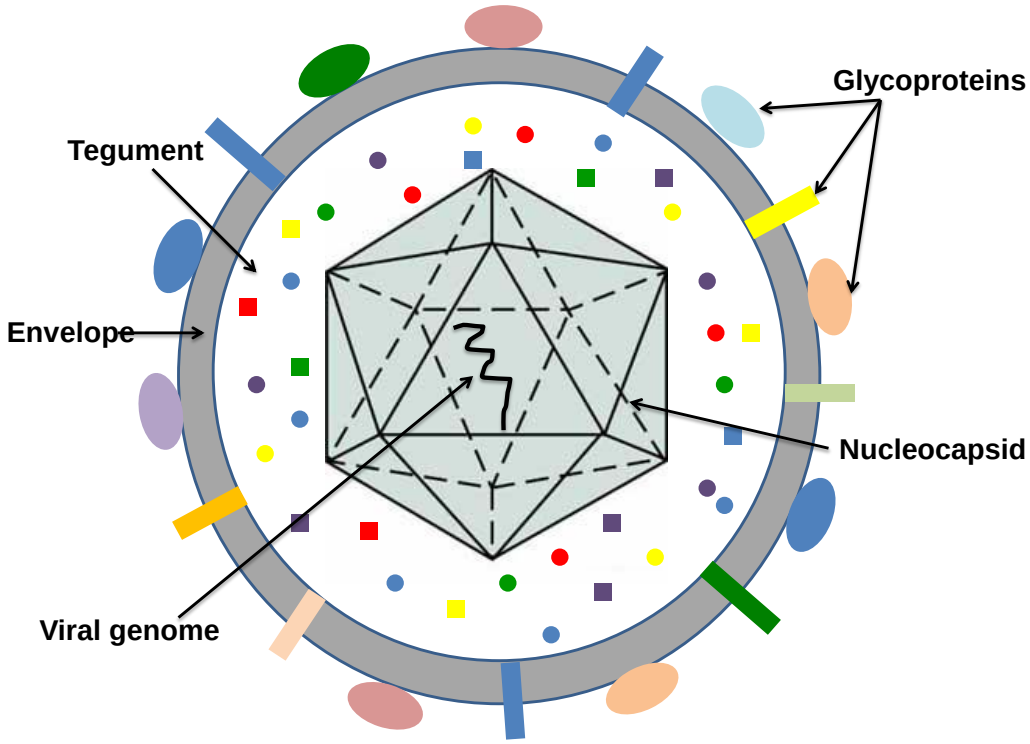


**Figure 1.** HSV-1 structure.

Some of these glycoproteins have been found to exist as heterodimers including the heterodimers gH-gL and gE-gI. Many associate with each other, and have the potential to function as oligomeric complexes (Handler et al., 1996). In addition, these glycoproteins are suggested to have distinct size, morphology and distribution in the viral envelope, based on studies that have used the electron microscope, and monoclonal antibodies against the viral glycoproteins gB, gC, and gD. Accordingly, gB forms the most prominent spikes that are about 14 nm long with a flattened T-shaped top, invariably clustered in protrusions of the viral envelope. While gC was up to 24 nm long distributed randomly, and widely spaced. gD seemed to be 8-10 nm long, clustering in a distinct irregular pattern (Stannard et al., 1987).

### 1.3 HSV-1 Lytic Life Cycle

HSV-1 life cycle (Fig. 2) starts when the virion attaches to the host cell plasma membrane and binds to cell surface receptors through its glycoproteins. Virions penetrate the host cell by the fusion of the viral envelope with cell plasma membrane. The envelope becomes part of the plasma membrane of the cell, and the nucleocapsid is released to the cytoplasm. Nucleocapsids are then transported through the cellular cytoskeleton to the nuclear pore of the nuclear membrane for viral transcription and DNA replication (Ojala et al., 2000, Nagel et al., 2008). Viral DNA is then released from the capsid and enters the nucleus, where it circularizes. One tegument protein that is released from the virion stops protein synthesis of the host cell. While another tegument protein VP16 is transported to the nucleus and serves as a transinducing regulatory protein. VP16 induces the transcription of immediate-early (IE) genes which results in the generation of IE messenger ribonucleic acids (mRNAs). These mRNAs are transported to the cytoplasm and get translated into IE proteins, which induce the transcription of early (E) genes.

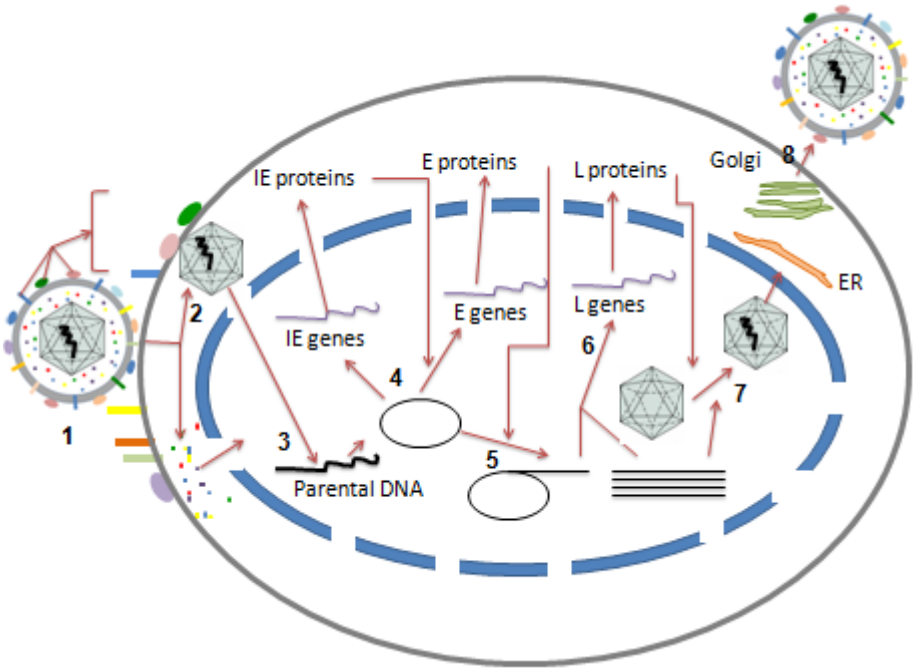


**Figure 2.** HSV-1 lytic replication cycle. This schematic represents the steps of HSV-1 lytic replication cycle in a host cell: (1) Receptor binding and membrane fusion; (2) Release of the viral nucleocapsid and tegument proteins into the cytoplasm of the host cell and transport of the nucleocapsid to the nuclear pore; (3) Release of viral DNA into the nucleus; (4) Transcription and translation of the viral immediate early (IE) and early (E) genes; (5) viral DNA synthesis; (6) Transcription and translation of the viral late (L) genes; (7) capsid assembly and DNA packaging; and (8) egress of progeny virions.

E mRNAs then are translated into E proteins in the cytoplasm. E proteins are involved in DNA synthesis by rolling circle mechanism yielding concatamers or viral DNA. E proteins also induce the transcription of the late (L) genes, which when translated produce viral structural proteins. Nucleocapsids are then generated by the assembly of capsids proteins and the packaging of viral DNA into these capsids (Liesegang, 1992).

Two Scenarios have been suggested to describe the egress of the viral capsid from the nucleus. First Scenario is the single envelopment hypothesis. In this hypothesis, capsids get enveloped at the inner nuclear membrane, and transported within the exocytic or secretory pathway until the virion-containing vesicle fuses with the plasma membrane, where the virion is released. Virions released from this pathway maintain the tegument acquired in the nucleus in addition to the envelope acquired from the inner nuclear membrane, but the oligosaccharides of the viral glycoproteins get modified in the secretory compartments (Nagel et al., 2008, Campadelli-Fiume 2007b).

The second Scenario is the deenvelopment-reenvelopment hypothesis. This hypothesis suggests that progeny capsids get enveloped at the inner nuclear membrane, then undergo a deenvelopment step when fuse with the outer nuclear membrane or the endoplasmic reticulum (ER), which is a continuation of the nuclear membrane, releasing cytosolic capsids. These capsids then acquire tegument from the cytoplasm, and bud into a Golgi-derived compartments. When a capsid buds from these compartments, it undergoes reenvelopment by acquiring membranes from these compartments, and it is released with a vesicle surrounding it. Mature virions then exit the cell by the fusion of the vesicle surrounding the virion with the cell plasma

membrane (Mettenleiter et al., 2006b, Nagel et al., 2008, Campadelli-Fiume 2007b). Although both scenarios have their supporters, recently, there has been an increased support and favor to the second scenario (deenvelopment-reenvelopment) (Mettenleiter et al., 2006a, Campadelli-Fiume 2007b).

### 1.4 HSV Entry

HSV entry into host cell is a multistep process (Fig. 3) that is a result of fusion between the viral envelope and a host cell membrane. It is mediated and modulated by the action of seven HSV glycoproteins along with their interactions with their cognate receptors. These glycoproteins are gB, gC, gD, gH, gK, gL, and gM (Heldwein et al., 2008). However, only four of these glycoproteins (gB, gD, gH, and gL) are necessary and sufficient to allow virus fusion with the plasma membrane of the host cell (Turner et al., 1998, Pertel et al., 2001, Muggeridge et al., 2000, Campadelli-Fiume et al., 2007a).

The first step in HSV entry is the attachment of HSV through the envelope glycoproteins gB and/or gC to heparan sulfate proteoglycans (HSPG) on the surface of the host cell (Spear et al., 2004). The purpose of this interaction is thought to tether the virus to cells in order to concentrate the virus at the cell surface (Connolly et al., 2011). Although gC enhances HSV binding through its interaction with heparan sulfate (HS), it is not essential for entry (Spear et al., 2003). The next step in entry is specific interaction between HSV gD and a gD receptor (Shukla et al., 2001). Several gD receptors have been identified, including: Herpesvirus entry mediator (HVEM), Nectin-1 and nectin-2, and 3-O-Sulfated heparan sulfate proteoglycan (3-OS HS)

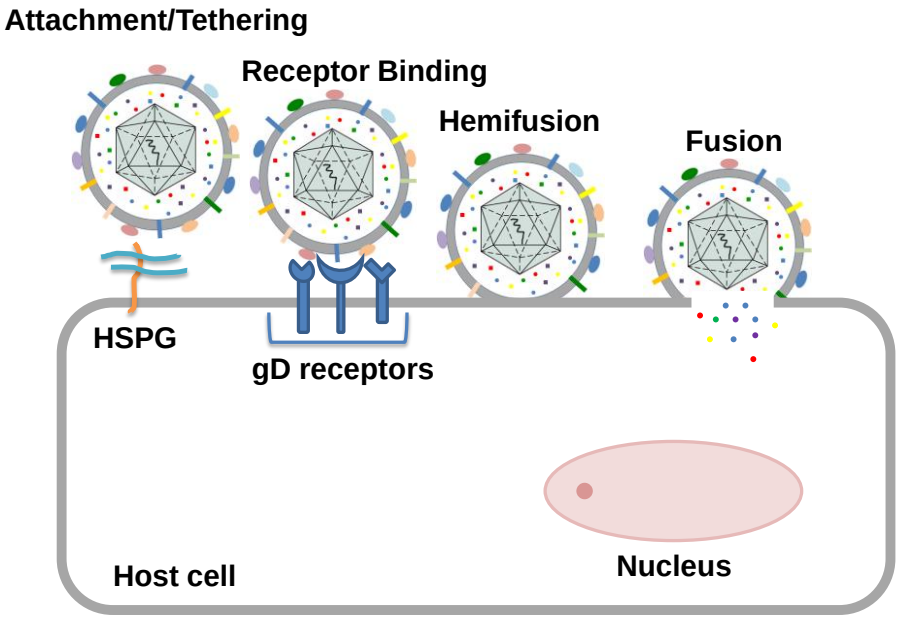


**Figure 3.** HSV-1 Entry. HSV-1 entry is a multistep process. HSV-1 binds to HSPG to tether the virus to the host cell via HSV-1 gB and gC. Next step is the receptor specific binding where gD binds to its receptors. Specific binding triggers conformational changes in HSV-1 fusion glycoproteins mediating membrane fusion through a hemifusion intermediate. This results in full fusion, in which the inner membrane leaflets mix and a fusion pore is formed, resulting in the release of the viral nucleocapsid into the host cell cytoplasm.

(Montgomery et al, 1996, Krummenacher et al., 1998, Geraghty et al., 1998, Warner et al., 1998, Lopez et al., 2000, Shukla et al., 1999). This interaction allows for tight anchoring of the virion particle to the plasma membrane of the host cell, and brings both the viral envelope and the cell plasma membrane into close juxtaposition (Campadelli-Fiume et al., 2000). It is thought that the interaction of gD with one of its receptors triggers a cascade of events that lead to membrane fusion. Structural studies of gD prior to receptor binding and in complex with a receptor suggest that gD undergoes conformational change upon receptor binding, which may transmit an activation signal to gB, and gH/gL leading to membrane fusion. Thus, fusion requires the formation of a multiprotein complex (a fusogenic complex) comprised of gD, gB, and gH/gL (Carfí et al., 2001, Subramanian et al., 2007, Gianni et al., 2009, Atanasiu et al., 2007, Avitabile et al., 2007). A proline-rich region (PRR) of gD has been shown to be important for this process (Fusco et al., 2005). Whether this region becomes exposed to contact gB and gH/gL upon receptor binding, or this region functions as a flexible joint to expose an unidentified region is still unknown.

gD crystal structure reveals that its ectodomain consists of a V-like immunoglobulin (IgV) core that is wrapped by two topologically and structurally distinct extensions: N-terminus which has the receptors binding sites, and the C-terminus that has a domain required for triggering viral membrane fusion (Carfí et al., 2001, Whitbeck et al., 1999, Zago et al., 2004). Various gD receptors bind distinct binding sites on the N-terminus (Whitbeck et al., 2001, Yoon et al., 2003, Manoj et al., 2004). Soluble gD has been shown to be sufficient to allow the entry of gD-null virus into the host cell (Cocchi et al., 2004). In addition, soluble forms of gD receptors have been also shown to be sufficient to allow wt virus entry into cells lacking gD receptors

(Kwon et al., 2006). These observations suggest that gD binding to its receptor is important to modify gD so that it can trigger fusion. A number of studies support the idea that the C-terminus of gD binds to the N-terminus resulting in an autoinhibitory closed conformation. gD receptor binding results in conformational change where the C-terminus is displaced adopting an open conformation, and thus activating the fusion machinery (Fusco et al., 2005, Krummenacher et al., 2005).

Although gB does not promote membrane fusion by itself, its crystal structure reveals that gB shares some properties with other class I and class II fusion proteins. gB belongs to a newly defined class of fusogens: class III. It is a multidomain trimmer that is suggested to undergo a complex and ordered refolding process to drive fusion (Heldwein et al., 2006). Currently solved gB structure is predicted to represent the post-fusion conformation of the protein (Lin et al., 2007). It possesses five domains and two linker regions in each protomer of the trimeric ectodomain: (Domain I) has the fusion loop, (domain II) is located in the middle, (domain III) is an α-helical coiled coil that represents the core of the protein, (domain IV) is the crown domain that has the epitopes for HSV specific neutralizing antibodies, and (domain V) is the arm domain that consists of a long extension spanning the full length of the protomer and contact to the other two protomers (Connolly et al., 2011, Lin et al., 2007, Hannah et al., 2009, Galdiero et al., 2008). The long linker regions are suggested to allow gB conformational change during fusion.

Recently, several gB receptors have been identified including paired immunoglobulin-like type 2 receptor-α (PILRα), non-muscle myosin heavy chain IIA (NMHC-IIA), and myelin-

associated glycoprotein (MAG). Expression of PILRa in HSV resistant Chinese Hamster Ovary (CHO) cells renders these cells susceptible to the virus. Moreover, treating susceptible cells with anti-PILRa or anti-HVEM blocked HSV-1 infection, indicating that both gB receptor and gD receptor are required for HSV infection (Satoh et al., 2008). MAG confers susceptibility to HSV-1 in MAG-transfected promyelocytes (Suenaga et al., 2010). Moreover, NMHC-IIA has been shown to physically interact with HSV-1 gB, and mediate HSV-1 infectivity both *in vitro* and *in vivo* (Arii et al., 2010). The discovery that gB interaction with its receptor is capable of triggering fusion is of great interest, as it differs from the current model where gD is the receptor binding glycoprotein and gB is strictly a fusion protein (Connolly et al., 2011).

Some investigators suggest gH/gL to have a fusogenic activity by initiating hemifusion (Subramanian et al., 2007). However, a recently resolved crystal structure of HSV-2 gH/gL revealed that gH/gL structure does not resemble any known viral fusogen (Chowdary et al., 2010). Moreover, a recent study, using cell fusion assay demonstrated that gD, a gD receptor and gH/gL heterodimer are unable to induce hemifusion formation (Jackson et al., 2010). Consistent with these results, a model was proposed where conformational changes in gD, upon its receptor binding, enable it to activate the heterodimer gH/gL into a form that binds to and activates gB fusogenic activity (Atanasiu et al., 2010). Thus gH/gL is suggested to act as a regulator of the fusion process by gB rather than a fusogenic glycoprotein (Atanasiu et al., 2010). The interaction of the heterodimer gH/gL with gB does not seem to require the presence of these glycoproteins on the same membrane; since cell-cell fusion has been reported when gB and gH-gL were expressed in trans on different cells (Atanasiu et al., 2010).

## 1.5 HSV-1 Spread

HSV-1 may infect cells by multiple spread strategies, including: HSV-1 induced cell-to-cell fusion, lateral cell-to-cell spread, and transmission of free virions. These HSV-1 transmission strategies are discussed below:

### 1.5.1 HSV-1 Induced Cell-to-Cell Fusion

HSV-1 induced cell-to-cell fusion is a process that resembles the virus membrane fusion. It involves the fusion of plasma membrane of an infected cell with that of a neighboring uninfected cell as a way for virus spread (O'Donnell et al., 2008). Upon virus entry, viral glycoproteins are expressed on the surface of infected cells. This allows the binding and fusion of the viral glycoproteins on the surface of infected cells with neighboring uninfected cells that express the receptors of HSV-1 glycoproteins, forming large multinucleated cells called syncytia (Fig. 4) (Akhtar et al., 2009). HSV-1 mediated cell-to-cell fusion has been observed *in vivo* through the detection of syncytia in herpetic corneal lesions (Farhatullah et al., 2004). Recurrent herpetic corneal lesions may result in scar formation which might lead to significant vision loss, and possibly blindness.

The requirements for virus induced cell-to-cell fusion are the same as those for virus entry: the glycoprotein gB, gD, gH, and gL, as well as a gD receptor (Pertel et al., 2001). Cell-to-cell fusion allows the virus spread into surrounding cells without the need to be released

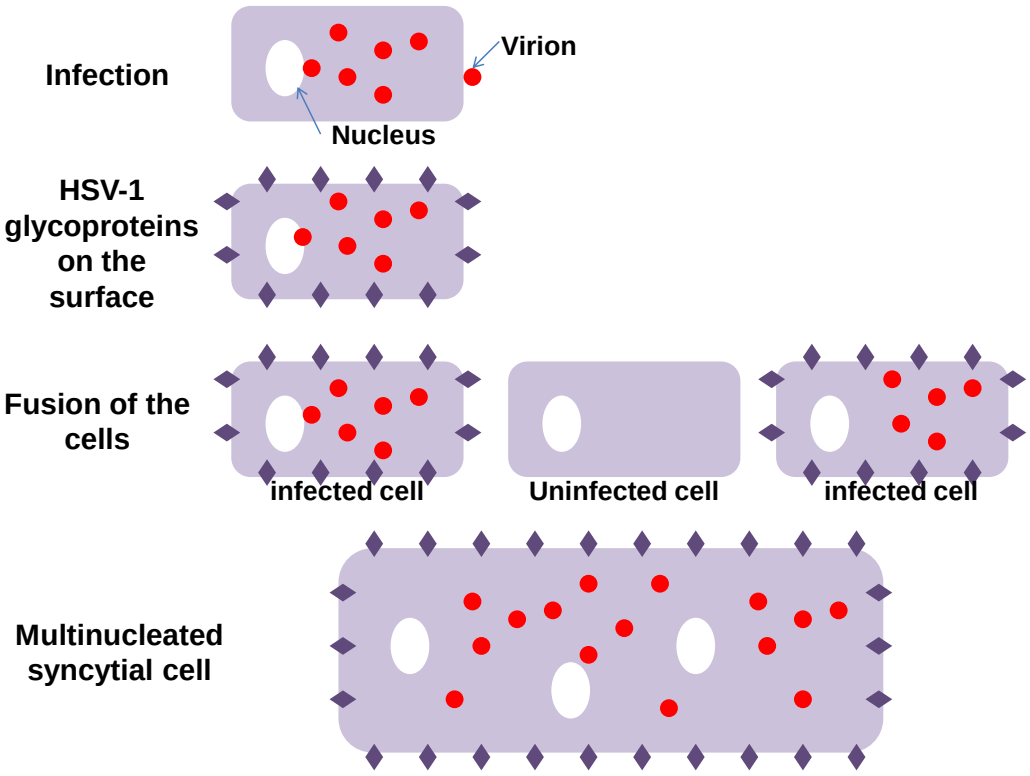


**Figure 4.** HSV-1 mediated cell-cell fusion. Upon virus entry, viral glycoproteins are expressed on the surface of the host cell (viral particles: red circles, viral glycoproteins: purple diamonds). This allows the binding and fusion of the viral glycoproteins (minimum requirement for fusion: gB, gD, gH-gL) on the surface of infected cells with neighboring uninfected cells that express HSV-1 glycoproteins' receptors (minimum requirement: a gD receptor), forming large multinucleated cells called syncytia.

outside the cell, allowing efficient transmission and escaping the host immune system (Even et al., 2006). Other glycoproteins have been shown to be involved in this process, but yet not required, including gE, gI, gM and gK. While gE, gI, and gM are needed for efficient virus mediated cell-to-cell fusion, gK has an inhibitory effect on cell fusion (Avitabile et al., 2003, Davis-Poynter et al., 1994). The spread of HSV-1 is relatively poorly understood and virtually nothing is known about the role of HSPGs in this process.

### 1.5.2 HSV-1 Cell-to-Cell Spread

HSV cell-to-cell spread involves transition of HSV virions across cell junctions, escaping the effects of virus-neutralizing antibodies (Johnson et al., 2001). HSV-1 spread from cell-to-cell requires the same requirements as HSV-1 initial entry into a host cell, which are: HSV-1 glycoproteins gB, gD, gH, and gL, in addition to a gD receptor (Mettenleiter et al., 2003). Other HSV-1 glycoproteins have been shown to be important for virus cell-to-cell spread, including the heterodimer gE-gI, and gK. During HSV infection, the heterodimer gE-gI participates in sorting HSV particles to cell junctions enhancing virus spread, while only few virions reach apical surfaces of polarized epithelial cell (Johnson et al., 2001). gK has been shown to be important for efficient viral replication and spread in the corneal epithelium and trigeminal ganglia neuroinvasion in mice (David et al., 2008). Importantly, Mutant HSV-1 gB that lacks a lysine-rich (pK) sequence (residues 68 to 76) that is suggested to be responsible for gB interaction with HS resulted in the formation of smaller HSV-1 plaques compared to wt virus. This suggests that HSPG binding to HSV-1 gB is required for efficient virus spread from cell-to-cell (Laquerre et al., 1998).

### 1.5.3 Transmission of Free Virions

HSV-1 is capable of spreading by exiting one cell, and infecting another cell as a free virion. The requirements of free virion transmission are the same as the requirements of virus entry (Pertel et al., 2001, Jenssen et al., 2005, Mettenleiter 2003). The release of infectious virus from infected cells is an important virus spread mechanism to reach uninfected sites. In addition, asymptomatic virus release from infected sites is a significant risk for transmission between hosts (Sacks et al., 2004). Viral culture studies have shown that HSV-1 was shed in the oral cavity 6% of the time (Sacks et al., 2004). Interestingly, HSV-1 asymptomatic shedding rate increases with immunosuppression (Sacks et al., 2004).

## 1.6 Heparan Sulfate Role in HSV-1 Infection

HS is a linear sulfated polysaccharide consisting of alternating uronic acid and N-acetylglucosamine residues. The polysaccharide chains are modified by sulfation, epimerization and N-acetylation at various sites making HS structurally heterogeneous (Kato et al., 1994, Gallagher et al., 1986). HS is abundantly expressed on the surface of almost all cell types as HSPG, where HS chains are covalently attached to a core protein via a trisaccharide link on a serein residue (Fig. 5) (O'Donnell et al., 2008, Barash et al., 2010). HS binds to various ligands including growth factors, cytokines, enzymes, protease inhibitors, extracellular matrix (ECM) proteins, bringing these ligands to closer proximity to the cell surface (Bernfield et al., 1999). By binding to these various ligands, HS regulates many biological processes including morphogenesis, axon guidance, blood coagulation, lipid metabolism, tissue repair, inflammation,

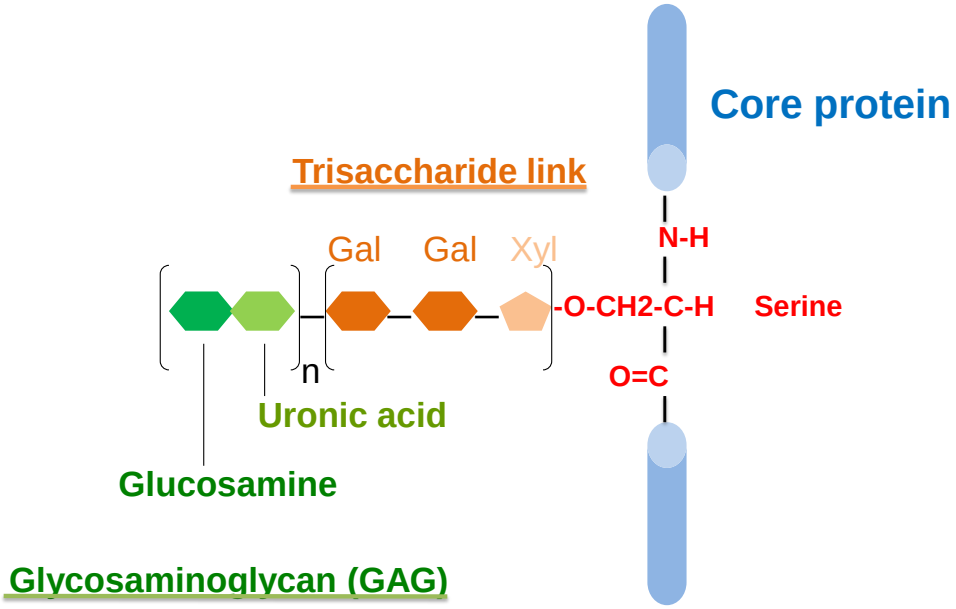


**Figure 5.** Heparan sulfate proteoglycan (HSPG) structure. HSPG are composed of HS polysaccharide side chains that are covalently linked to a core protein via a trisaccharide link on a serine residue. HS polysaccharide side chains consist of alternating uronic acid and N-acetylglucosamine residues that can be repeated n times. The polysaccharide chains are modified by sulfation, epimerization and N-acetylation at various sites making HS structurally heterogeneous.

Vascularization and cancer metastasis (Inatani et al., 2003, Lindahl et al., 1998, Barash et al., 2010).

The highly sulfated HS possesses negative charges making it suitable to interact with the positively charged viral glycoproteins (Trybala et al., 2000). Evidence for this interaction stems from the observations that HSV attachment to cell lines that are defective in HS biosynthesis, but not chondroitin sulfate (CS) biosynthesis is reduced by 85% causing a significant reduction in infectivity in these cell lines (Gruenheid et al., 1993). Moreover, soluble heparin, which is closely related to HS, binds HSV, causing an inhibitory effect on HSV binding to host cells (Nahmias et al., 1964). Enzymatic digestion of HS reduces HSV infection (WuDunn et al., 1989). The viral glycoproteins gB and gC are involved in the attachment to HS. The affinity of gB and gC to HSPG is not the same for HSV-1. HSV-1 gC has more critical role in HSPG attachment during HSV-1 entry compared to HSV-1 gB (Gerber et al., 1995). Although viral attachment to HS enhances the infection, the lack of gC on the viral envelope, or the lack of HS on cells lowers the efficiency of the infection, but does not prevent it (Herold et al., 1994b, Herold et al., 1994a). The lack of gB prevents the infection, primarily because of its critical role during membrane fusion of the virus.

A modified form of HS (3-OS HS) has been shown to serve as an entry receptor for HSV-1 (Shukla et al., 1999). Using soluble 3-OS HS, it has been shown that 3-OS HS is capable of triggering not only virus entry, but also HSV-1 induced cell-cell fusion (Tiwari et al., 2007). Furthermore, the downregulation of a prerequisite for the formation of 3-OS HS; 2-O-sulfation, was found to significantly inhibit HSV-1 binding, entry and virus induced cell-to-cell fusion

(O'Donnell et al., 2010). HS binding can also play a role in the virus's ability to form larger plaques since a mutant virus deleted for a putative HS binding lysine-rich sequence in gB (residues 68-76) showed reduced plaque sizes (Laquerre et al., 1998). Moreover, HS plays negative role in cell fusion; as cell-to-cell fusion increases in the absence of HS on the cell surface (O'Donnell et al., 2009).

### 1.7 Syndecan Family of HSPG

Syndecans are single transmembranous HSPGs with the HS chains covalently attached to the extracellular portion of the core protein (Fig. 6) (Multhaupt et al., 2009). Syndecans family constitutes the most abundant HSPGs expressed on the surface of mammalian cells (Muto et al., 2007, Schofield et al., 1999, Tumova et al., 2000). Four members in the syndecan family have been described in the mammalian cells (syndecan-1 to 4). The syndecan core protein is linearly organized into three regions: the N-terminal ectodomain that is unique for each syndecan, conserved transmembrane domain that supports syndecan oligomerization, and the cytoplasmic domain that consists of two conserved regions and one variable region specific for each syndecan (Multhaupt et al., 2009, Tumova et al., 2000). The ectodomain has HS attachment sites. The cytoplasmic domain binds to several intracellular proteins supporting signaling (Lambaerts et al., 2009). *In vivo* studies have shown that syndecans-1-2-3 and -4 are expressed on specific cell types. For example, syndecan-1 is expressed predominantly in epithelial and mesenchymal tissues, syndecan -2 in cells of mesenchymal origin, neuronal and epithelial cells, and syndecan-3 in neuronal and musculoskeletal tissue, whereas syndecan-4 is expressed in virtually every cell type (Couchman et al., 2003, Tkachenko et al., 2005).

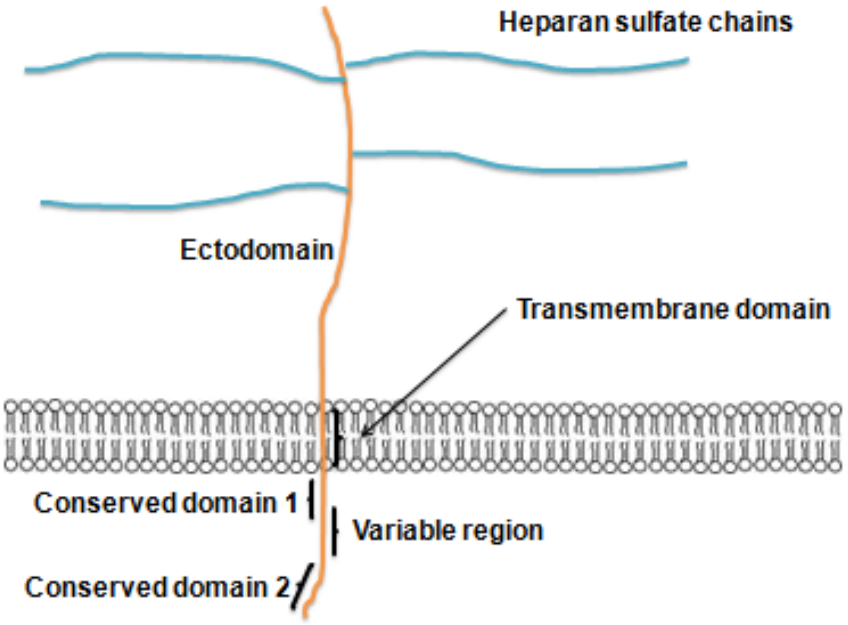


**Figure 6.** Syndecan core protein structure. Four members in the syndecan family have been described in the mammalian cells (syndecan-1 to 4). The ectodomains of the four vertebrate syndecans have several sites for heparan sulfate chain attachment, and is unique for each syndecan. The transmembrane domain is highly conserved among the family members, it supports syndecan oligomerization. The cytoplasmic domains consist of two highly conserved regions (C1 and C2), except for a conservative substitution of arginine for lysine in syndecan-2. C1 and C2 flank a variable (V) region specific for each family member. Cytoplasmic domain binds to several intracellular proteins supporting signaling.

Syndecans on the cell surface modulate ligands activities by acting as a coreceptor, or internalizing physiological extracellular ligands as well as some viruses, bacteria, and other positively charged ligands into the host cell (Lambaerts et al., 2009). This internalization involves clustering of syndecans, followed by endocytosis translocating macromolecular cargo to intracellular vesicles (Wittrup et al., 2009). Proteoglycan-binding ligands have been shown to be efficiently trafficked to late endosomes (Payne et al., 2007). Suggesting the trafficking of the proteoglycans (PGs) to lysosomes where it get degraded with their cargo. An alternative endocytic route is suggested where syndecan can be targeted for plasma membrane recycling (Zimmermann et al., 2005). Syndecans has the ability to function as soluble HSPGs as they undergo proteolytic cleavage at the juxtamembrane position in a process called shedding. This results in the release of the syndecan ectodomain in a soluble form to the extracellular space. Syndecan shedding is a constitutive process as part of the PG normal turnover. However, this process can be accelerated in response to certain stimuli including growth factors, chemokines, bacterial virulence factors, and cell stress (Lambaerts et al., 2009). Syndecan shedding is considered a regulatory mechanism of many processes, because it reduces the levels of HS on the cell surface, and generates soluble ectodomains that have the ability to function as paracrine or autocrine effectors or competitive inhibitors (Manon-Jensen et al., 2010). Multiple signal transduction pathways are involved in the activation of syndecan shedding. These pathways include protein kinase C, protein tyrosine kinase, mitogen-activated protein kinases, and the nuclear factor kappaB pathways (Fitzgerald et al., 2000, Teng et al., 2012).

Syndecans are involved in many diseases including different types of cancers, bacterial and viral infections, as well as, in injuries and wound healing (Fears et al., 2006). They

participate in many events during cell adhesion, migration, proliferation and differentiation, through their interaction with extracellular and cytoplasmic ligands (Tumova et al., 2000). For the purpose of this study, the focus will be on the functions of one member of the syndecan family: syndecan-1. Syndecan-1 binds to many factors that initiate and regulate the inflammatory response. It is involved in leukocyte recruitment, where data suggest that syndecan-1 negatively regulate leukocyte adhesion and migration, probably by inhibiting the interactions between leukocyte integrins and Intercellular adhesion molecule 1(ICAM-1) and Vascular cell adhesion protein 1 (VCAM-1) on endothelial cells (Teng et al., 2012). In addition, syndecan-1 regulates the initiation and activity of chemokines gradients in inflammatory diseases (Teng et al., 2012, Handel et al., 2005, Kuschert et al., 1999). Syndecan-1 has been also shown to have a role in the resolution of inflammation through the shedding of its ectodomain, removing sequestered CXC chemokines (or α-chemokines) (Hayashida et al., 2009). An additional role of syndecan-1 during inflammation is matrix remodeling to restore the normal structure and function of inflamed tissue (Teng et al., 2012).

Another important function of syndecan-1 is its involvement in many important processes of tumorigenesis. This includes a role of syndecan-1 in cancer cell proliferation and apoptosis, where cell surface syndecan-1 and shed syndecan-1 mediate cell growth and survival in certain cancer cells, and yet also stimulate apoptosis in other cancer cells (Maeda et al., 2004, Su et al., 2007, Sun et al., 2008). Syndecan-1 affects tumorigenesis also by mediating cancer cell adhesion to ECM and retarding the migration of cancer cell (Ishikawa et al., 2010). Moreover, syndecan-1 has the ability to bind to pro-angiogenic factors including vascular endothelial growth factor (VEGF), and present these factors to their receptors on endothelial cells, mediating endothelial

cells invasion (Purushothaman et al., 2010). In many of these tumorigenesis processes integrins have been shown to cooperate with syndecan-1 in initiating these events (Purushothaman et al., 2010, Teng et al., 2012).

Syndecan-1 (-/-) mice are viable and fertile, with no major pathologies. However, when syndecan-1 (-/-) mice are challenged with physiological stress, multiple defects appear. These include defected wound healing, angiogenesis, and inflammation (Lambaerts et al., 2009). In addition, syndecan-1 (-/-) mice are protected from tumor development (McDermott et al., 2007). Syndecan-1 has been targeted by many microbial pathogens as a coreceptor, or even direct entry receptor to enter into the host cell. Further, syndecan-1 shedding has been shown to be exploited by some bacteria to inhibit the antibacterial mechanisms and evade the immune system (Teng et al., 2012). Accordingly, syndecan-1 (-/-) mice exhibit significant resistance to certain microbial infections including *P. aeruginosa* following thermal injury, and *S. aureus* in mouse model of scarified corneal infection (Haynes et al., 2005, Hayashida et al., 2011).

## 1.8 Regulation of HSPG by Heparanase

Heparanase is an endo-β-D-glucuronidase that has the ability to cleave HS side chains at a limited number of sites, producing appreciable sized HS fragments that are resistant to further degradation by the enzyme (Ogren et al., 1975). Heparanase cleavage to HS is thought to facilitate the disassembly of the ECM and basement membrane underlying epithelial and endothelial cells, resulting in structural alterations (Fux et al., 2009). Under normal conditions, Heparanase activity is limited to the placenta, skin tissue, and to some blood cells including

neutrophils, monocytes, T-lymphocytes, while epithelial cells express low or undetectable levels of Heparanases (Arvatz et al., 2011). However, Heparanase levels are induced in all major types of human cancer including carcinomas, sarcomas, and hematological malignancies (Fux et al., 2009, Arvatz et al., 2011).

Heparanase is synthesized as a non-active (or latent) precursor (65 kDa), which undergoes proteolytic cleavage, resulting in 50 kDa and 8 kDa protein subunits that heterodimerize to form the active enzyme (Fairbanks et al., 1999). For Heparanase activation, it has to be uptaken by the cell, and delivered to lysosomes, where it undergoes proteolytic processing and activation by cathepsin L (Gingis-Velitski et al., 2004b, Zetser et al., 2004, Abboud-Jarrous et al., 2008). Active Heparanase possesses the ability to cleave HS, which result in the subsequent ECM structural remodeling. This enzymatic activity is implicated in tumor metastasis, angiogenesis, embryo implantation, wound healing, inflammation, and autoimmunity (Barash et al., 2010, Zhang et al., 2011). Latent Heparanase exhibits enzymatic activity-independent functions including facilitating adhesion and migration of primary endothelial cells, as well as mediating phosphorylation of diverse molecules including Src substrates, and the signaling molecules Akt and Src, which affects gene transcription (Barash et al., 2010). These activity independent functions were supported by the identification of the C-terminus of Heparanase as the vital domain for Heparanase signaling function (Barash et al., 2010).

Heparanase has the ability to modulate HSPG beyond the cleavage of HS chains. Heparanase can transform syndecan into highly selective surface interaction protein. For example, an epithelial prosecretory mitogen called lacritin targets syndecan-1 N terminus only if

it has been modified by the removal of HS by Heparanase as a prerequisite for mitogenesis (Ma et al., 2006). In addition, Heparanase uptake has been shown to be mediated by syndecans, where syndecan-1 and -4 have been shown to be internalized and colocalized with Heparanase in endocytic vesicles following the treatment with exogenous Heparanase (Gingis-Velitski et al., 2004b). This suggests that Heparanase mediates syndecan-1 internalization. Moreover, studies have shown that Heparanase mediates syndecan-1 shedding from the cell surface as a way to fine-tune the tumor microenvironment (Yang et al., 2007). It has been suggested that Heparanase induces syndecan-1 shedding by cleaving HS chains, exposing syndecan-1 core protein to the proteases, since mutated forms of Heparanase that lacks HS degrading activity, failed to enhance syndecan-1 shedding (Yang et al., 2007, Lambaerts et al., 2009).

## 1.9 Objectives of This Study

HSV-1 glycoproteins interact with various receptors on the surface of host cell to facilitate its attachment and entry into the cell. Understanding these molecular interactions will help the development of antivirals against HSV-1. HSV-1 interaction with HSPG on the cell surface is critical for HSV-1 infection, as it is involved in virus attachment, and sometimes in entry if HSPG underwent specific modifications within its HS chains. While most studies focused on the role of HS chains on HSV-1 infection, little is known about the contribution of the core protein of HSPG on the infection. The first part of the study is focused on the role of one member of the syndecan family of HSPG: syndecan-1 during HSV-1 infection, particularly, during virus entry and spread (Chapter 3). Strategies including downregulation, and

overexpression of syndecan-1 were utilized. Moreover, a HS-deficient cell line was used to evaluate the role of the core protein of syndecan-1 in HSV-1 spread in HS-independent manner.

HSPG levels on the cell surface are regulated by the activity of Heparanase, which has been shown to affect HSPG internalization, clustering and shedding. It is not known whether these modulations on HSPG would affect HSV-1 infection. Likewise, very little is known about the modulation of HSPG by Heparanase and its resultant effects on HSV-1 infection. The second part of the study focuses on identifying the effects of Heparanase on various aspects of HSV-1 infection (Chapter 4).

Together, this study will enhance our understanding of the role of modulating HSPG, and particularly syndecan-1 on HSV-1 entry, spread and egress from the cell. Moreover, it will provide additional information on how the virus exploits HSPG for efficient viral infection and spread.

# 2. Material and Methods

## 2.1 Cell Culture and Viruses

Wild type CHO cells (CHO-K1), mutant CHO-745 cells, African green monkey kidney cells (Vero) cells and gL expressing Vero cells (79B4) were provided by P. G. Spear (Northwestern University). Human cervical (HELA) cells were provided by B. S. Prabhakar (University of Illinois at Chicago, Chicago, IL, USA). The human corneal epithelial (HCE) cell line (RCB1834 HCE-T) was provided by Kozaburo Hayashi (National Eye Institute, Bethesda, MD) (Araki-Sasaki et al., 1995). All CHO cell lines were grown in Ham's F-12 medium (Gibco/BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin (P/S) (Sigma). Vero, 79B4 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and P/S (Sigma). HCE cells were grown in minimum essential medium (MEM) supplemented with 10% FBS and P/S. Wild type HSV-1 (KOS) virus strain, β-galactosidase expressing recombinant HSV-1 (KOS) gL86, and syncytial plaque forming HSV-1 (KOS) 804 virus were provided by P.G. Spear (Northwestern University). HSV-1 (KOS) K26GFP virus strain was provided by P. Desai (the Johns Hopkins University) Jellyfish green fluorescent protein (GFP) was fused in frame with the UL35 open reading frame generating K26GFP virus whose capsids express GFP (Desai et al., 1998). Virus stocks were propagated and titered on Vero cells, and stored at–80°C.

## 2.2 Plasmids

HSV-1 (KOS) glycoproteins expressing plasmids used were pPEP98 (gB), pPEP99 (gD), pPEP100 (gH), and pPEP101 (gL) (Pertel et al., 1997). Wild type human syndecan-1 (SDC1) and human syndecan-1 mutants including $FcR^{ecto}$hS1 (a chimera comprised of the ectodomain of

human Immunoglobulin G (IgG) Fcγ receptor Ia/CD64 fused to the transmembrane and cytoplasmic domains of human SDC1), hS1$^{\Delta cyto}$ (lacking the 33 C-terminal amino acids) and hS1$^{pLeuTM}$ (transmembrane domain replaced with leucine residues) were provided by Alan Rapraeger (University of Wisconsin-Madison) (McQuade (2003)). Plasmids used for Luciferase reporter assay were pCAGT7 (T7 RNA polymerase) and pT7EMCLuc were described previously (Pertel et al., 1997). Human Heparanase expression plasmid pIRES2 EGFP-HPSE1 and the control empty vector pIRES2 EGFP were provided by Dr. Ralph Sanderson (University of Alabama at Birmingham, Brimingham, AL, USA) (Yang et al., 2007). Other plasmids used including PBG38 (Nectin-1) (Geraghty et al., 2000), plasmid expressing cyan fluorescent protein (CFP) fused to a nuclear localization signal (NLS) from Clontech (Mountain View, CA), and Plasmid expressing red fluorescent protein (RFP)-expressing plasmid fused to a nuclear export signal (NES) (Hu et al., 2003).

## 2.3 Antibodies

The following antibodies were used in these studies: Polyclonal rabbit antibodies (Abs) for syndecan-1 (Santa Cruz Biotechnology, Santa Cruz, CA USA; Catalog #: sc-5632), monoclonal Abs (mAbs) for syndecan-1 (Santa Cruz Biotechnology; Catalog #: sc-12765), mouse anti-β-actin mAb (Sigma-Aldrich; Catalog # A-5316), rabbit polyclonal anti-HSV-1 (Dako; Catalog# N1562), mouse anti-vp16 mAb (Santa Cruz Biotechnology; Catalog #: sc-7545), mouse anti-heparan sulfate mAb 10E4 (US Biological; Catalog# H1890), rabbit polyclonal anti-heparanase (Santa Cruz Biotechnology; Catalog #: sc-25825), horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West

Grove, PA; Catolog# 73102), horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories; Catalog# 115-035-062), FITC-conjugated anti-mouse IgG (Sigma-Aldrich; Catalog# F9137).

## 2.4 Small Interfering RNA (siRNA) Constructs

siRNA constructs used in these studies were all prepared by Sigma-Aldresh: Two siRNA constructs against human syndecan-1 (5´-CCAUUCUGACUCGGUUUCU-3´, 5´-GCCAAGGUUUUAUAAGGCU-3´) (Shimada et al., 2010).

## 2.5 Herpes Simplex Virus Entry Assay

Standard entry assays were used as described previously (Shukla et al., 1999). Cells were grown in 96 well plates until subconfluency. After 16 h cells were transfected with Heparanase expression plasmids using Lipofectamin 2000 (Invetrogen). Media was changed 6 h post transfection. 24 h post transfection, cells were infected in a twofold serial dilution with the recombinant HSV-1 virus expressing β-galactasidase enzyme HSV-1(gL86). Cells were incubated with virus for 6 h at 37°C. Cells were then washed twice with PBS-ABC, and the soluble substrate for the β-galactasidase enzyme o-nitrophenyl-β-d-galactopyranoside (ONPG) was added. Enzymatic activity was measured at 410 nm using an ELISA plate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA).

For entry assay after siRNA treatment, cells were plated in 96 well plates until reaching 40-50% confluency. Cells were mock treated or transfected with syndecan-1, or control scrambled siRNA using Lipofectamine 2000. Media was changes 6 h post transfection. After 48 h, Infection was started as described above.

**2.6 Nuclear transport of VP-16**

50% confluent HeLa cells were either left untransfected or were transfected with either control scrambled siRNA or syndecan-1 specific siRNA. 72 h post transfection, cells were infected with HSV-1 (KOS) at an MOI of 10 for 2 h at 4°C to allow virus binding. Infection was then continued for 30 min at 37°C to allow virus entry. Cells were then washed and treated with citrate buffer (PH = 3.0) for 1 min to inactivate any bound but nonpenetrant virus. Cells were then washed, and overlaid with fresh medium, and incubation was continued for another 2 h at 37°C. Cells were then collected and cytoplasmic and nuclear extracts were prepared using a nuclear extraction kit (Active Motif, Carlsbad, CA, USA, Cat # 40010), and the nuclear transport of VP16 was measured by Western blotting as a marker for HSV-1 entry.

**2.7 Cell-Cell Fusion Assay**

Standard cell-to-cell fusion assay was used as previously described (Tiwari et al., 2004). Cells were split into two populations. "Target" cells were transfected with plasmid expressing Nectin-1 as a gD receptor (1.0 μg) and the luciferase gene (0.5 μg). "Effector" cells were transfected with plasmids expressing HSV-1 glycoproteins gD, gB, gH, and gL and T7 RNA

polymerase (0.5 µg each). After 16 h, target and effector cells were mixed in a 1:1 ratio and replated in 24-well dishes. Luciferase activity was measured after 16 h. As a negative control, target cells were mixed with effector cells that lack HSV-1 gB.

For cell fusion experiment after overexpressing syndecan-1, or syndecan-1 mutants, target or effector cells were additionally transfected with 0.5 µg of a plasmid expressing human syndecan-1 or control GFP plasmid.

For cell fusion experiment after syndecan-1 knockdown, target cells were first either mock treated or transfected with syndecan-1 siRNA. After 24 h, target and effector cells were transfected as described above.

### 2.8 Syncytia Assay

Assay was performed as previously described (O'donnell et al., 2009). Target cells were additionally transfected with 0.5 µg of a plasmid expressing cyan fluorescent protein (CFP) fused to a nuclear localization signal (NLS). Effector cells were also transfected with a red fluorescent protein (RFP)-expressing plasmid fused to a nuclear export signal (NES). Target and effector cells were mixed in a 1:1 ratio and replated in 8-chamber slides (Lab-Tek Corp.). Syncytia images were captured after 72 h using microscopy at the 40× objective on a confocal microscope (Leica DMIRE2) equipped with a camera (Leica TCSSP2). Syncytia size and number were compared after 72 h at the 10× objective (Zeiss Axiovert 200). Number of syncytia was normalized to the number of syncytia detected in the negative control wells where the effector cell population lacks gB.

## 2.9 Immunoblotting

For experiments after syndecan-1 or heparanase overexpression, cells were lysed 24-48 h post transfection. For experiments after syndecan-1 downregulation, cells were lysed 48-72 h post siRNA transfection with cell lysis buffer supplemented with protease inhibitor.

For immunoblotting experiments after HSV-1 infection, cells were infected with HSV-1 at a multiplicity of infection (MOI) of 10 for 2 h at 37°C. Cells were then washed with phosphate buffered saline (PBS) 3×, and complete media was added, and incubation continued. At 8 h and 24 h post infection, cells were lysed with cell lysis buffer supplemented with protease inhibitor.

Western blot assay was performed according to protocols described previously (Shukla et al., 2009). Briefly, Whole cell lysates were denatured in NuPAGE LDS Sample Buffer (Invitrogen, NP0007) and heated to 86 °C for 8 min before gel loading. Equal amounts of protein were subjected to 4-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a nitrocellulose membrane. Nonspecific binding was blocked using 5% nonfat milk in tris buffered saline (TBS) for 2 h at 37°C. The membranes were then incubated with primary rabbit polyclonal antibodies to sydecan-1, or heparanase overnight at 4°C. The blots were rinsed 5 times with 0.1% TTBS (0.1% Tween 20 in TBS) for 5 min followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG. Protein bands were detected using SuperSignal West Femto maximum sensitivity substrate (Pierce, 34096), and visualized using ImageQuant LAS 4000 imager (GE Healthcare Life Sciences). Protein bands were quantified using ImageQuant TL image analysis software (version: 7). For repeated probing, the blots were stripped for 30 min at room temperature with

Restore™ Western Blot stripping buffer (Thermo scientific, 21059). β-actin was measured as a loading control. Syndecan-1, syndecan-1 or heparanase were quantified by calculating the relative intensity of each band to that of β-actin.

**2.10 Flowcytometry**

Assay was performed as described previously (Shukla et al., 2009). Confluent monolayer of cells were washed 2× with PBS, dissociated from the culture dishes using Hank's based disassociation buffer (Gibco), pelleted, and incubated with primary Ab diluted in PBS with 1% Bovine serum albumin (BSA) for 1 h at 4°C. After primary Ab incubation, cells were washed 3× with PBS with 1%BSA, and incubated for 30 min with Fluorescein isothiocyanate (FITC)-conjugated secondary anti-IgGs. Cells stained only with FITC-conjugated secondary anti-IgGs were used as background control. Cells were then examined by fluorescence-activated cell sorter (FACS) analysis.

For flow cytometry experiment to determine syndecan-1 cell surface expression after syndecan-1 overexpression, cells were either mock treated or transfected with human syndecan-1 plasmid for 48 h.

**2.11 Cytotoxicity Assays**

Cytotoxicity assays were performed after syndecan-1, syndecan-1 mutants, or heparanase overexpression, as well as after syndecan-1 siRNA transfection. For overexpression experiments, approximately $1.5\times10^4$-$2.5\times10^4$ cells were plated in 96 well plate. Cells were transfected with syndecan-1, syndecan-1 mutants, heparanase, or control GFP expression plasmids. After 6 h,

complete media (supplemented with FBS and P/S) was added to cells. For downregulation experiments, approximately $1\times10^4$ cells were plated in 96 well plate. Cells were transfected with syndecan-1 siRNA, or control nonspecific scrambled siRNA. After 6 h, complete media was added to cells. 24-48 h post expression plasmid transfection, and 48-72 h post siRNA transfection, growth medium was removed, and 100uL of complete media was added to each well in addition to 20ul of CellTiter 96 Aqueous One solution (Promega, Madison, WI, USA). Plates were incubated at 37°C for 1-4 h. Absorbance was then measured at 490 nm using microplate reader (Spectra Max 190 Molecular Devices, Sunnydale, CA USA). Background absorbance was subtracting from the reading which represent cell-free wells filled only with media and the CellTiter 96 Aqueous One solution.

### 2.12 Plaque Assays

Monolayer of cells in 24 well plates overexpressing syndecan-1, syndecan-1 mutants, Heparanase or GFP control plasmid (0.8 µg using Lipofectamine 2000 reagent), or transfected with syndecan-1 siRNA, or scrambled siRNA, were infected with 10-fold serial dilutions of syncytial plaque forming HSV-1 (KOS) 804 virus stocks. Infected cells were fixed with 100% methanol for 5 min, stained with crystal violet, and plaques were counted with naked eye or at the 10× objective (Zeiss Axiovert 200).

### 2.13 Plaque Size Determination

Monolayer of HCE cells in 4-well chamber slides (Lab-Tek Corp.) overexpressing syndecan-1, syndecan-1 mutants, or GFP control plasmid, or monolayer of HCE cells in 24-well plate transfected with syndecan-1 siRNA, or scrambled siRNA, were infected with 0.1 MOI of HSV-1 (KOS). After 2 h of adsorption at 37°C, inoculums were removed; cells were washed 2×

with PBS. Infected cells were overlaid with 0.5% methylcellulose (sigma). The overlay was removed at 3 day post infection (dpi), and cells were fixed with a 1:1 dilution of methanol:acetone, or 100% methanol. Plaques were stained using rabbit anti-HSV-1 (Dako), horseradish peroxidase-conjugated secondary antibody (Amersham), and 3, 3'-diaminobenzidine tetrahydrochloride (DAB) substrate (Sigma). Plaques were measured with a micrometer at the 10× objective (Zeiss Axiovert 200), and the area was calculated by measuring the outline of each plaque using Axioversion software (version 4). The average area was determined by measuring 22 plaques from each of the two experiments performed in duplicate.

## 2.14 Growth Curves

Monolayers of HCE cells in 6 well plate transfected with syndecan-1 siRNA, or scrambled siRNA, were infected with HSV-1 (KOS) at a multiplicity of infection (MOI) of 0.1. After 2 h of adsorption at 37°C, inoculums were removed, cells were washed 2× with Hanks' balanced salt solution, and growth medium was added back to each culture. At the indicated time points in each experiment post infection, cells were scraped into medium, and cell suspensions were transferred to tubes, sonicated, clarified by centrifugation (800 × *g* for 10 min), and stored at -80º C. Infectious virus in the supernatant was quantified by standard plaque assay on HCE or Vero cell monolayers.

## 2.15 Virus Spread Assay

Virus spread assay was designed using previously described protocols (Roller et al., 1997), Jenssen et al., 2008). Monolayer of HCE cells were exposed to HSV-1 (KOS) or HSV-1

(KOS) K26GFP strains at MOI of 5 at 37 °C to allow virus entry. 2 h post-infection, cells were washed 1× with PBS, and then incubated for 1 min with 0.1M citrate buffer (PH 3.0) to inactivate residual virus particles. Cells were then washed 3× with PBS and overlaid with 0.5% methylcellulose (sigma) MEM medium. After 4 h, cells were washed 3× with PBS, and dissociated using Hanks'-based enzyme free dissociation buffer (Gibco). Approximately 600 cells were plated onto 80% confluent monolayers of uninfected HCE cells that have been transfected with either scrambled siRNA or syndecan-1 siRNA, for 72 h in 0.5% methylcellulose MEM medium. The spread of HSV-1(KOS) from infected cells to the siRNA transfected HCE cells was evaluated by staining and counting the number of plaques formed at the 10 × objectives (Zeiss Axiovert 200). Whereas the spread of HSV-1 (KOS) K26GFP from the infected cells to the siRNA transfected HCE cells was qualitatively assessed by capturing images of the green virus at the 10× objective (Zeiss Axiovert 200).

### 2.16 HSV-1 Egress Assay After Hepraranase Overexpression

HeLa and HCE cells grown in 6-well culture plates were transfected with Heparanase expression plasmid pIRES2 EGFP-HPSE1, or the control empty vector pIRES2 EGFP. After 18 h, HeLa cells and HCE cells were infected with HSV-1 (KOS) at MOI of 0.1 and 0.01 respectively for 2 h at 37°C. Cells were then washed three times with Hanks' balanced salt solution, and growth medium was added back to the cells, and incubation continued at 37°C. At 6, 12, 24, 36, 48, 60, and 72 h post infection, 100 µl of cell culture supernatant were collected, and tittered using Vero cells monolayers in 24 well plates. Each time the supernatant was collected, 100 µl complete medium was add to maintain the total volume of growth media in the wells.

## 2.17 HSV-1 Release After Heparinase Treatment

HeLa and HCE cells grown in 6-well culture plates were infected with HSV-1 (KOS) at MOI of 0.1 and 0.01 respectively for 2 h at 37°C. Cells were then washed 3X with Hanks' balanced salt solution, then treated for 1 min with Citrate buffer (PH=3.0) followed by cells washing. Cells were then treated with either Heparinase II, Heparinase III (15 and 30 IU/ml), or a combination of both heparinases II and III (15 and 30 IU/ml) for 2 h at 37°C. After 2 h, cells were washed, and growth medium was added back to the cells, and incubation continued for 20 h at 37°C. HSV-1 release was measured by titering the supernatant of the cultured cells on Vero cells. For a control, some cells were infected with HSV-1 (KOS), but were not treated with the Heparinases.

## 2.18 HSV-1 Release After Phorbol 12-Myristate 13-Acetate (PMA) Treatment

HeLa and HCE cells were infected at MOI of 0.01 with HSV-1 (KOS) for 2h. After 2 h, cells were washed 3X with Hank's balanced salt solution, and cells were overlaid with complete medium and incubation was continued for 8 h at 37°C. Cells were then washed 3X with serum free medium, and were serum starved for 12 h. After 12 h, cells were treated with PMA in serum free medium for 0.5, 1, 2, and 4 h. After each time point, 100 µl of the cell culture supernatant was tittered on Vero cells monolayer. HSV-1 infected cells treated with dimethyl sulfoxide (DMSO) were used as control.

## 2.19 Statistical Analyses

The data shown are the means ± 1standard deviation (SD) values. Statistical analyses were performed with GraphPad Prism software (version 4.0). Data were assessed using unpaired student's *t* test. $*P < 0.05$ and $**P < 0.0001$ were regarded as significant differences between treated and mock-treated groups.

# 3.AN IMPORTANT ROLE FOR SYNDECAN-1 IN HERPES SIMPLEX VIRUS TYPE-1 INDUCED CELL-TO-CELL FUSION AND VIRUS SPREAD

## 3.1 Rationale

The functions of cell surface HSPGs have been centered on the role of HS chains on the extracellular cell surface, where the PGs were given the passive role of carrying HS chains. More recently, however, the core proteins of different HSPGs have also been shown to be involved in HSPG functions independently of the HS chains including cell signaling (Lopes et al., 2006). They participate in many events during cell adhesion, migration, proliferation and differentiation (Tumova et al., 2000). Although the role of HS as an attachment receptor for HSV-1 has been intensively studied, little is known about the contribution of the core protein that carries the HS chains in the infection. Several families of HSPG have been identified, and one major family is the syndecan family of HSPG (Tumova et al., 2000). It has been shown that two members of the syndecan family of HSPG (syndecan-1 and syndecan-2) play a role during HSV entry into HeLa cells (Bacsa et al., 2011). The downregulation of these syndecans using specific siRNA resulted in a significant reduction in HSV entry and plaque formation. These results were confirmed using antibodies blocking assay, where antibodies against syndecans were capable of inhibiting viral entry (Bacsa et al., 2011). Interestingly, HSV infection resulted in the upregulation of syndecan-1 and syndecan-2 expression on the cell surface and at the protein level (Bacsa et al., 2011). This observation strongly suggests that these HSPGs are involved in the infection, and most probably beyond the attachment step of the infection.

The aim of this study was to investigate the role of syndecan-1 in HSV-1 entry, virus mediated membrane fusion and viral cell-to-cell spread. Syndecan-1 knockdown reduced HSV-1 entry into cells at a step prior to viral nuclear transport, as syndecan-1 downregulation reduced virus entry, but did not affect viral nuclear transport. Moreover, using wild type CHO-K1 cells

and the mutant CHO-745 cells deficient in glycosaminoglycans (GAGs) synthesis (O'Donnell et al., 2009), we show that syndecan-1 is important for HSV-1 induced membrane fusion in HS independent manner. CHO-745 cells have an inactive form of the xylosyltransferase enzyme essential for GAG synthesis. Therefore, these cells express only the core protein of syndecan-1 without any of the GAGs including HS. In addition, using plaque assays performed in methylcellulose, which restricts virus spread through the medium allowing plaque formation due to virus spread from cell-to-cell, we show syndecan-1's role in HSV-1 cell-to-cell spread in HCE cells. HCE cells are natural target for HSV-1 infection. Evidence has shown that syndecan-1 exhibits very strong localization within the corneal epithelium that represents one of the major infection sites for HSV-1 that may precede infection of other sites within the eye (Filla et al., 2004, Farooq et al., 2011, Shah et al., 2010). We also demonstrate that the downregulation of syndecan-1 results in fewer plaques and therefore, less infectious virus production. Overall, our study demonstrates a new role for syndecan-1 in HSV-1 cell-to-cell fusion and spread.

## 3.2 Results

### 3.2.1 Syndecan-1 Overexpression or Downregulation Does Not Affect Cell Viability

In order to understand the significance of syndecan-1 during HSV-1 infection, three cell lines were used to examine its role in different aspects of HSV-1 infection. The cell lines used were the wild type CHO cell line (CHO-K1), a GAG-deficient CHO cell line (CHO-745) (Pertel et al., 2001, Esko et al., 1988), and HCE cells which are prime targets for HSV-1 infection (Shah et al., 2010). The cell lines were subjected to syndecan-1 overexpression or selective downregulation by siRNA. While Human syndecan-1 plasmid was used to enhance syndecan-1

production, two sets of siRNAs were used to selectively knockdown its expression. The downregulation of syndecan-1 in CHO-K1, CHO-745, and HCE cells was confirmed at the protein level using Western Blot analysis. Densitometric analysis showed that treatment with syndecan-1 specific siRNA resulted in a significant decrease (approximately 50%) in protein production, confirming the specific downregulation of syndecan-1 (Fig. 7A). Increase in syndecan-1 level was confirmed using flowcytometric analysis on CHO-K1, CHO-745, and HCE cells, and resulted in approximately 20-25 fold increase. Relative mean fluorescence of syndecan-1 on the surface of CHO-K1, CHO-745, and HCE cells is shown in Fig. 7B. To evaluate whether alterations in syndecan-1 levels affect cell viability, an MTS assay was performed 3-days post syndecan-1 downregulation, and 1-day post syndecan-1 overexpression. Neither the former nor the latter affected cell viability compared to control cells that were either transfected with scrambled siRNA or transfected with the control GFP plasmid (Fig. 7C-D). These results demonstrated that syndecan-1 downregulation and overexpression were successful in CHO-K1, CHO-745, and HCE cells, and neither affected cell viability.

### 3.2.2 Syndecan-1 Knockdown Reduces HSV-1 Entry into HCE Cells.

After verifying syndecan-1 knockdown by siRNA transfection, the effect of reduced syndecan-1 on HSV-1 entry into HCE cells was examined. HCE cells were transfected with either scrambled siRNA or syndecan-1 specific siRNA. 72 h post transfection, cells were infected with the recombinant β-galactosidase expressing HSV-1 (KOS) gL86 reporter virus. HSV-1 entry was measured after 6 h of viral infection. As shown in Figure 8, a significant, 36.6±6.8% inhibition

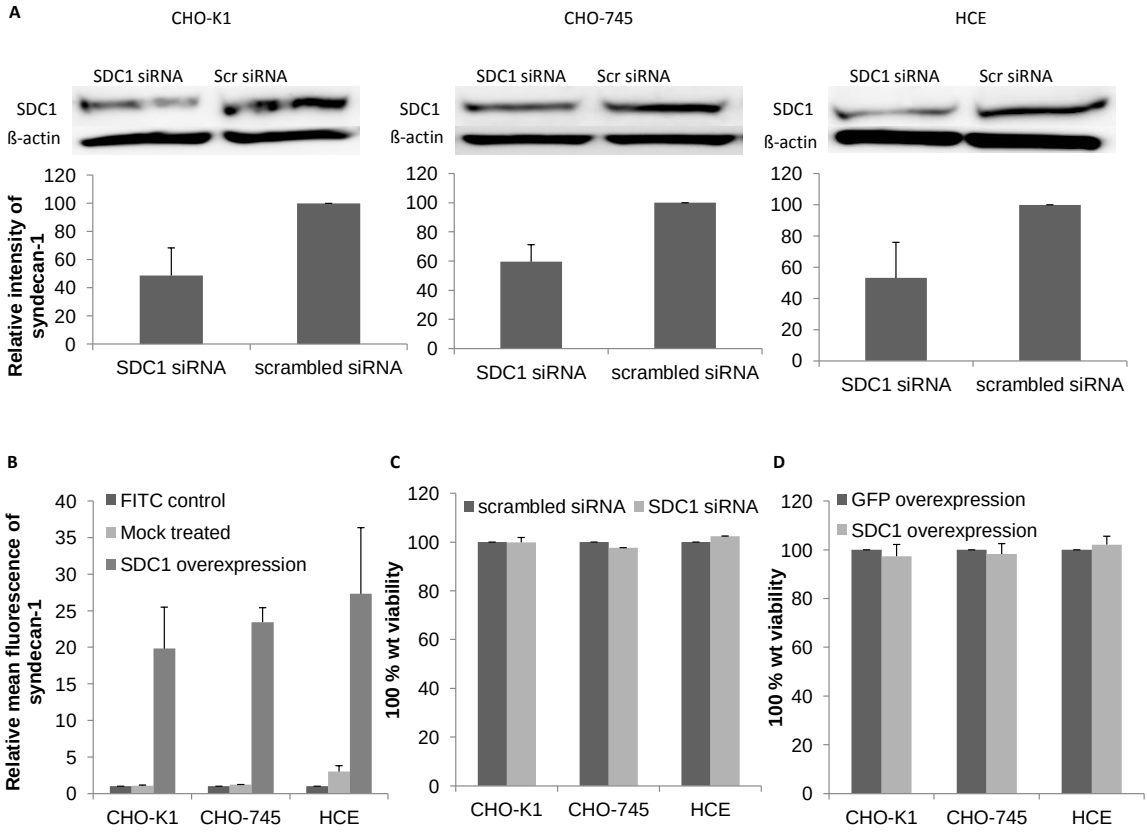


**Figure 7.** Syndecan-1 knockdown or overexpression do not affect cell viability. (A). CHO-K1, CHO-745, and HCE cells were transfected with scrambled (scr) siRNA or syndecan-1 (SDC1) siRNA. 72-96 h after transfection, immunoblots of cell lysates were prepared and probed with anti-SDC1 polyclonal Ab. β-actin protein expression was measured as loading control. Representative blots are shown. Protein bands were quantified using ImageQuant TL image analysis software (version: 7). SDC1 protein expression (mean ± 1SD), normalized to that of β-actin, of at least three independent experiments was quantified by calculating the relative intensity of each syndecan-1 band relative to the control scrambled siRNA treated bands, and presented as bar graph. (B) Cells were grown in 6-well plates, mock treated or transfected with human SDC1 plasmid for 48 h. Cell surface expression of SDC1 was evaluated by flowcytomety. FITC stained cells were used as background control. Results are representative of two independent experiments (C, D). Cells were grown in 96-well plates, transfected with scrambled siRNA or SDC1 siRNA for 48 h (C), or transfected with control GFP plasmid or human SDC1 plasmid for 24 h (D). Triplicate wells were evaluated for cell viability using MTS assay. Results are expressed as 100% wild type (wt) viability where they represent the percent corrected absorbance after subtracting the background absorbance, relative to scrambled siRNA transfected cells (C), or relative to GFP transfected cells (D), and are mean ± 1SD of at least 2 independent experiments.

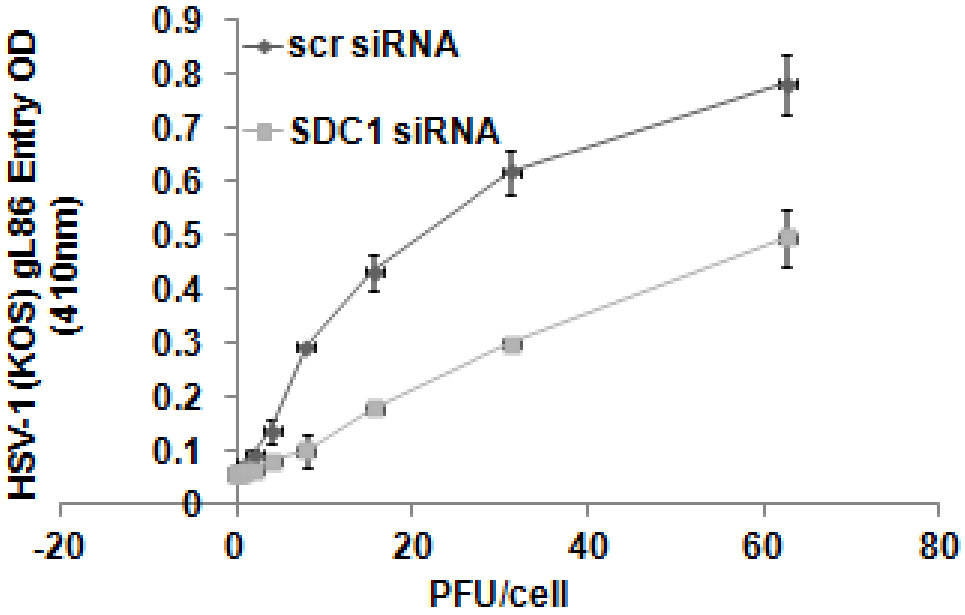


**Figure 8.** Syndecan-1 knockdown reduces HSV-1 entry into HCE cells. 50% confluent HCE cells were transfected with either control scrambled siRNA or syndecan-1 specific siRNA. 72 h post transfection, cells were infected with a twofold serial dilution of the recombinant HSV-1 virus expressing β-galactasidase enzyme HSV-1(gL86). Cells were incubated with virus for 6 h at 37°C. Cells were then washed twice with PBS-ABC, and the soluble substrate for the β-galactasidase enzyme o-nitrophenyl-β-d-galactopyranoside (ONPG) was added. Enzymatic activity was measured at 410 nm using an ELISA plate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA). Results are representative of two independent experiments. Values in the figure were plotted as the mean of three determinations (± 1 SD).

of HSV-1 entry was observed in cells transfected with syndecan-1 siRNA compared to HCE cells transfected with the control scrambled siRNA.

### 3.2.3 Syndecan-1 Knockdown Does Not Affect Viral Nuclear Transport.

Since syndecan-1 knockdown reduces HSV-1 entry into cells, we sought to investigate if the effect of knocking down syndecan-1 on HSV-1 entry is a result of a defective viral nuclear transport. The nuclear transport of VP16, a tegument protein, following syndecan-1 knockdown was evaluated by preparing Western blots of nuclear and cytoplasmic extracts. As controls, non transfected, and scrambled siRNA transfected cells were used. The results demonstrate that there was no defect in VP16 nuclear transport after knocking down syndecan-1 (Fig. 9). The vast majority of VP16 appeared in the nuclear extract but not in the cytoplasmic extract in non transfected, scrambled siRNA transfected, as well as syndecan-1 siRNA transfected cells, suggesting successful nuclear transport of VP16. Although in all tested conditions a fixed virus MOI was used (MOI=10), syndecan-1 siRNA transfected cells showed smaller VP16 band compared to the controls. This correlates with the reduced virus entry after syndecan-1 downregulation observed in Figure 8. These results suggest that the down regulation of syndecan-1 does not affect viral nuclear transport.

### 3.2.4 Increase in Syndecan-1 Level on Target Cells Promotes, and its Loss on Target Cells Inhibits HSV-1 Induced Cell-to-Cell Fusion

HSV-1 induced cell-to-cell fusion can be studied by co-cultivating two populations of cells: “Target” and “Effector” cell populations. Target cells express gD receptor and the luciferase reporter gene under the control of T7 promoter. Effector cells express HSV-1 glycopr-

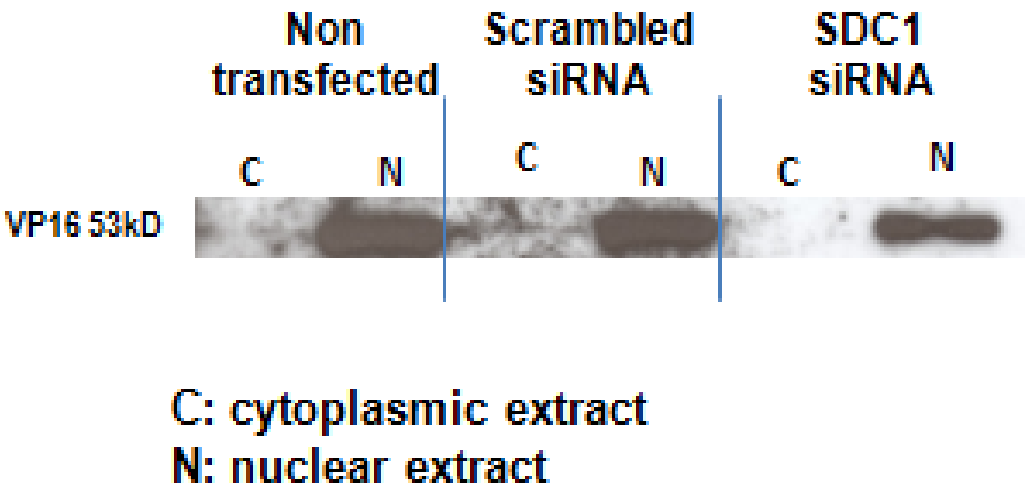


**Figure 9.** Syndecan-1 does not affect HSV-1 nuclear transport. 50% confluent HeLa cells were either left untransfected or were transfected with either control scrambled siRNA or syndecan-1 specific siRNA. 72 h post transfection, cells were infected with HSV-1 (KOS) at an MOI of 10 for 2 h at 4°C to allow virus binding. Infection was then continued for 30 min at 37°C to allow virus entry. Cells were then treated with citrate buffer (PH = 3) for the removal of bound virus, and incubation was continued for another 2 h at 37°C to allow synchronized virus entry. Cells were then collected and cytoplasmic and nuclear extracts were prepared, and the levels of VP16 were measured by Western Blotting as a marker for HSV-1 entry.

-roteins that are absolutely required for virus fusion (gB, gD, gH, and gL) plus T7 polymerase (Pertel et al., 2001). Luciferase reporter gene activity is determined 16 h post mixing the two populations to quantify cell-to-cell fusion. As a negative control, target cells are mixed with effector cells that lack HSV-1 gB, where cell-to-cell fusion is expected to be reduced dramatically because of the absence of one of the four absolutely required HSV-1 glycoproteins for cell-to-cell fusion; gB (Fig. 10A). Using this co-cultivating system, we aimed to understand the contribution of the core protein of syndecan-1 during HSV-1 induced cell-to-cell fusion. Two cell lines were used, the wild type CHO-K1 and a GAG-deficient CHO cell line CHO-745 that has an inactive form of xylosyltransferase enzyme, which is required for the initiation of GAG chain by transferring xylose to the GAG core protein (Pertel et al., 2001, Esko et al., 1988). We have previously shown that HS plays negative role in cell fusion; as cell-to-cell fusion increases in the absence of HS on the cell surface (O'Donnell et al., 2009). Thus using CHO-745 cells enables the elimination of the effect of HS chains on cell fusion. Enhancement of syndecan-1 on target CHO-K1 and CHO-745 cells resulted in a significant increase (37.9±6.9% and 34.5±9.9% increase respectively $P < 0.0001$) in cell-to-cell fusion compared to wild type cells that were transfected with GFP control plasmid (Fig. 10B). Moreover, Syndecan-1 downregulation on target CHO-K1 and CHO-745 cells inhibited HSV-1 induced cell-to-cell fusion (41.3±9.5% and 43.8±4.3% inhibition respectively $P < 0.0001$) compared to wild type cells that were transfected with control scrambled siRNA (Fig. 10C). These results suggest that syndecan-1 may play a role during HSV-1 induced cell-to-cell fusion, and this role is independent of HS chains.

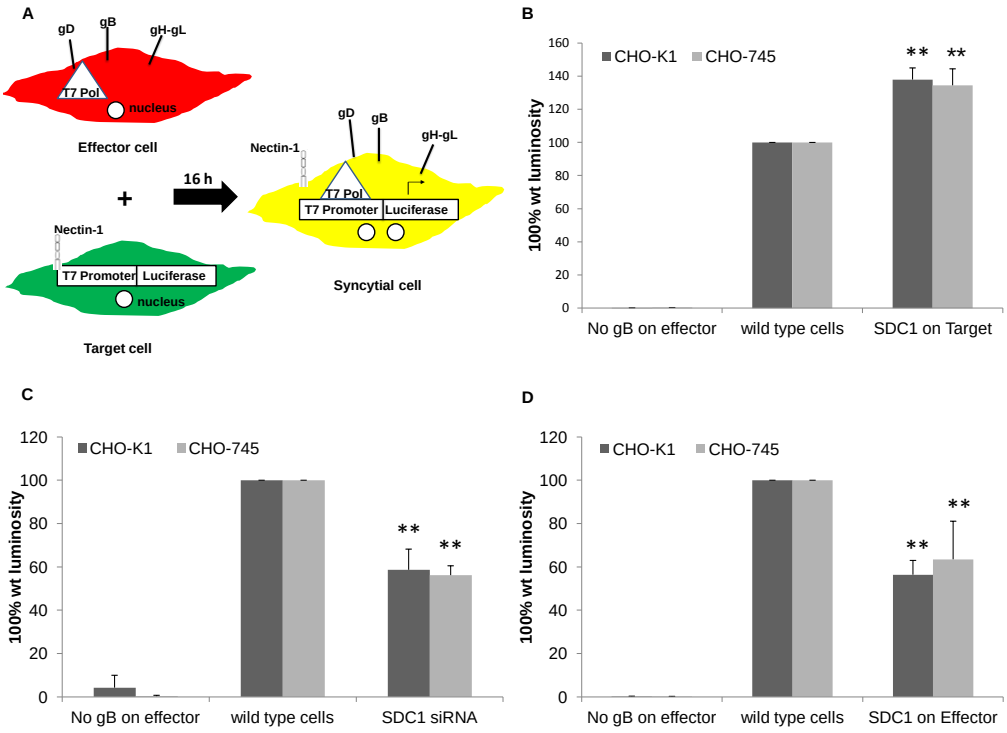


**Figure 10.** Syndecan-1 overexpression on target cells enhances cell fusion, while its overexpression on effector cells inhibits cell fusion. (A). An illustration of cell fusion assay that was exploited to understand the contribution of syndecan-1 during HSV-1 induced cell-to-cell fusion. Effector cell population that expresses HSV-1 fusion glycoproteins plus T7 polymerase is mixed with the target cell population that expresses nectin-1 as a gD receptor and the luciferase reporter gene under the control of T7 promoter. Luciferase reporter gene activity is determined to quantify cell-to-cell fusion. (B). Target cells for CHO-K1 and CHO-745 cells were either transfected with GFP control plasmid (wild type cells) or transfected with syndecan-1 plasmid and mixed with effector cells 24 h post transfection. As a negative control, target cells were mixed with effector cells that lack HSV-1 gB. (C). Target cells for CHO-K1 and CHO-745 cells were transfected with either control scrambled siRNA (wild type cells) or syndecan-1 specific siRNA and mixed with effector cells 48 h post transfection. As a negative control, target cells were mixed with effector cells that lack HSV-1 gB. (D). Effector cells for CHO-K1 and CHO-745 cells were transfected with either GFP control plasmid (wild type cells) or human syndecan-1 plasmid and mixed with target cells 24 h post transfection. As a negative control, target cells were mixed with effector cells that lack HSV-1 gB. (B, C, D). Fusion was measured 16 h post mixing. Results are presented as mean ± 1 SD of at least 3 independent experiments. *SDC1*, syndecan-1

### 3.2.5 Enhanced Syndecan-1 on Effector Cells Inhibits Cell-to-Cell Fusion

The fusion of the virus envelope with the host cell plasma membrane results in the expression of the virus glycoproteins along with the cellular proteins on the cell surface including syndecan-1. To understand the effect of an increase in syndecan-1 level with the virus glycoproteins on the same cell, we overexpressed syndecan-1 on the effector cell population that also expresses HSV-1 fusion glycoproteins. Interestingly, syndecan-1 overexpression on effector CHO-K1 and CHO-745 cells inhibited HSV-1 induced cell-to-cell fusion significantly compared to wild type cells that were transfected with GFP control plasmid ($P < 0.0001$) (Fig. 10D). This result was observed in CHO-K1 cells (43.6±6.0% inhibition) as well as CHO-745 cells (36.4±17.5% inhibition) that lack all GAGs including HS, suggesting that the observed inhibition is HS independent.

### 3.2.6 Enhancement of Syndecan-1 Production in Target Cells Induces the Syncytial Cell Formation, Whereas the Same on Effector Cells Inhibits Syncytial Cell Formation and Reduces the Average Number of Nuclei Per Syncytia

HSV-1 induced cell-to-cell fusion results in the formation of large, multinucleated syncytial cells (O'Donnell et al., 2008). To compare the number and size of syncytia after overexpressing syndecan-1 on target or effector cells, a cyan fluorescent protein (CFP) construct attached to a nuclear localization signal (NLS) for limiting the CFP to the nuclei was additionally transfected into target cells. Likewise, the effector cells were also additionally transfected with a red fluorescent protein (RFP) attached to a nuclear export signal (NES), limiting the expression of RFP to the cytoplasm (O'Donnell et al., 2009, Hu et al., 2003). Syncytia were then identified

as cells expressing red cytoplasm and at least one blue nucleus (Fig. 11A). The *top panels* show representative syncytia formed in CHO-K1 cells after overexpressing syndecan-1 on target or effector cells. The *bottom panels* show representative syncytia formed in CHO-745 cells after overexpressing syndecan-1 on target or effector cells. The positive controls consist of target cells mixed with effector cells where both populations express normal levels of syndecan-1. The negative controls consist of target cells mixed with effector cells missing gB, and thus no syncytia formation is observed (Fig. 11B).

Table 1 lists the average number of syncytia formed in CHO-K1 and CHO-745 cells in each condition and the size of syncytia formed indicated by the average number of syncytia that had 2 nuclei, 3-5 nuclei, or more than 5 nuclei. Table 1 shows that overexpressing syndecan-1 on target CHO-K1 or CHO-745 cells formed a significantly greater number of syncytia than the positive control that has target and effector cells expressing normal levels of syndecan-1 ($P < 0.05$). However, overexpressing syndecan-1 on effector CHO-K1 or CHO-745 cells formed a significantly smaller number of syncytia compared to the positive control ($P < 0.05$). The number of nuclei per syncytia cell was also examined. Overexpressing syndecan-1 on target cells did not result in a significant increase in the number of nuclei per syncytial cell. However, when syndecan-1 was overexpressed on effector cells, syncytia formed had significantly fewer nuclei per syncytial cell ($P < 0.05$).

### 3.2.7 The Ectodomain and the Cytoplasmic Domains of Syndecan-1 are Important for Inhibiting HSV-1 Induced Cell-to-Cell Fusion When Syndecan-1 Co-Exists with HSV-1 Glycoproteins on Effector Cells

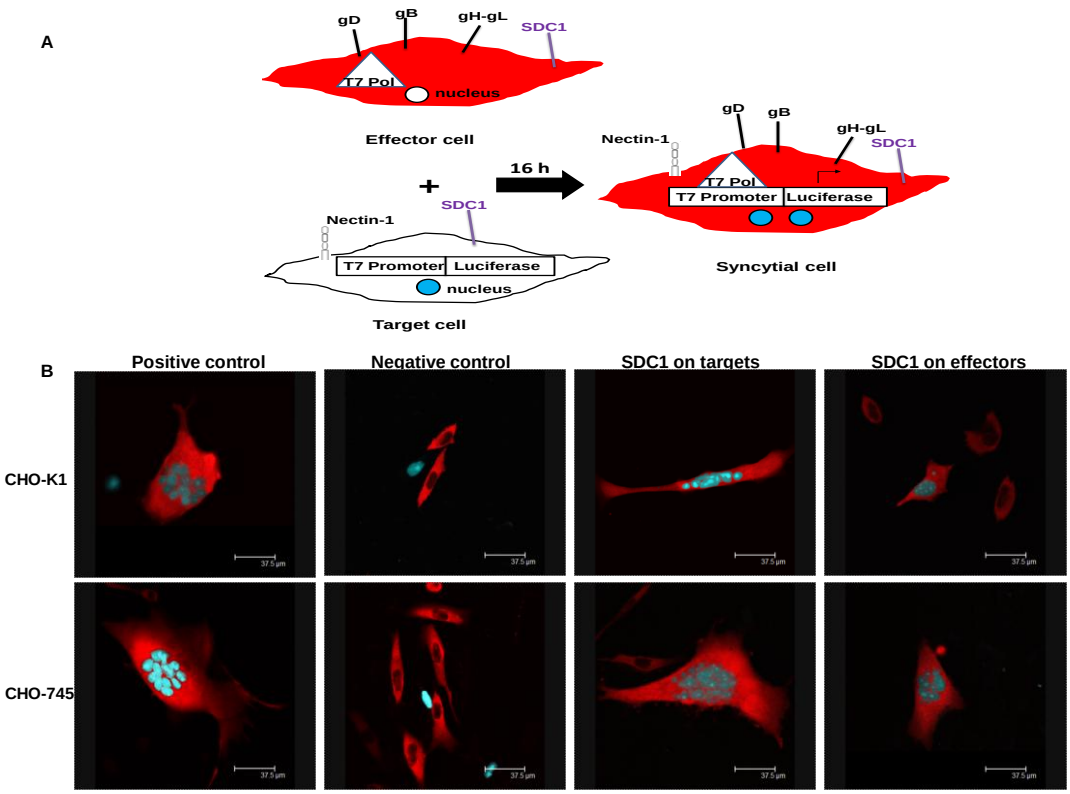


**Figure 11.** Syncytial cell formation in CHO-K1 and CHO-745 after syndecan-1 overexpression. (A). An illustration of syncytia assay that was exploited to understand the contribution of syndecan-1 during HSV-1 induced cell-to-cell fusion. Effector cell population that expresses HSV-1 fusion glycoproteins, T7 polymerase as well as a RFP-NES construct that restricts the expression of RFP to the cytoplasm is mixed with the target cell population that expresses nectin-1 as a gD receptor, the luciferase reporter gene under the control of T7 promoter as well as a CFP-NLS construct that restricts the expression of CFP to the nucleus. (B). Syncytia formation was observed in cells 72 h after the mix of target cells with effector cells. *Top panels* show representative syncytia formed in CHO-K1 cells after overexpressing syndecan-1 in target, or effector cell. *Bottom panels* show representative syncytia formed in CHO-745 cells after overexpressing syndecan-1 in target, or effector cells. Positive controls are target cells mixed with effector cells where both populations have the normal expression level of syndecan-1. Negative controls are target cells mixed with effector cells missing gB. *(scale bar = 37.5 μm).* SDC1, syndecan-1

**Table 1.** Comparison of syncytia number and nuclei count after syndecan-1 overexpression in each CHO cell type.

| Cell type | Condition | Average number syncytia per well | 2 nuclei per syncytia | 3–5 nuclei per syncytia | >5 nuclei per syncytia |
|---|---|---|---|---|---|
| CHO-K1 | Positive control | 142.5±9.8 | 30±13.9 | 63±14.6 | 50.88±16.5 |
| | SDC1 on targets | 196.8±15.0 | 66±7 | 72.7±22.0 | 49.6±16.7 |
| | SDC1 on effectors | 72.8±19.3 | 43.3±24.9 | 22.2±6.5 | 4.5±5.7 |
| CHO-745 | Positive control | 213.8±4.6 | 38.5±17.0 | 89.5±3.5 | 85.8±25.1 |
| | SDC1 on targets | 338.3±13.8 | 129.3±6.7 | 131.3±8.8 | 77.8±15.9 |
| | SDC1 on effectors | 46.8±8.8 | 17.3±1.8 | 23.5±4.9 | 6±5.7 |

**Table 1.** Average number of syncytial cells, as well as, the average number of nuclei per syncytia was counted in CHO-K1 and CHO-745 cells after overexpressing syndecan-1 on target cells or effector cells. Positive controls are target and effector cells expressing normal levels of syndecan-1. Syncytial cells were counted 72 h post mixing. Syncytia were classified as any red cell having two or more nuclei. Number of syncytia was normalized to the number of syncytia detected in the negative control wells where the effector cell population lacks gB. The average is based on results from two independent experiment performed in duplicate (mean ± 1SD).

To determine whether specific syndecan-1 domain(s) are required for the observed inhibition of HSV-1 induced cell-to-cell fusion when syndecan-1 is overexpressed on the effector cells along with HSV-1 fusion glycoproteins, a series of syndecan-1 molecules with specific truncations and mutations were expressed and analyzed. A diagram summarizing the mutant constructs used is shown in Fig. 12A. Cell fusion assay after overexpressing syndecan-1 mutants reveals that while the constructs $FcR^{ecto}$-hS1 (a construct in which the ectodomain of syndecan-1 is replaced by that of the human Fc) and $hS1^{\Delta cyto}$ (a construct that lacking the 33 C-terminal amino acids) were able to allow the fusion to near positive control levels that express normal levels of syndecan-1, overexpressing the construct $hS1^{pLeuTM}$ (a construct in which the transmembrane domain is replaced with leucine residues) on effector cells resulted in similar inhibition of HSV-1 induced cell fusion caused by overexpressing the wild type syndecan-1 (Fig. 12C). The cell surface expression of syndecan-1 mutants has been confirmed previously (McQuade et al., 2006). Identical observations were made in both CHO-K1 and CHO-745 cell lines, and were not due to cytotoxic effect of overexpressing syndecan-1 mutants, as that did not affect cell viability measured by MTS assay (Fig. 12B). These results demonstrate that syndecan-1 ectodomain and cytoplasmic domains but not the transmembrane domain are required for reduced cell fusion when syndecan-1 is overexpressed along with HSV-1 fusion glycoproteins on effector cells.

### 3.2.8 Syndecan-1 Knockdown Reduces Plaque Formation in HCE Cells

HSV-1 has the ability to produce visible plaques on HSV-1 susceptible cells which results in central clearing as the virus spreads (Zhe et al., 2008). One way of its spread is lateral

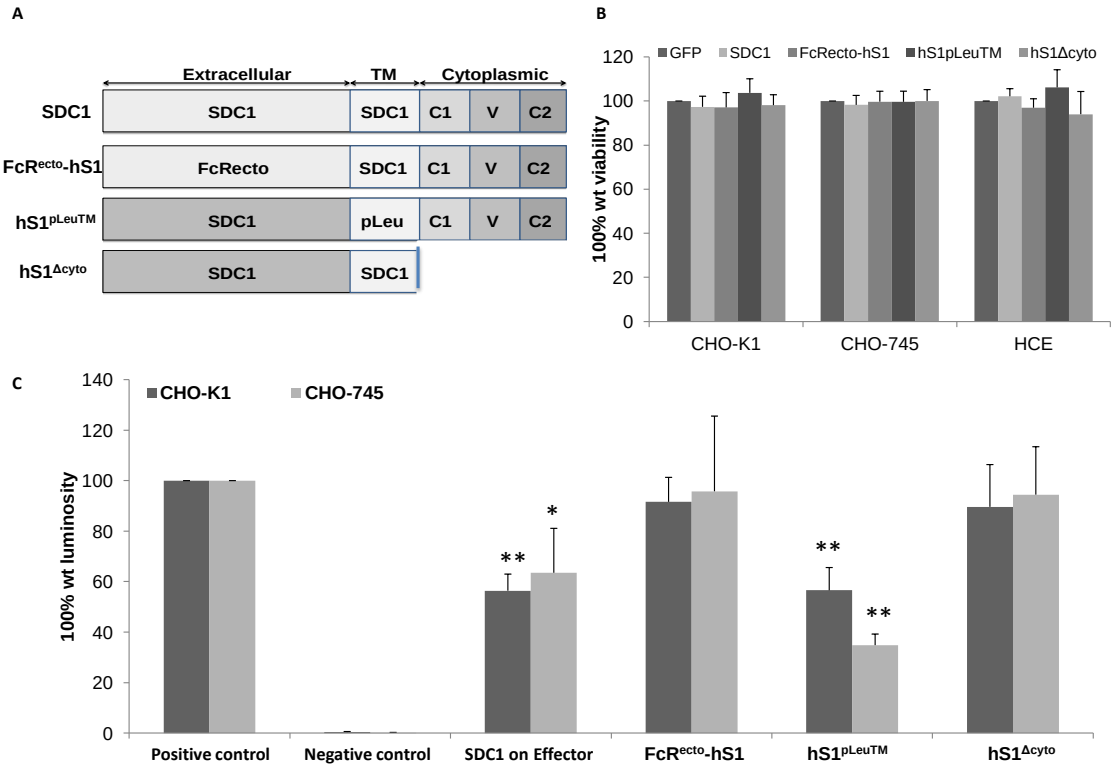


**Figure 12.** syndecan-1 ectodomain and cytoplasmic domains are important for inhibiting cell fusion when overexpressed on effector cells. (A). Syndecan-1 truncation and mutants used in the study are illustrated including the full-length wild type (*wt*) core protein syndecan-1 (SDC1) that includes an extracellular domain, transmembrane domain (TM), and COOH-terminal cytoplasmic domain. Also illustrated are the construct $FcR^{ecto}hS1$ that is a chimera comprised of the ectodomain of human IgG Fcγ receptor Ia/CD64 fused to the transmembrane and cytoplasmic domains of human syndecan-1, the construct $hS1^{pLeuTM}$ that has the transmembrane domain replaced with leucine residues, and a truncation mutant $hS1^{\Delta cyto}$ that lacks the 33 C-terminal amino acids. (B) Cells were grown in 96-well plates, transfected with control GFP plasmid, full-length *wt* human SDC1 plasmid, the construct $FcR^{ecto}hS1$, the construct $hS1^{pLeuTM}$, or the construct $hS1^{\Delta cyto}$ for 24 h. Triplicate wells were evaluated for cell viability using MTS assay. Results are expressed as 100% wild type (wt) viability where they represent the percent corrected absorbance after subtracting the background absorbance, relative to control GFP plasmid transfected cells, and are mean ± 1SD of at least 3 independent experiments. (C). Effector cells for CHO-K1 and CHO-745 cells were transfected with either control GFP plasmid, full-length *wt* syndecan-1, the construct $FcR^{ecto}hS1$, the construct $hS1^{pLeuTM}$, or the construct $hS1^{\Delta cyto}$ and mixed with the target cells 24 h post transfection. Fusion was measured 16 h post mixing. Results are presented as mean ± 1 SD of at least 3 independent experiments. As a negative control, target cells were mixed with effector cells lacking HSV-1 gB.

virus cell-to-cell spread by the fusion of infected cells with neighboring uninfected cells to form large multinucleated syncytial cells that can then slough off forming plaques. HCE cells were chosen to examine the contribution of syndecan-1 during HSV-1 plaque formation because corneal epithelium represents one of the major infection sites for HSV-1 and may precede infection of other parts within the eye (Farooq et al. 2011). Plaquing efficiency of HSV-1 was examined in HCE cells after either overexpressing or downregulating syndecan-1. Confluent monolayers of HCE overexpressing syndecan-1, or have syndecan-1 downregulated were infected with serial dilutions of virus stocks and overlaid with 0.5% methylcellulose growth medium. Several days post-infection, the monolayers were fixed and stained and plaques were counted. Surprisingly, the number of plaques formed after syndecan-1 overexpression on HCE cells resulted in an insignificant reduction in plaque formation. However, as seen with cell fusion assay, the downregulation of syndecan-1 resulted in 57.37±3.96 % reduction in plaque formation ($p < 0.0001$) (Fig. 13). Overexpressing syndecan-1 mutants did not show significant difference in plaque number. However one mutant showed smaller plaque size.

### 3.2.9 Increase in Syndecan-1 Production Increases Plaque Size, while a Reduction in its Expression and Overexpression of a Syndecan-1 Mutant that Lacks the Ectodomain form Smaller Size Plaques

To further investigate the role of syndecan-1 in lateral HSV-1 transmission, plaque size was examined after overexpressing wild type syndecan-1 and syndecan-1 mutants, or downregulating syndecan-1 in HCE cells. As demonstrated in Fig. 14A, the average plaque size formed in HCE cells after syndecan-1 overexpression was larger than that of the positive control

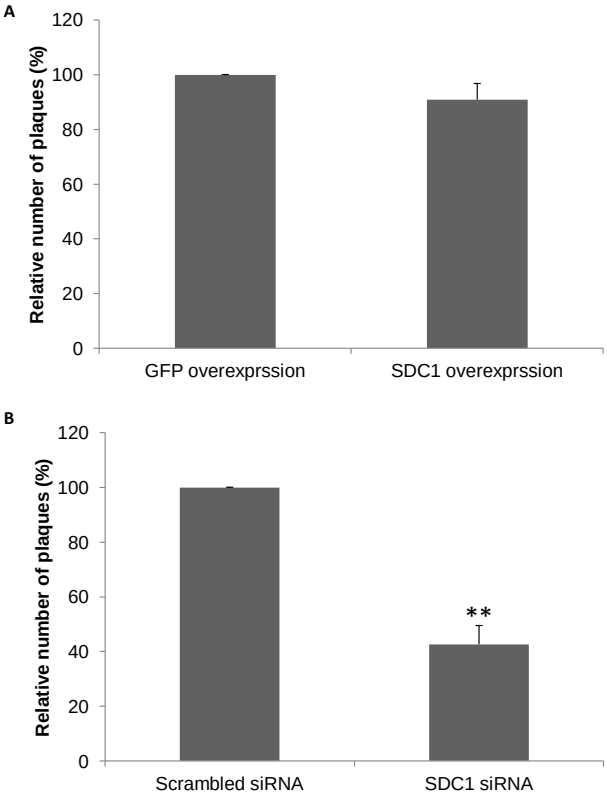


**Figure 13.** Syndecan-1 knockdown reduces plaque formation in HCE cells. (A). Monolayers of HCE cells were transfected with either control plasmid GFP, or with human syndecan-1. 24 h post transfection cells were infected with serial dilution of HSV-1(KOS) stocks. (B). 50% confluent HCE cells were transfected with either control scrambled siRNA or syndecan-1 specific siRNA. 72 h post transfection, cells were infected with serial dilution of HSV-1(KOS) stocks. (A, B). 72 h post-infection cells were fixed and stained with crystal violet stain. Infectivity was measured by the number of plaque forming units (PFUs). Number of PFUs was counted at the 10× objective (Zeiss Axiovert 200). Plaques consist of 15 or more nuclei were counted. Results are means ± 1 SD of three independent experiments conducted in duplicate. *SDC1*, syndecan-1.

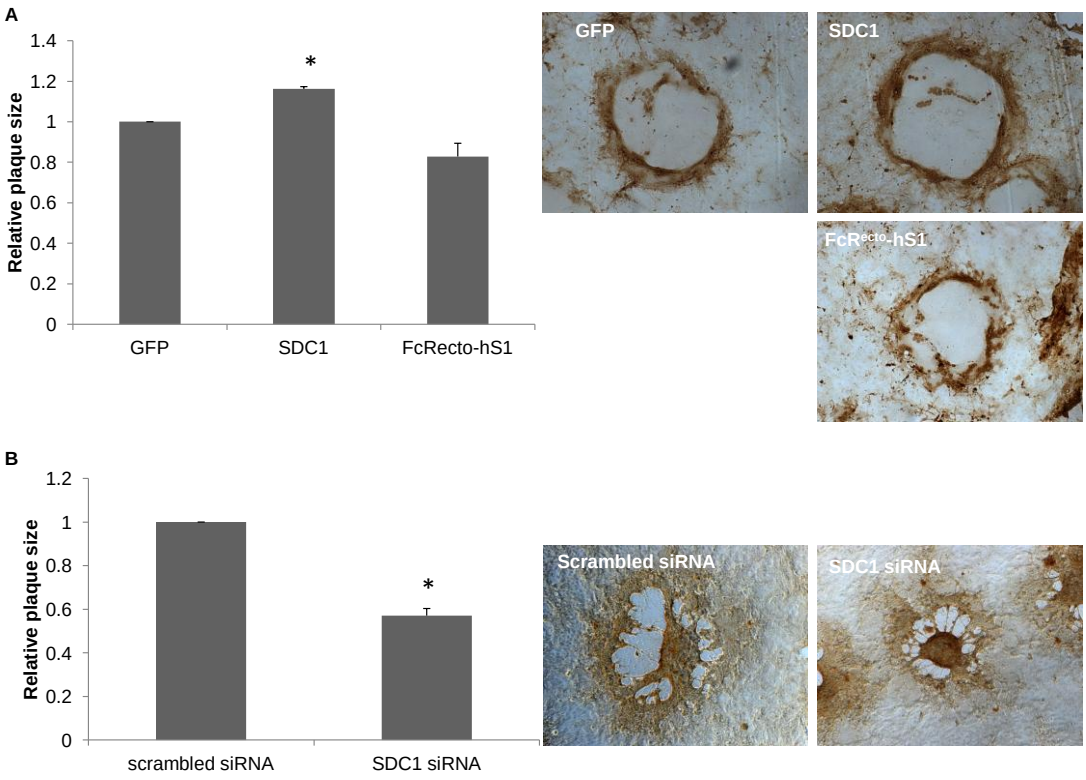


**Figure 14.** Effect of syndecan-1 overexpression, or downregulation on HSV-1 plaque size. Monolayers of HCE cells were transfected with either control GFP plasmid, full-length *wt* syndecan-1 (SDC1), or the construct $FcR^{ecto}hS$. 24 h post transfection, cells were infected with HSV-1 (KOS) at an MOI of 0.1 and overlaid with 0.5% methylcellulose medium. (B) 50% confluent HCE cells were transfected with either control scrambled siRNA or syndecan-1 specific siRNA. 72 h post transfection, cells were infected with HSV-1 (KOS) at an MOI of 0.1 and overlaid with 0.5% methylcellulose medium. (A, B). 72 h post-infection cells were fixed and stained using rabbit anti-HSV-1, horseradish peroxidase-conjugated secondary antibody, and 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate. Plaques were measured with a micrometer at the 10× objective (Zeiss Axiovert 200), and the area was calculated by measuring the outline of each plaque using Axioversion software (version 4). Results are expressed as relative plaque size (means ± 1 SD) of two independent experiments conducted in duplicate. Representative plaques from each condition are shown. *SDC1*, syndecan-1

HCE cells that express normal levels of syndecan-1 (16.19% ± 1.13, $P < 0.05$), showing that wild type syndecan-1 induced lateral virus spread, confirming the cell fusion results where enhancement of syndecan-1 production showed increase in cell fusion which is one major way the virus can laterally spread (Fig. 14A). Interestingly, overexpressing the mutant $FcR^{ecto}$-hS1 that lacks the ectodomain of syndecan-1 showed smaller size plaques compared to the positive control (17.16% ± 6.54, $P$=0.065) indicating that the ectodomain of syndecan-1 might play a role in the virus lateral spread (Fig. 14A). Representative plaques from each condition are shown in Fig. 6A. Since the increase insyndecan-1 resulted in larger size plaques, the effect of syndecan-1 loss on plaque size was examined. Syndecan-1 downregulation reduced plaques sizes 42.92% ± 3.27 ($P< 0.05$) compared with HCE cells transfected with the control scrambled siRNA supporting the involvement of syndecan-1 in HSV-1 lateral cell-to-cell spread (Fig 14B). Representative plaques from each condition are shown in Fig 14B.

### 3.2.10 Syndecan-1 Downregulation Reduces HSV-1 Spread

Since syndecan-1 downregulation showed significantly smaller plaque size compared to control cells transfected with scrambled siRNA, we aimed to confirm the involvement of syndecan-1 in virus lateral cell-to-cell spread by performing virus spread assay. HSV-1(KOS) infected HCE cells, which were treated with low pH citrate buffer to inactivate residual virus, were mixed with syndecan-1 siRNA transfected cells, and the virus spread was evaluated by counting the number of plaques formed 48-72 h post mixing. Syndecan-1 knockdown resulted in less virus spread compared to HCE cells transfected with scrambled siRNA (26.93 ± 4.62, $P< 0.0001$) (Fig. 15A). A qualitative fluorescent approach was also utilized to examine the effect of syndecan-1 downregulation on virus spread. HSV-1 (KOS) K26GFP virus strain that has the jellyfish GFP fused in frame with the UL35 open reading frame generating K26GFP virus whose

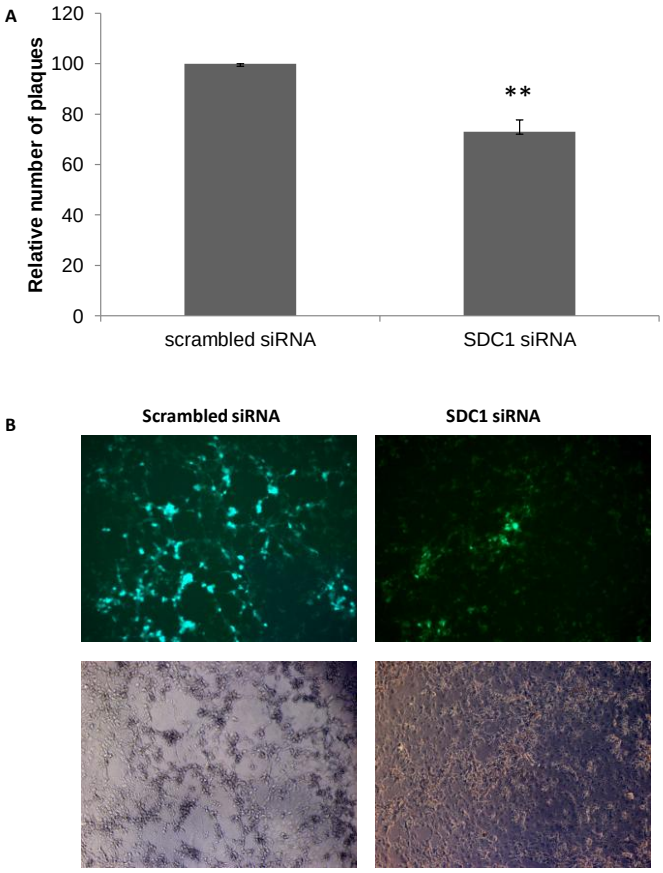


**Figure 15.** Syndecan-1 downregulation reduces HSV-1 spread. Monolayers of HCE cells were exposed to (A) HSV-1 (KOS) or (B) HSV-1 (KOS) K26GFP strains at MOI of 5 at 37 °C to allow virus entry. 2 h post infection, cells were washed, incubated for 1 min with 0.1M citrate buffer (PH 3.0), then washed with PBS and overlaid with 0.5% methylcellulose medium. After 4 h, cells were washed, dissociated, and approximately 600 cells were plated onto 80% confluent monolayers of uninfected HCE cells that have been transfected with either scrambled siRNA or syndecan-1 siRNA for 72 h in 0.5% methylcellulose medium. (A). The spread of HSV-1 (KOS) from infected cells to the siRNA transfected HCE cells was evaluated by staining and counting the number of plaques formed at the 10 × objective (Zeiss Axiovert 200). Results are presented as relative number of plaques (mean ± 1SD) of four independent experiments performed in duplicates. (B) HSV-1 (KOS) K26GFP spread was qualitatively assessed at the 10× objective (Zeiss Axiovert 200). Representative images from one experiment performed in triplicate are shown. *SDC1*, syndecan-1.

capsids express GFP (Desai et al., 1998) was used in the spread assay (Fig.15B). The top panels show representative HSV-1 (KOS) K26GFP virus spread to HCE cells treated with either syndecan-1 siRNA or the control scrambled siRNA. Syndecan-1 downregulation resulted in less virus spread as indicated by the less green fluorescence compared to control scrambled siRNA transfected cells. The bottom panels show HCE cells under the bright field. Together these results strengthen the conclusion that syndecan-1 is involved in HSV-1 lateral cell-to-cell spread.

### 3.2.11 Syndecan-1 Downregulation Reduces Infectious Virus Production

Since knocking down syndecan-1 on HCE cells resulted in reducing the plaquing efficiency of HSV-1, we sought to verify whether this reduction also translates into a loss of infectious virus produced as well. To answer this question, infectious virus production was analyzed after syndecan-1 downregulation. HCE cells transfected with either scrambled siRNA or syndecan-1 specific siRNA were infected 72 h post-transfection with HSV-1 (KOS) at an MOI of 0.1. At various times post-infection, cells and media were harvested, and virus titers were measured. Consistent with the results of plaquing efficiency, the downregulation of syndecan-1 severely impaired infectious virus production ($P < 0.05$) (Fig. 16). Taken together, these results suggest that downregulation of syndecan-1 also negatively impacts infectious virus production.

## 3.3 Discussion

HSV-1 entry into host cells starts with the attachment of HSV-1 gB and gC with HSPGs on the surface of the host cell. While substantial work has focused on delineating the role of HS as an

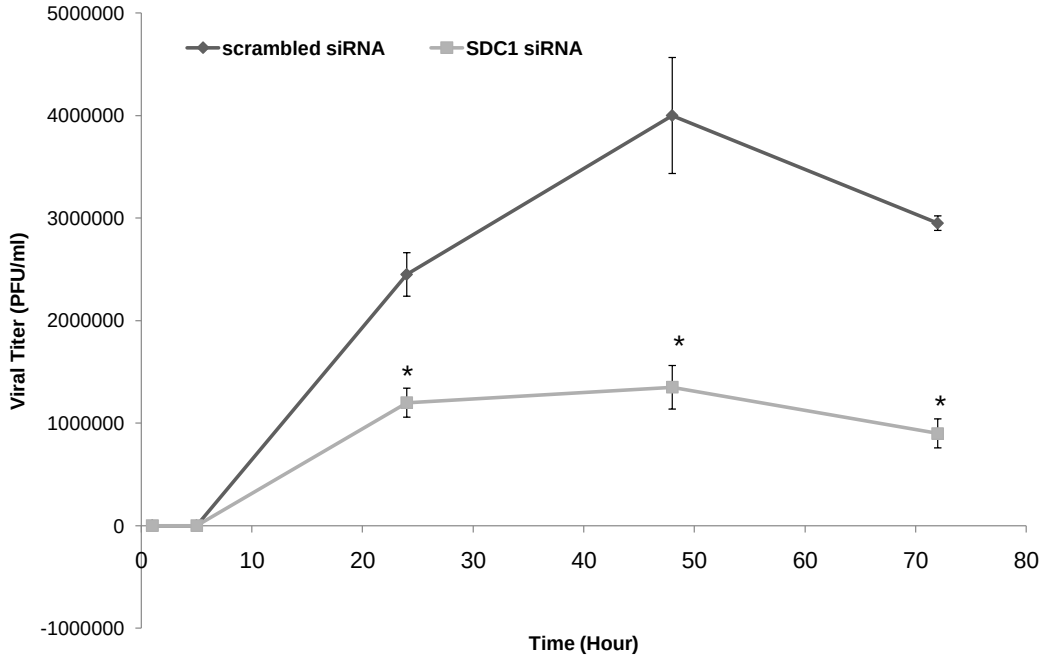


**Figure 16.** Downregulation of Syndecan-1 results in reduced production of infectious virus. HCE cells were transfected with either control scrambled siRNA or syndecan-1 siRNA. 72 h post transfection cells were infected with HSV-1 (KOS) at an MOI of 0.1. At 0, 5, 24, 48, and 72 h post infection, infectious virus was quantified by a standard plaque assay on HCE cell monolayers. The titers shown are the mean ± 1 SD of a representative experiment of two independent experiments performed in duplicates. *SDC1*, syndecan-1.

attachment receptor for HSV-1, far less is known about the contribution of the HSPG core protein in HSV-1 infection. Our study demonstrates a novel role for the core protein of HSPG during HSV-1 infection. Specifically, we demonstrate that the downregulation of syndecan-1 inhibits HSV-1 entry into the cell at a step prior to viral nuclear transport. Moreover, syndecan-1 contributes to HSV-1 induced cell-to-cell fusion and lateral spread. Syndecan-1 core protein contribution to virus induced cell-to-cell fusion is independent of HS chains as evident by using HS-deficient CHO-745 cells. The increase of syndecan-1 in target cells enhanced HSV-1 induced cell-to-cell fusion, while its decrease reduced cell fusion as well as virus spread.

A related interesting finding of our study is that the overexpression of syndecan-1 in effector cells that express HSV-1 fusion glycoproteins (gD, gB, gH, and gL) showed significantly reduced HSV-1 induced cell-to-cell fusion. The observed reduction in cell-to-cell fusion is reminiscent of a previously described phenomenon called gD-mediated interference. In this phenomenon, expressing gD in HSV-1 susceptible cells results in resistance to viral entry due to sequestering gD receptors and preventing their accessibility by cell-associated gD (Scanlan et al., 2003, Geraghty et al., 2000). This raises the possibility that there might be a direct interaction between syndecan-1 and at least one of the fusion glycoproteins, which in turn sequesters at least one of the glycoproteins preventing them from being fully functional during cell-to-cell fusion. Our results showed similar levels of inhibition of cell-to-cell fusion in both CHO-K1 and CHO-745 cells. The latter differ from CHO-K1 cells in that CHO-745 cells are deficient in the synthesis of all GAGs (Pertel et al., 2001, Esko et al., 1988). This suggests that this inhibition in cell-to-cell fusion is HS independent and not due to interactions between HS and HSV-1 fusion glycoproteins. In this context it is well known that gB utilizes HS chains on

HSPGs as an attachment receptor to initiate the entry process of the virus into the host cell (O'Donnell et al., 2008).

Another possibility is that syndecan-1 affects cell fusion indirectly by physically binding other cellular protein(s) that HSV-1 glycoprotein(s) require for fusion. This binding could also prevent the cellular protein from interacting with HSV-1 fusion glycoproteins, and that somehow leads to less efficient cell-to-cell fusion. Syndecan-1 ectodomain and cytoplasmic domain, which our results indicate their importance for this phenomenon, are both been shown to interact with a wide range of cellular proteins (Couchman et al., 2010). For example syndecan-1 ectodomain has been shown to interact directly with β3 or β5 integrins, which in turn have been shown to be one of the cellular determinants of HSV-1 entry (Beauvais et al., 2009, Gianni et al., 2010). It is also possible that overexpressing syndecan-1 along with HSV-1 fusion glycoproteins may perturb cellular signaling mechanisms required for efficient cell-to-cell fusion. The core protein of syndecan-1 functions in a variety of regulatory events including actin cytoskeleton reorganization, cell adhesion, cell proliferation, and angiogenesis, many of which are involved in HSV-1 infection (O'Donnell et al., 2009, Carey et al., 1994, Beauvais et al., 2004, Xian et al., 2010, Bernfield et al., 1999, Zong et al., 2010, Oh et al., 2010a, Oh et al., 2010b, Zhou et al., 2009, Zheng et al., 2001). Further work will be critical to clearly understand the reason behind the reduced cell-to-cell fusion upon syndecan-1 overexpressing along with HSV-1 fusion glycoproteins in effector cells.

This study also demonstrates that syndecan-1 is required for HSV-1's ability to form plaques. Although syndecan-1 overexpression did not result in increased number of plaques, the

knockdown of syndecan-1 resulted in significant impairments in plaque formation. One possible explanation is that syndecan-1 might be a regulatory component of a multi-protein complex that affects virus plaque formation. In that case, loss of syndecan-1, and not so much the extra copies of syndecan-1, is critical for cell-to-cell fusion. It is also possible that for this multi-protein complex, over-expression of syndecan-1 alone may not lead to more complex formation since the other components of that complex are not overexpressed. Another explanation is that syndecan-1 is involved in signaling pathways important for virus plaque formation, so a reduction in syndecan-1 level resulted in less efficient plaque formation, but an enhancement in syndecan-1 production did not affect the virus's ability to form plaques.

Although the overexpression of syndecan-1 did not result in more efficient HSV-1 plaquing, it resulted in bigger size plaques. Moreover, the knockdown of syndecan-1 resulted in smaller size plaques compared to control siRNA transfected cells. Plaque assays were preformed in the presence of methylcellulose layer where plaque formation depended exclusively on cell-to-cell virus spread. This suggests that syndecan-1 is playing a key role during HSV-1 virus spread. This suggestion was strengthened by a spread assay where the knockdown resulted in less virus spread as evident by a quantitative and a qualitative spread assays.

We further analyzed the effect of syndecan-1 knockdown on infectious virus titers after HSV-1 (KOS) infection utilizing HSV-1 growth curve assay. Syndecan-1 knockdown severely reduced the titers of infectious virus. The effect of syndecan-1 downregulation on HSV-1 growth curve could be an accumulative effect of syndecan-1 playing role in HSV-1 entry, virus induced cell fusion, and HSV-1 spread. However, this does not exclude the possibility that at least part of

this observed reduction in HSV-1 growth curve might be due to less HSV-1 replication in HCE cells transfected with syndecan-1 specific siRNA.

Syndecan-1 binds to many factors that initiate and regulate the inflammatory response. It inhibits leukocytes adhesion to activated endothelial cells through negatively affecting the β2 intergrin-ICAM-1 interaction (Teng et al., 2012). Furthermore, syndecan-1 on endothelial cells facilitates leukocytes transendothelial migration by generating a chmokine gradient through chemokines binding (Teng et al., 2012, Handel et al., 2005, Kuschert et al., 1999). However, syndecan-1 shedding mediated by metalloproteinase-7 (MMP-7) in alveolar epithelial cells generates transepithelial migration of neutrophils through generating a chemokine gradient (Teng et al., 2012). Syndecan-1 shedding removes sequestered CXC chemokines (or α-chemokines), thus resolving inflammation (Hayashida et al., 2009). An additional role of syndecan-1 during inflammation is matrix remodeling to restore the normal structure and function of inflamed tissue (Teng et al., 2012). Studies have suggested that while cell surface syndecan-1 promotes tissue repair and thus attenuates fibrosis, shed syndecan-1 ectodomains induce fibrosis by reducing epithelial repair and inducing fibrotic factors by an unknown mechanism (Teng et al., 2012). Syndecan-1 is also implicated in multiple tumorigenesis events. It affects tumor cell growth, apoptosis, invasion, metastasis, and angiogenesis. There are several mechanisms by which syndecan-1 (cell surface and shed ectodomains) is involved in cancer which vary according to cancer cell type.

The role of syndecan-1 has been investigated during other viral and bacterial infections including human papillomavirus (HPV) infection, human immunodeficiency virus (HIV) infection, and *Neisseria gonorrhoeae* infection. In HPV infection, syndecan-1 was shown to

provide attachment receptor to HPV major capsid protein L1 (Shafti-Keramat et al., 2003). This attachment is suggested to induce conformational change in the minor capsid protein L2, exposing certain residues in L2 protein that bind to another entry receptor mediating HPV entry (Richards et al., 2006, Day et al., 2008). In the case of HIV infection, syndecans have been shown to provide attachment receptor for HIV gp 120 mediating viral adsorption to the surface of permissive cells. Further, syndecans by providing attachment receptor to HIV in nonpermissive cells that lack HIV entry receptors enhances the *in trans* infectivity of HIV in permissive cells (Bobardt et al., 2003). Syndecan-1 and syndecan-4 are direct entry receptor for *N. gonorrhoeae* with the cytoplasmic domain of syndecans being essential for this function. Mutagenesis studies with syndecan-4 have revealed that deleting C2 region of the cytoplasmic domain did not support *N. gonorrhoeae* invasion (Freissler et al., 2000). C2 region of the cytoplasmic domain is conserved among all members of the syndecan family, and it has a postsynaptic density-95/ disc large protein/zonula occludens-1 (PDZ) binding motif. PDZ binding proteins are implicated in organizing multiprotein complexes that function in signaling, as well as establishment and maintenance of cell polarity (Tumova et al., 2000, Harris et al., 2001).

Since syndecan-1 has been implicated in modulating cellular signaling, it is possible that syndecan-1 affects HSV-1 infection by integrating signals from the virus with other cellular systems. Syndecans do not appear to encode any intrinsic catalytic activity; however they interact with molecules including kinases, GTPases, integrins, and cytoskeletal molecules (Lambaerts et al., 2009). These molecular interactions are controlled by syndecans phosphorylation and clustering. Syndecan-1 ectodomain away from HS attachment sites at a part

of the ectodomain called synstatin interacts with αvβ3 and αvβ5 integrins blocking angiogenesis and tumorigenesis (Beauvais et al., 2009). The transmembrane domain of syndecan has a conserved motif that is required for multimerization, where ligand mediated clustering of syndecans is suggested to be a key event during signaling (Lambaerts et al., 2009). The cytoplasmic domain of syndecans contains several signaling motifs, including one invariant serine (Ser), and four invariant Tyrosine (Tyr) residues (Hayashida et al., 2008). Studies have shown that a mutation of a conserved Tyr in the V region of syndecan-1 abolishes its co-localization with microfilaments (Carey et al., 1996). C1 region is thought to be involved in binding to several cytoskeletal proteins, including ezrin, tubulin, and cortactin, in addition to other intracellular proteins like Src kinase (Tkachenko et al., 2005). C2 region contains a PDZ-binding site. Several syndecan-interacting PDZ domain–containing proteins have been identified including: syntenin, synectin, synbindin, and calcium/calmodulin dependent serine protein kinase (Tkachenko et al., 2005). Moreover, differential regulating of syndecan expression is one suggested mechanism of syndecans function. Syndecans' temporal and spatial expression is possibly how the various members of the syndecan family function specifically *in vivo*. Syndecan-1 expression in epithelial cells has been shown to be regulated by Transforming growth factor β (TGFβ) through protein kinase A (PKA) to phosphorylate Ser residue in the cytoplasmic domain of syndecan-1 (Hayashida et al., 2006).

Further studies must be done to determine the molecular mechanisms behind the contribution of syndecan-1 during HSV-1 infection. The emerging role of syndecan-1 during HSV-1 infection opens new doors that might add clarity to the picture of virus entry and infection Delineating the role of syndecan-1 during the various HSV-1 infection events,

especially those at the early stages of the infection is of significance as that might help the development of new antiviral agents or an HSV-1 vaccine.

# 4. AN IMPORTANT ROLE FOR HEPARANASE IN HERPES SIMPLEX VIRUS TYPE-1 RELEASE FROM INFECTED CELLS.

## 4.1 Rationale

Heparanase is an endo-β-D-glucuronidase that has the ability to cleave HS side chains at a limited number of sites, producing appreciable sized HS fragments that are resistant to further degradation by the enzyme (Ogren et al., 1975). Cleavage of HS by Heparanase results in the disassembly of the ECM and basement membrane underlying epithelial and endothelial cells, allowing structural modification. Heparanase is synthesized as a 65kDa non-active precursor that gets cleaved to produce the active form of Heparanase which is composed of a 50kDa and 8kDa heterodimer. Heparanase has functions that are related to its enzymatic activity including tumor metastasis, angiogenesis, embryo implantation, wound healing, inflammation, and autoimmunity (Barash et al., 2010, Zhang et al., 2011). Moreover, independent of its enzymatic activity, Heparanase facilitates adhesion and migration of primary endothelial cells, as well as mediates phosphorylation of diverse molecules including Src substrates, and the signaling molecules Akt and Src, which affects gene transcription (Barash et al., 2010). Another important way Heparanase modulates HSPG is through enhancing the shedding of syndecan-1 member of the syndecan family of HSPG (Yang et al., 2007). This effect on syndecan-1 shedding requires an active Heparanase, since mutated forms of Heparanase that lacks HS degrading activity, failed to enhance syndecan-1 shedding (Yang et al., 2007).

HSPGs provide attachment receptor for HSV-1 as a first step of the infection (O'Donnell et al., 2008). This step is followed by specific interaction of HSV-1 gD glycoprotein with its cognate receptors which results in the fusion of the viral envelope with the cell membrane, and subsequently, the release of the nucleocapsid into the cell cytoplasm (Connolly et al., 2011). Additionally, modified form of HS (3-OS HS) serves as an entry receptor for the virus (Shukla et

al., 1999). HS has also been implicated in fine tuning of HSV-1 induced cell-to-cell fusion (O'Donnell et al., 2009). More recently, the core protein of HSPG has been shown to play a role during HSV-1 infection. Syndecan-1 and Syndecan-2, which are two members of the syndecan family of HSPG, have been shown to be involved in HSV-1 entry into HeLa cells (Bacsa et al., 2011). Also, HSV-1 infection resulted in an induction of the cell surface expression, as well as the protein synthesis of syndecan-1 and syndecan-2, suggesting that these HSPG are involved in HSV-1 infection (Bacsa et al., 2011). Moreover, our results from the first part of this study (Chapter 3) suggest that syndecan-1 is important in HSV-1 spread by affecting virus induced cell-to-cell fusion and cell-to-cell spread.

Heparanase has the ability to modulate HSPG in various ways, suggesting that it might affect HSV-1 infection by modulating HSPG. The aim of this study is to understand the role of Heparanase during HSV-1 infection. The effect of overexpressing Heparanase in HCE and HeLa cells, as well as the treatment of exogenous Heparinases on HSV-1 infection was assessed. It has been reported that Heparanase expression was significantly higher in Hepatitis C virus (HCV) related hepatocellular carcinoma (HCC) compared with HCC patients who are HCV-negative (Dong et al. 2010, El-Assal et al., 2001). Therefore, the effect of HSV-1 infection on the expression level of Heparanase was evaluated. Our results suggest that overexpressing Heparanase has no effect on HSV-1 entry, while HSV-1 plaque formation was mildly reduced following Heparanase overexpression. Interestingly, Heparanase overexpression resulted in a significant increase in virus egress as compared to its egress from control cells transfected with an empty vector. Moreover, Treating HSV-1 infected cells with exogenous Heparinases showed similar enhancement in virus egress. This suggests that Heparanase is important for virus release

from infected cells. Additionally, HSV-1 infection resulted in an increase in the expression levels of active Heparanase. This suggests that HSV-1 exploits Heparanase to induce its release from infected cells by enhancing the expression of the active form of the enzyme. Our results also show that Heparanase induces HSV-1 egress not only by degrading HS chains, but also by inducing syndecans shedding, since treating HSV-1 infected cells with the syndecan shedding agonist: PMA, induced virus release from infected cells. The release of infectious virus from infected cells is an important virus spread mechanism to reach uninfected sites. Actually, asymptomatic virus release from infected sites is a significant risk for transmission between hosts (Sacks et al., 2004). Together, these results suggest a novel function for Heparanase during HSV-1 release from infected cells.

## 4.2 Results

### 4.2.1 Heparanase Overexpression Does Not Affect Cell Viability

In order to examine the importance of Heparanase in the various aspects of HSV-1 infection, human heparanase construct was used to induce the expression of heparanase in HeLa and HCE cells. To evaluate the effect of inducing Heparanase expression on cell viability, MTS assay was performed 1 day post Heparanase overexpression. Enhancing Heparanase expression did not affect cell viability in both HeLa and HCE cells (Fig. 17).

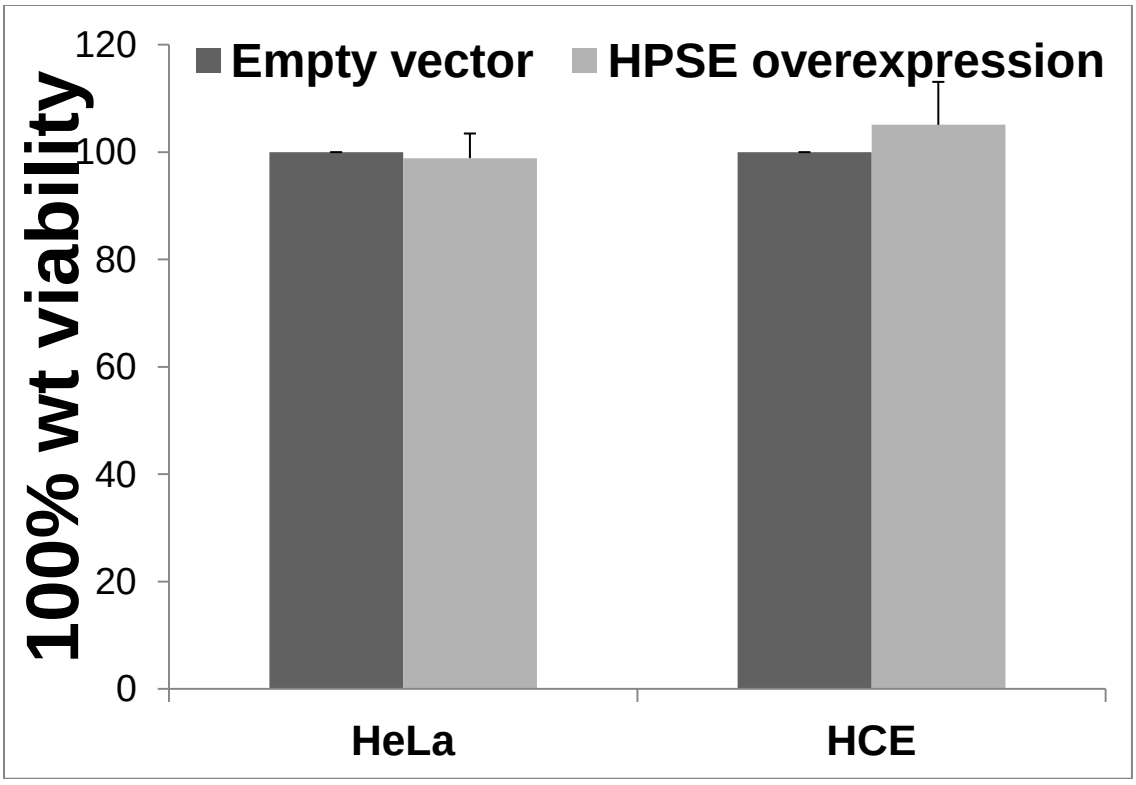


**Figure 17.** Heparanase overexpression does not affect cell viability in HeLa and HCE cells. HeLa and HCE cells were transfected with human Heparanase (HPSE) expression plasmid. 24 h post transfection, triplicate wells were evaluated for cell viability using MTS assay. Results are expressed as 100% wild type (wt) viability where they represent the percent corrected absorbance after subtracting the background absorbance, relative to empty vector transfected cells, and are mean ± 1SD of 3 independent experiments.

### 4.2.2 Heparanase overexpression does not affect HSV-1 entry into HeLa and HCE cells.

It is well established that HSV-1 utilizes HS on the surface of the host cell as an attachment receptor preceding virus entry into the host cell (O'donnell et al., 2008). Moreover, Heparanase has the important role of cleaving HS chains as part of the remodeling process of the ECM (Fux et al., 2009). To investigate whether enhanced Heparanase expression would affect HSV-1 ability to enter into host cells, Heparanase was overexpressed in HeLa and HCE cells and HSV-1 entry was evaluated by standard ONPG assay. Enhanced Heparanase expression did not affect the ability of HSV-1 to enter into HeLa and HCE cells compared to cells overexpressing an empty vector (Fig. 18).

### 4.2.3 Enhanced Heparanase reduces HSV-1 plaque formation in HCE cells.

Since overexpressing Heparanase did not affect HSV-1 entry into HeLa and HCE cells, we sought to examine whether enhanced Heparanase would affect virus plaque formation. Plaque formation reflects the ability of the virus to enter, replicate, and cell-to-cell spread. HCE cells were transfected with either Heparanase expression plasmid or with an empty vector. 24 h post transfection, cells were infected with HSV-1 (KOS) 804. 4 dpi, cells were fixed and stained, and plaques were counted. Surprisingly, overexpressing Heparanase in HCE cells showed reduced plaque numbers, compared to cells transfected with an empty vector (Fig. 19). This suggests that Heparanase might have a role in HSV-1 infection at a step that is beyond the viral entry.

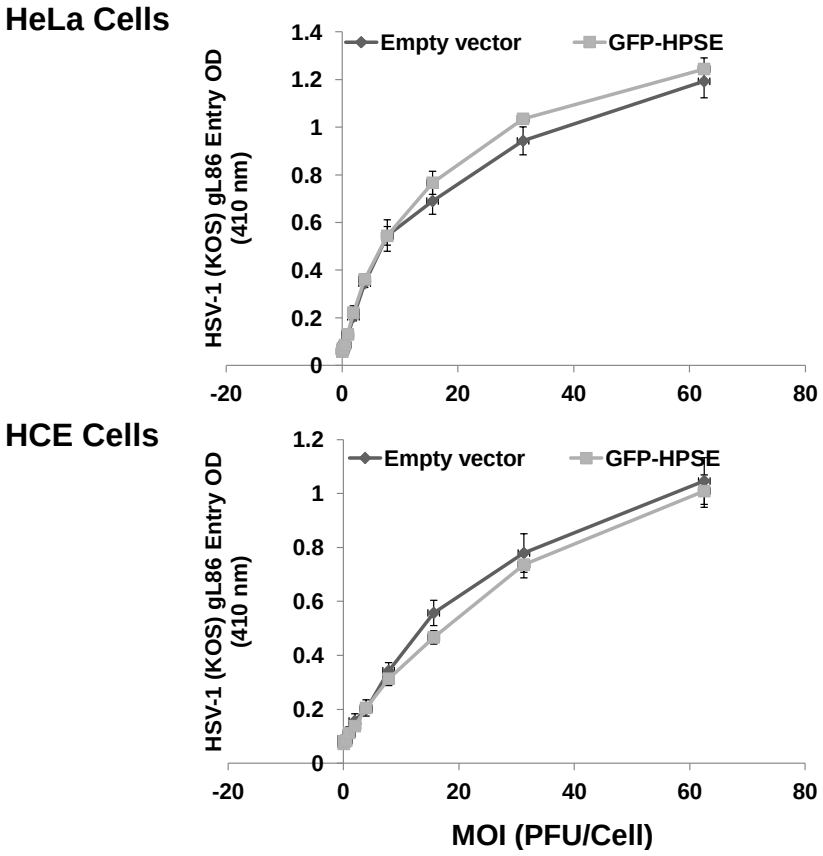


**Figure 18.** Heparanase overexpression does not affect HSV-1 entry into HeLa and HCE cells. HeLa and HCE cells were transfected with either Heparanase expression plasmid or an empty vector. 24 h post transfection, cells were infected in a twofold serial dilution with the recombinant HSV-1 virus expressing β-galactasidase enzyme HSV-1(gL86). Cells were incubated with virus for 6 h at 37°C. Cells were then washed, permeabilized and incubated with ONPG substrate. Enzymatic activity was measured at 410 nm using an ELISA plate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA). Results are representative of at least three independent experiments. Values in the figure were plotted as the mean of four determinations (± 1 SD).

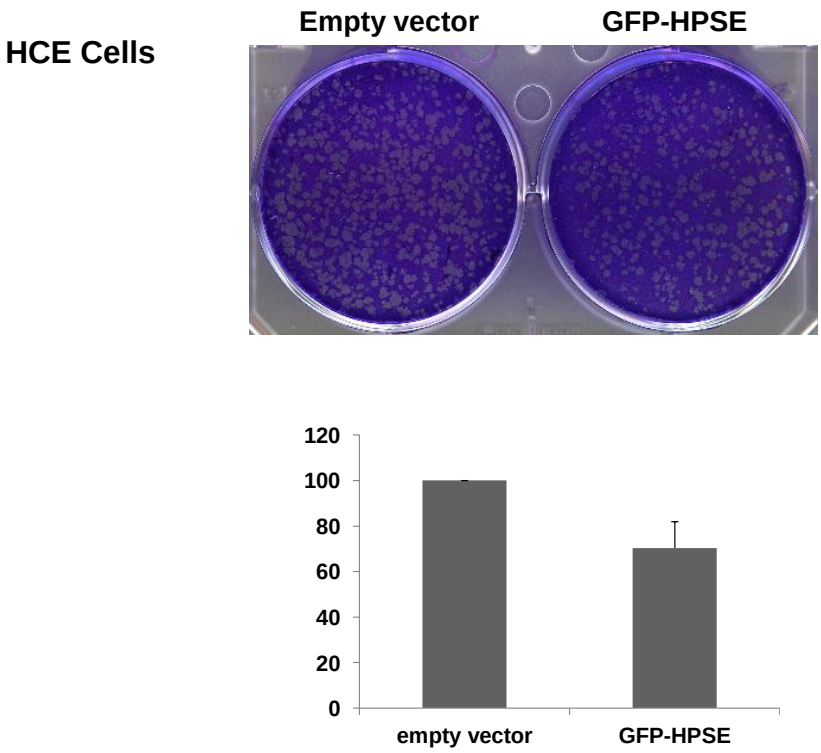


**Figure 19.** Heparanase overexpression inhibits HSV-1 plaque formation in HCE cells. HCE cells were transfected with either Heparanase expression plasmid or an empty vector. 24 h post transfection, cells were infected with syncytial plaque forming HSV-1 (KOS) 804 virus at MOI of 0.001. 4 days post infection, cells were fixed and stained with crystal violet stain. Infectivity was measured by the number of plaque forming units (PFUs). Results are means (±1 SD) of three independent experiments conducted in duplicate.

### 4.2.4 Heparanase overexpression induces HSV-1 release from infected HeLa and HCE cells

HSV-1 attaches to HS via it glycoproteins: gB and gC. HSV-1 release from infected cells might be facilitated by less HS chains on the surface of infected cells. In order to evaluate the effect of enhanced Heparanase expression on virus ability to exit from infected cells, virus egress assay was performed in HeLa and HCE cells overexpressing Heparanase plasmid. HeLa and HCE cells were either transfected with an empty vector or with Heparanase expression plasmid. 24 h post transfection, cells were infected with HSV-1 (KOS) for 2 h. Cells were then washed and growth media was added, and incubation was continued. At the indicated time points, 100 µl from the growth medium were collected and titered to measure virus release from the cells. Overexpressing Heparanase resulted in significantly higher HSV-1 release into the culture supernatant both in HeLa and HCE cells (Fig. 20). These results suggest that Heparanase play an important role in virus egress, possibly through cleaving HS chains from infected cells.

### 4.2.5 Recombinant Heparinase treatment to HSV-1 infected HeLa and HCE cells induces HSV-1 release from infected cells to the culture supernatant.

Since enhanced Heparanase expression increased HSV-1 egress from infected cells, the effect of the addition of exogenous recombinant Heparanase on HSV-1 egress was evaluated. HeLa and HCE cells were infected with HSV-1 (KOS) at MOI of 0.1 and 0.01 respectively for 2 h at 37°C. Cells were then washed, and bound virus was removed by Citrate buffer (PH=3) treatment, followed by cell washing. Cells were then treated with either heparinase III (15 and 30 IU/ml), or a combination of both heparinases II and III (15 and 30 IU/ml) for 2 h at 37°C. After 2

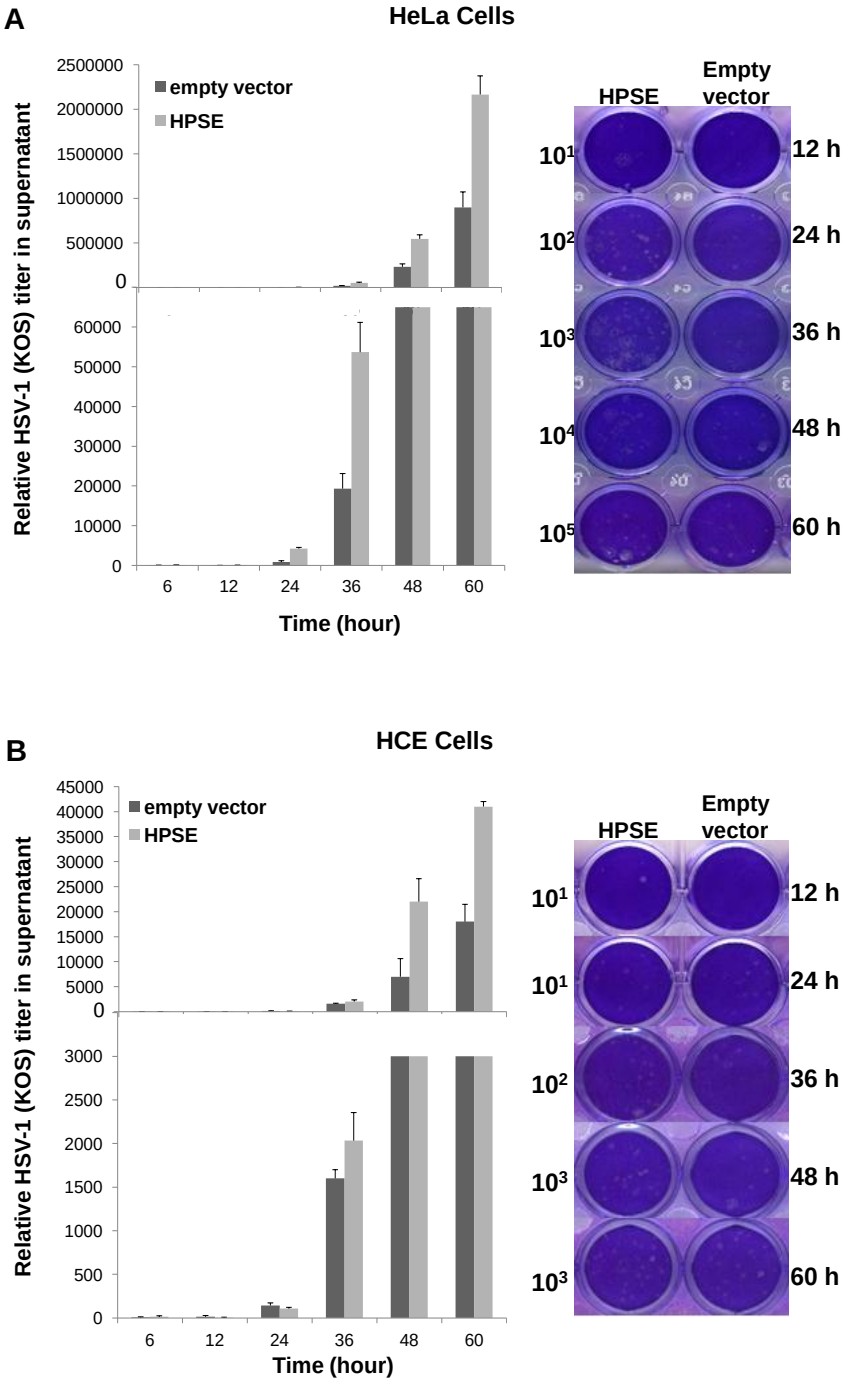


**Figure 20.** Heparanase overexpression enhances HSV-1 release from infected cells to the culture supernatant. HeLa (A) and HCE (B) cells were transfected with either Heparanase expression plasmid or an empty vector. 24 h post transfection, cells were infected with HSV-1 (KOS) for 2 h. After 2 h, cells were washed and growth medium was added back to the cells. At time points: 6, 12, 24, 36, 48, and 60 h post infection, HSV-1 release from infected cells was examined by titering 100 µl from the culture supernatant using plaque assay on Vero cells.

h, cells were washed, and growth medium was added back to the cells, and incubation was continued for 20 h at 37°C. HSV-1 release was measured by titering the supernatant of the cultured cells on Vero cells. As a control, some cells were infected with HSV-1 (KOS), but were not treated with the heparinases. Treating cells with Heparinase II, Heparinase III, or a combination of both Heparinases at both concentrations used resulted in an induction in released virus from infected cells to the culture medium (Fig. 21). These results strengthen the notion that Heparanase has a significant role in HSV-1 release from infected cells.

### 4.2.6 HSV-1 infection induces the expression of active Heparanase

Enhanced Heparanase expression and the addition of recombinant Heparinases to HeLa and HCE cells resulted in an induction in HSV-1 release from infected cells. Released infectious virus from infected cell is one way the virus can spread to reach uninfected cells. Since Heparanase enhanced virus spread, the effect of HSV-1 infection on Heparanase expression level was investigated. Western Blot was performed to evaluate the expression level of Heparanase after HSV-1 infection. HeLa and HCE cells were infected with HSV-1 (KOS) at MOI of 1 for 2 h at 37°C. Cells were then washed, growth media was added, and incubation was continued for 24 h. Non-infected cells were used as a control. After 24 h, Heparanase protein levels were evaluated. Heparanase is synthesized as a non-active precursor (65 kDa), which undergoes proteolytic cleavage, resulting in 50 kDa and 8 kDa protein subunits that heterodimerize to form the active enzyme (Fairbanks et al., 1999). HSV-1 infection resulted in an increase in the active (50 kDa) Heparanase which was associated with a decrease in the non-active (65 kDa)

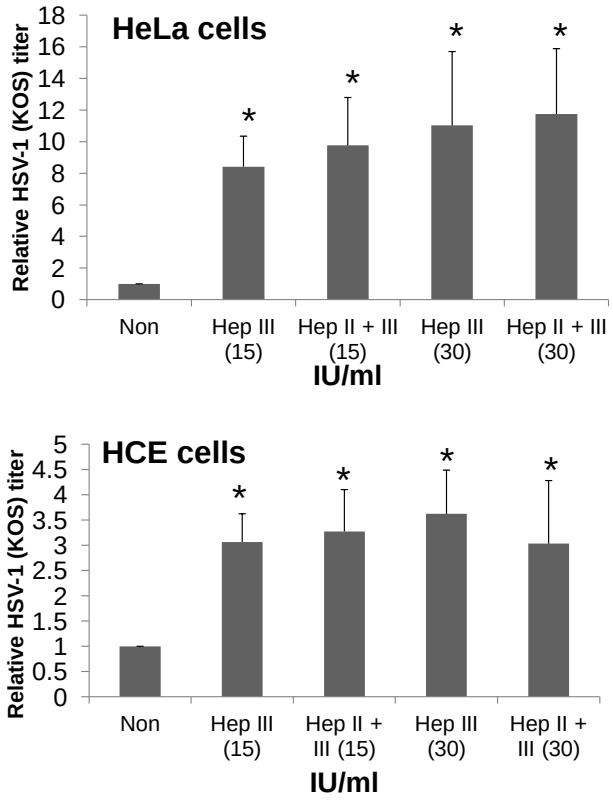


**Figure 21.** Heparanase treatment enhances HSV-1 release from infected cells to the culture supernatant. HeLa and HCE cells were infected at MOI of 1 with HSV-1 (KOS) for 2 h. After 2 h, cells were treated with citrate buffer (PH=3) for 1 min, followed by cell washing. Cells were then either mock treated of treated with heparanase III or a combination of heparanase II and III, at 15 or 30 IU/ml for 2 h at 37°C. After 2 h, cells were washed and growth medium was added back to the cells. HSV-1 release from infected cells was examined after 20 h incubation at 37°C post cell treatment by plaque assay using Vero cells. Results are means ± 1SD of three independent experiments conducted in duplicate (*$P$<0.05).

Heparanase compared to non-infected cells (Fig. 22). These results were observed in HeLa and HCE cells. β-actin was used as a loading control.

### 4.2.7 PMA treatment to HSV-1 infected HeLa and HCE cells induces virus release from infected cells to the culture media

Results suggest an increase in active Heparanase expression in HSV-1 infected cells, as well as an involvement of Heparanase in HSV-1 release from infected cell. Additionally, it has been reported that Heparanase enhances the shedding of syndecan-1 from tumor cells (Yang et al., 2007). Therefore, enhanced virus release from HSV-1 infected cell by Heparanase overexpression and exogenous Heparinases might be, at least partially, influenced by the induced syndecan-1 shedding by Heparanase. In order to understand the effect of enhancing syndecan-1 shedding on virus release from infected cells, HSV-1 release from infected cells was evaluated following PMA treatment. Phorbol esters, including PMA, have been considered the best characterized shedding agonists (Fitzgerald et al., 2000). Moreover, PMA has been shown to activate syndecan-1 shedding (Fitzgerald et al., 2000). HeLa and HCE cells were infected at MOI of 0.01 with HSV-1 (KOS) for 2 h. After 2 h, complete media was added and incubation was continued for 8 h. Cells were then serum starved for 12 h, followed by PMA treatment (0.5µM and 1µM) in serum free media for 0.5, 1, 2, and 4 h. Control cells were treated with DMSO. After each time point, HSV-1 released to the culture supernatant was evaluated by titering 100 µl of culture supernatant on Vero cells. PMA treatment induced HSV-1 release from infected cells to the culture media, with the most significant increase to be at later time points (2, and 4 h) (Fig. 23). Both PMA concentrations (0.5µM and 1µM) showed similar effect on virus

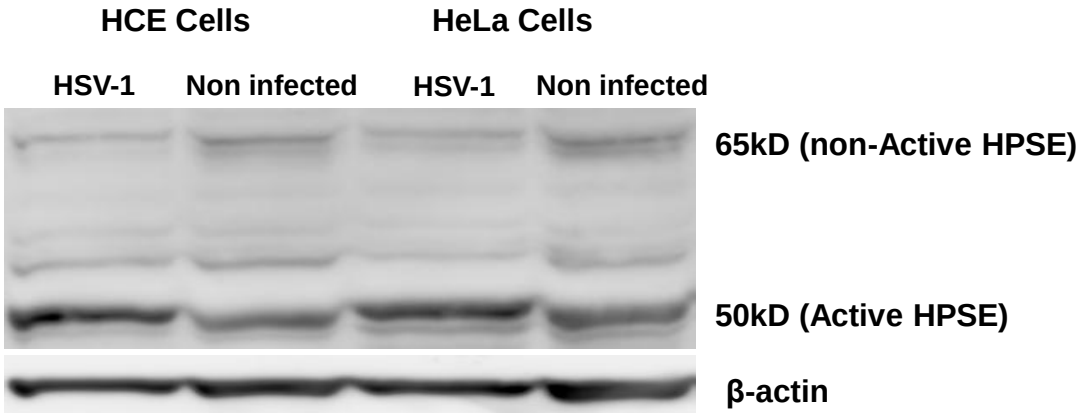


**Figure 22.** HSV-1 infection induces active Heparanase protein expression in HeLa and HCE cells. HeLa and HCE cells were infected with HSV-1 (KOS) for 2 h. After 2 h, cells were washed and growth medium was added back to the cells. 24 h after infection, immunoblots of cell lysates were prepared and probed with anti-HPSE polyclonal Ab., β-actin protein level was measured as loading control. *HPSE,* Heparanase.

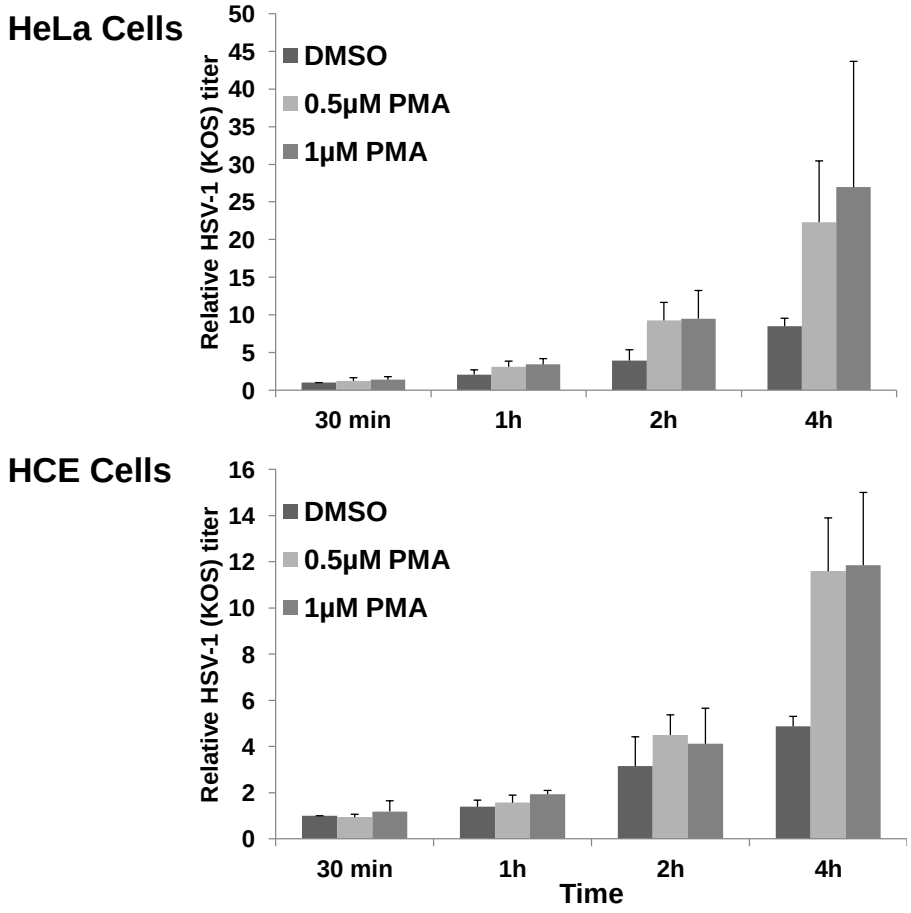


**Figure 23.** PMA treatment enhances HSV-1 release from infected cells to the culture media. HeLa and HCE cells were infected at MOI of 0.01 with HSV-1 (KOS) for 2 h. After 2 h, complete media was added and incubation continued for 8 h. Cells were then serum starved for 12 h, followed by PMA treatment in serum free media for 0.5, 1, 2, and 4 h. After each time point, 100 µl of the culture supernatant was titered on Vero cells monolayer. Results are means ± 1SD of at least three independent experiments.

release from infected cells. A confirmation of the effect of PMA on syndecan-1 shedding was carried out by measuring shed syndecan-1 after PMA treatment using Dot Blot analysis (Fig. 24).

### 4.3 Discussion

Heparanase modulates HSPG in multiple ways. Not only Heparanase has the function of degrading HS chains, but also it enhances the shedding of HSPGs including syndecan-1 from the cell surface. HSPGs, which are the attachment receptor for HSV-1, contribute to HSV-1 infection both at the level of HS chains and at the level of the protein core. How Heparanase actions on HSPG affect HSV-1 infection has remained poorly understood. This study demonstrates, for the first time, a novel role for Heparanase in HSV-1 release from the infected cell. Heparanase overexpression, and exogenous Heparinases treatment significantly induced the virus release from infected cell to the supernatant. These results suggest that Heparanase plays an important role in virus spread, and possibly, transmission.

Although HSPGs provide attachment receptors for HSV-1 as a first step of virus infection, our study shows that Heparanase overexpression has no effect on HSV-1 entry into the host cell. A previous study from our lab has shown that HSV-1 infection induces the cell surface expression and the protein synthesis of two members of the syndecan family of HSPG: syndecan-1 and syndecan-2. Additionally, this increase in syndecans expression was associated with an increase in HS expression on the cell surface (Bacsa et al., 2011). This suggests that multiple factors affect HSPG expression in cells with enhanced Heparanase expression that have been infected with HSV-1. It is possible that the increased expression of HSPG by HSV-1 infection masked

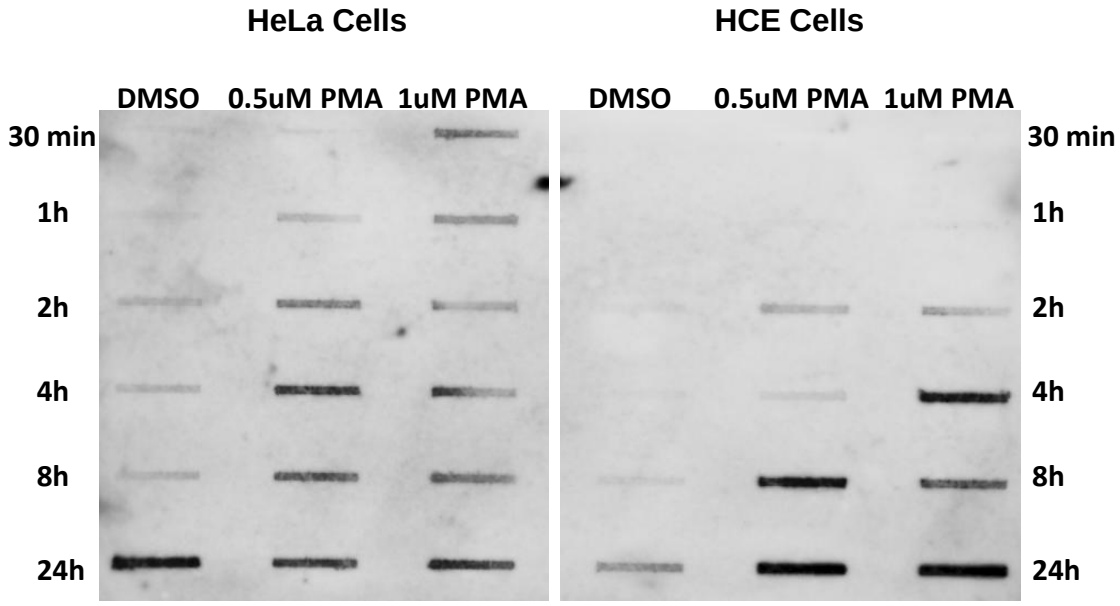


**Figure 24.** PMA treatment enhances syndecan-1 shedding to the culture media. HeLa and HCE cells were serum starved for 16 h. Cells were then treated with PMA (0.5µM and 1µM) in serum free media. At 0.5, 1, 2, 4, 8, and 24 h time points, culture media was collected. Shed syndecan-1 was evaluated by dot blot analysis. Results are representative of two independent experiments.

the Heparanase effect on virus entry into cells with enhanced Heparanase expression. Further analysis of HS cell surface expression level after Heparanase overexpression in the context of HSV-1 infection is required to better understand the role of Heparanase during early events of the infection.

We further studied the effect of enhanced Heparanase expression on HSV-1 plaque formation. Heparanase overexpression resulted in 30% less plaques compared to cells overexpressing an empty vector. Since Heparanase overexpression has no effect on virus entry, but yet induces virus egress significantly, it is plausible that the induced virus release from infected cells disrupted the efficiency of virus plaque formation. Plaque assays were performed with methylcellulose overlaying the cells. Therefore, plaque formation is dependent on virus lateral cell-to-cell spread. In this case, cells with enhanced Heparanase expression have fewer viruses on the cell surface available for lateral cell-to-cell spread, and more virus released to the cell culture media that get trapped in the methylcellulose mush. Plaquing efficiency depends also on the efficiency of virus replication. However, since enhanced Heparanase expression in infected cells showed significantly higher virus titer in cell culture supernatant compared to the cell culture supernatant of control cells transfected with an empty vector, virus replication is probably not affected by inducing Heparanase expression.

This study also demonstrates that HSV-1 infection induces the expression of active Heparanase. The increase in active Heparanase correlated with a decrease in the inactive form of Heparanase, with an increase in total Heparanase. Heparanase exerts enzyme-dependent and enzyme-independent functions. The active form of Heparanase accounts for the HS degrading

enzyme-dependent functions. This suggests that HSV-1 infection induces HS degradation, which might be exploited by the virus to facilitate HSV-1 release, and thus subsequent spread to uninfected cells, and possibly transmission to new host. This effect on Heparanase expression was also observed in HCV infection, where Heparanase expression was elevated in HCV related HCC compared to HCV negative HCC. This observation is important in the sense that not only HSPG on the surface affects HSV-1 infection, but it is also the other way around where HSV-1 modulates HSPG expression levels by inducing syndecans expression, HS expression, as was shown by previous study (Bacsa et al., 2011), as well as Heparanase expression shown in this study. Moreover, this provides an interesting mechanism for the virus to enhance its spread, where it induces Heparanase at later time points of the infection to facilitate virus egress from the cell.

Heparanase modulates HSPG by degrading HS chains, and also by enhancing the shedding of syndecans from the cell surface. Accordingly, we found that enhancing syndecan-1 shedding by treating infected cells with the shedding agonist PMA resulted in an increase in virus release to the cell culture supernatant. This suggests that Heparanase enhances virus release not only through degrading HS chains, but also through increasing syndecans shedding from the surface of the infected cell. In a previous study, PMA and forskolin treatment has been found to trigger active Heparanase secretion. PMA is a strong Protein Kinase C (PKC) inducer, while forskolin is a Protein Kinase A (PKA) inducer. Utilizing PKC and PKA inhibitors; it has been shown that active Heparanase secretion is accelerated by extracellular cues activating PKA and C signaling pathways (Shafat et al., 2006). Therefore, the observed induction of HSV-1 release may also be a result of enhanced active Heparanase secretion.

Interestingly, PKC activation has been reported in HSV-1 infection at 8h and 12 h postinfection. HSV-1 activates and recruits PKC to the inner nuclear membrane to phosphorylate lamin B to help modify the nuclear lamina and mediate budding of nucleocapsids at the inner nuclear membrane during virus egress (Park et al., 2006). Therefore, it is possible that HSV-1 mediated PKC activation facilitates further egress of the viral particles at the cellular plasma membrane by inducing active Heparanase secretion.

Heparanase dependent degradation of HS chains on HSPG affects the integrity and functional state of tissues. Consequently, Heparanase is implicated in several biological and pathological processes. Active Heparanase is expressed by activated immune cells including T cells and macrophages, which upon secretion degrades HS in the ECM and basement membrane facilitating diapedesis. Heparanase also function during embryo implantation where it allows embryo implantation to the maternal uterine epithelia. Degradation of HS chains by Heparanase releases HS bound growth factors making them available for growth factor required events including angiogenesis and wound healing (Elkin et al., 2001, Zhang et al., 2011). In addition, Heparanase dependent degradation of HS chains liberates attached cytokines and chemokines, amplifying the immune reaction (Vaday et al., 2000). Heparanase functions in other biological processes independent of its enzymatic activity. The 65-kD latent Heparanase promotes phosphatidylinositol 3-kinase dependent endothelial cell migration and invasion through the activation of Akt signaling pathway (Gingis-Velitski et al., 2004a). Also, latent Heparanase enhances the expression of the ECM-resident angiogenic growth factor: vascular endothelial growth factor (VEGF) by the activation of Src family (Zetser et al., 2006). It is suggested that

this up-regulation of VEGF by Heparanase could be one mechanism by which Heparanase enhances angiogenesis (Zetser et al., 2006).

Heparanase has been shown to be an important player in tumor progression and metastasis. Heparanase expression has been shown to be elevated in virtually all human carcinomas examined (Barash et al., 2010). This is attributed to enhanced cell dissemination as a result of HS cleavage and remodeling of the ECM and basement membrane underlying epithelial and endothelial cells which act as barrier to tumor cell invasion and spread (Zhang et al., 2011). Furthermore, Heparanase mediates the establishment of a vascular network that promotes primary tumor growth and metastatic cells invasion (Barash et al., 2010). It is possible that HSV-1 exploits Heparanase disseminating functions to induce its spread the same way how tumor cells utilize Heparanase functions to induce their invasion and metastasis.

Further studies are needed to better understand the role and regulation of Heparanase during HSV-1 infection. While several Heparanase inhibitors are under clinical trials as novel anti-cancer therapeutics, it is tempting to study the effect of these inhibitors on HSV-1 infection in *in vivo* model of infection.

# 5. CONCLUSIONS AND FUTURE WORK

Together, the results from our study have provided important data about the contribution and modulation of HSPG during HSV-1 infection. Specifically, our study has improved our understanding of the role of a specific member of the syndecan family of HSPG: syndecan-1 during HSV-1 infection. Syndecan-1 is relatively a common HSPG expressed on target cell types for HSV-1 infection, including corneal epithelium. Moreover, our results show how HSV-1 modulates HSPG, and how these modulations influence HSV-1 infection. Expanding our understanding of the various aspects of HSV-1 infection is critical for better understanding of HSV-1 pathogenesis and transmission. Additionally, the more knowledge we gain on virus' mechanisms of entry, and spread would help in the development a of anti-HSV-1 agents that can target these events, and prevent the infection.

HSPG are known to serve as attachment receptor for HSV-1, while modified HS chains are capable of serving as an entry receptor for HSV-1. In this study, we show that syndecan-1 is actually important for virus penetration. Downregulation of syndecan-1 expression using siRNA treatment results in approximately 36% reduction in HSV-1 entry into HCE cells. This effect on virus entry happens at a step prior to viral nuclear transport, as that was not affected by syndecan-1 expression downregulation. Interestingly, this effect on virus entry occurs at 50% efficiency in syndecan-1 downregulation, indicating that virus entry might decrease even more with a more efficient downregulation of syndecan-1. One of our future directions will be to measure HSV-1 entry into syndecan-1 knockout cells to examine how much syndecan-1 accounts for the viral entry, and whether other syndecan family members overlap with syndecan-1 in its contribution to HSV-1 entry into the host cell.

An interesting observation in our study is that syndecan-1 core protein has an important role during HSV-1 induced cell-to-cell fusion. Inducing the expression of syndecan-1 in HS-deficient cells (CHO-745) showed enhanced cell-to-cell fusion, while the downregulation of syndecan-1 expression in CHO-745 inhibited virus induced cell-to-cell fusion. This study shows for the first time the core protein independently from HS chains being implicated in HSV-1 infection. Similar results were obtained in wild type CHO-K1 cells that have the ability to express HS. These results were strengthened by a syncytial cell assay, where enhancing syndecan-1 expression in CHO-K1 and CHO-745 cells resulted in more syncytia formation compared to cells expressing normal levels of syndecan-1. The mechanism by which syndecan-1 core protein influences HSV-1 mediated cell-to-cell fusion is currently unknown. It is possible that the core protein is involved in signaling pathways that result in the induction of cell-to-cell fusion. Pathways including Rho-GTPases activation that leads to the cytoskeleton reorganization have been suggested to be important in HSV-1 induced cell-to-cell fusion. Further analysis of the role of Rho-GTPases during virus mediated cell-to-cell fusion in the context of syndecan-1 downregulation and overexpression will be an important future direction to better understand the mechanism by which syndecan-1 affects cell fusion.

One observation of critical importance is that syndecan-1 overexpression on effector cells, which express HSV-1 fusion glycoproteins (gB, gD, gH, and gL) during virus free cell fusion assay, results in significant reduction in HSV-1 induced cell-to-cell fusion. Using a panel of syndecan-1 mutants, this reduction in cell fusion was mapped to both the cytoplasmic domain and the ectodomain of syndecan-1. This result was observed in both CHO-K1 cells, and the HS-deficient CHO-745 cells. This result raises several possibilities including a possible direct

interaction between syndecan-1 core protein and at least one of HSV-1 fusion glycoproteins, making these glycoproteins less available for cell fusion. If this possibility is proven true, it will be a direct evidence for syndecan-1 core protein involvement in HSV-1 infection. Another possibility to explain our results is that syndecan-1 core protein interacts indirectly with HSV-1 fusion glycoproteins through physically binding another cellular protein(s). This interaction might result in sequestering HSV-1 fusion glycoproteins, or sequestering that cellular protein from functioning in the fusion process. It is also possible that overexpressing syndecan-1 with HSV-1 fusion glycoproteins on effector cells interferes with signaling pathways that are important during cell fusion, affecting its efficiency. Further work is needed to test these possibilities, in order to better explain our results.

More studies will help understand the role that syndecan-1 core protein plays during HSV-1 induced cell-to-cell fusion. Our study has already made the initial observation that syndecan-1 core protein interacts in some way with HSV-1 fusion glycoproteins. Studying the activation of signaling pathways by the interaction between syndecan-1 core protein and HSV-1 fusion glycoproteins during virus mediated cell-to-cell fusion is a fascinating area that would shed light on the mechanism by which syndecan-1 core protein affects cell fusion. Syndecan-1 core protein has been shown to be involved in several biological processes including actin cytoskeleton reorganization, cell adhesion, cell proliferation, and angiogenesis, many of which have been implicated in HSV-1 infection (O'Donnell et al., 2009, Carey et al., 1994, Beauvais et al., 2004, Xian et al., 2010, Bernfield et al., 1999, Zong et al., 2010, Oh et al., 2010a, Oh et al., 2010b, Zhou et al., 2009, Zheng et al., 2001).

In addition to syndecan-1 role in HSV-1 spread by inducing virus mediated cell-to-cell fusion, our study provides evidence for the importance of syndecan-1 during virus lateral cell-to-cell spread. While syndecan-1 overexpression resulted in larger HSV-1 plaques, the downregulation of syndecan-1 expression resulted in smaller plaques. Plaque sizes were measured while methylcellulose was added to the cells, thus virus spread is exclusively through cell-to-cell spread. The role of syndecan-1 was strengthened by a spread assay. Cells were infected with HSV-1 and the bound virus inactivated by citrate buffer (PH=3.0) treatment were co-cultured with the cells transfected with either control scrambled siRNA or syndecan-1 siRNA. Virus spread to syndecan-1 downregulated cells was significantly lower compared to control scrambled transfected cells. The mechanism by which syndecan-1 affects lateral cell-to-cell spread is not clear yet. HSV-1 lateral cell-to-cell spread share common requirements with virus entry including the requirement of glycoproteins gB, gD, gH, and gL, as well as a gD receptor (Mettenleiter et al., 2003). However, Lateral virus cell-to-cell spread has other players including the glycoproteins gE-gI heterodimer, and gK (Johnson et al., 2001, David et al., 2008). Since our study shows that syndecan-1 is important in virus entry, it is possible that syndecan-1 affects cell-to-cell spread by the same mechanism it affects virus entry. Further studies are needed to identify the mechanism by which syndecan-1 affects virus lateral cell-to-cell spread. Interestingly, a study has shown that the interaction between HSV-1 gB and HS is important for plaque formation, as a mutant gB that lacks a lysine-rich (pK) sequence which is important for gB interaction with HS, resulted in the formation of smaller HSV-1 plaques compared to wild type virus. This effect on plaque size was not observed in mutant virus with gC deletion (Laquerre et al., 1998).

Syndecan-1 downregulation not only resulted in smaller virus plaques, but also resulted in fewer plaques compared to cells transfected with control scrambled siRNA. Plaquing efficiency depends on virus entry, cell-to-cell spread, and replication. Therefore, fewer plaques after syndecan-1 downregulation could be explained by the reduced viral entry and cell-to-cell spread. However, this does not exclude the possibility that at least part of the observed reduction in plaquing efficiency after syndecan-1 downregulation might be due to less HSV-1 replication. Less infectious virus production following the downregulation of syndecan-1 expression might also be explained by the same way, as it could be due to reduced entry, virus induced cell-to-cell fusion, and cell-to-cell spread, and possibly due to a reduction in virus replication. Interestingly, while syndecan-1 downregulation reduced plaquing efficiency, syndecan-1 overexpression showed no significant change compared to control GFP transfected cells. This observation is critical as it gives some insight to the possible mechanism of action of syndecan-1 during HSV-1 plaque formation. One possible explanation is that overexpressing syndecan-1 results in enhancing the expression of syndecan-1 that does not carry HS chains, or that carries partially modified HS chains when fully modified HS chains are required for efficient plaque formation. Another possibility is that syndecan-1 might be a component of a multi-protein complex, and thus enhancing syndecan-1 expression alone is not enough to induce plaquing efficiency since other components are not overexpressed. Another way to explain the absence of enhancing HSV-1 plaquing efficiency after syndecan-1 overexpression is that syndecan-1 might be involved in a signaling pathway that is important for virus plaque formation. In this case, a reduction in syndecan-1 expression would result in lower plaquing efficiency, because of reduced/lost signal, while enhancing syndecan-1 expression would not affect signal transduction during plaque

formation. Further work must be done to determine the mechanism by which syndecan-1 affects plaquing efficiency.

Heparanase modulates HSPG by degrading HS chains, transforming syndecans into highly selective surface binding protein, mediating syndecan-1 internalization, and by enhancing the shedding of HSPG including syndecan-1 from the cell surface (Ma et al., 2006, Gingis-Velitski et al., 2004b, Yang et al., 2007). These modifications on HSPG by Heparanase affect the expression and function of HSPG on the cell surface. The results in this study show that changing syndecan-1 cell surface expression by transfecting cells with syndecan-1 expression plasmid, or syndecan-1 specific siRNA affects the various aspects of HSV-1 infection. Studies have shown that HSV-1 infection modulates HSPG expression on the cell surface. Following HSV-1 infection, syndecan-1 cell surface expression, and protein synthesis, and HS chains cell surface levels were all elevated (Bacsa et al., 2011). This raises the critical question of what is the effect of Heparanase role in modulating HSPG during HSV-1 infection.

A novel function of Heparanase during HSV-1 infection based on our study is its involvement in virus release from the cell. Cells with enhanced Heparanase expression showed significant increase in virus release compared to cells expressing an empty vector. This enhancement in virus egress was observed at time points after 12 h post infection. This suggests that measured virus titers in these experiments are titers of virus progeny not from the initial infection. This function was further examined by applying exogenous Heparinases to infected cells, where virus release was shown to be significantly enhanced by this treatment. These results suggest that Heparanase facilitates the virus release from already infected cell, to reach other far

away uninfected cell. Since these results were shown in two different cell lines, this effect of Heparanase is probably cell type unspecific.

This is the first report of the involvement of HSPG during virus release from the cell. Previously, HSPG function during HSV-1 infection has been focused on its contribution at early viral infection events including attachment and entry. This indicates that HSV-1 has the ability to utilize the same cell surface receptor during its entry process and egress. Additionally, Heparanase role in HSV-1 egress provides an important spread mechanism for the virus. Viral progeny benefit from leaving an already infected and probably dying cell, to reach a healthy cell to start a new round of infection. Moreover, participation of Heparanase in virus egress might facilitate virus transmission from one host to another. In fact, asymptomatic virus release from infected sites is a significant risk for transmission between hosts (Sacks et al., 2004). Viral culture studies have shown that HSV-1 was shed in the oral cavity 6% of the time (Sacks et al., 2004). Interestingly, HSV-1 asymptomatic shedding rate increases with immunosuppression (Sacks et al., 2004). Since HSPGs serve as an attachment receptor for many viruses including HCV, human papillomavirus, and Dengue virus, it would be interesting to test whether HSPGs are also involved in the release of these viruses from infected cells (Barth et al., 2003, Giroglou et al., 2001, Chen et al., 1997).

Our study has further characterized the effect of modulating HSPG by Heparanase on HSV-1 infection. Enhancing Heparanase expression has no effect on HSV-1 entry into cells. This might be explained by the previously reported induction in syndecans, and HS chains expression on the cell surface following HSV-1 infection. Therefore, it is plausible that the

increased expression of HSPG after HSV-1 infection masked the effect of enhancing Heparanase expression which resulted in no change in viral entry into cells. Also, it is possible that although Heparanase expression is enhanced, its function on HSPG is regulated in somehow, so that at early events of virus infection, it does not exert its functions. Interestingly, Heparanase overexpression resulted in a moderate 30% reduction in HSV-1 plaquing efficiency. Induced virus egress after Heparanase overexpression might have resulted in inefficient plaque formation, as virus particles were released from infected cells rather than being involved in plaque formation. Since plaque assays were performed in methylcellulose medium, released virus has a limited ability to move freely in the culture medium to reach and infect far away cells. Thus, plaque formation is dependent solely on cell-to-cell spread. Virus replication influences plaque formation. However, it's probably unaffected by Heparanase overexpression because of the detected increase in virus release observed in cells with enhanced Heparanase expression compared to cell expressing an empty vector.

An observation of great significance in this study is that HSV-1 infection induces the levels of active Heparanase. Since HSV-1 utilizes HSPG in early steps of the infection including attachment as well as in later steps including virus egress, it has to regulate its interaction with HSPG throughout the infection. This might be achieved by regulating Heparanase expression and activity in infected cells. While Heparanase expression levels in epithelial cells are almost undetected under normal conditions, its expression levels are elevated in all major types of human cancers (Fux et al., 2009, Arvatz et al., 2011). Using Western Blot analysis to cell lysates from infected cell, we show that active Heparanase expression level was elevated in HSV-1 infected cells, compared to uninfected cells. This elevation in active Heparanase was associated

with a reduction in the in-active Heparanase expression after the infection, with an increase in the total expression of Heparanase. This effect on active Heparanase expression might be one of the mechanisms by which HSV-1 exploits Heparanase activity to induce viral egress and spread from infected cell. However, how the virus affects Heparanase expression and activation is still not known. Previously, it has been reported that Heparanase expression was significantly higher in Hepatitis C virus (HCV) related hepatocellular carcinoma (HCC) compared with HCC patients who are HCV-negative (Dong et al. 2010, El-Assal et al., 2001). This suggests that regulating Heparanase expression might be a general mechanism used by some pathogens during infection. Future directions will include studying the mechanism by which HSV-1 induces Heparanase expression and activation, which will improve our understanding of Heparanase physiology as well.

Furthermore, our study suggests that enhanced virus release from infected cells by Heparanase overexpression and exogenous Heparinases treatment is a result of Heparanase activity not only degrading HS chains but also enhancing HSPG shedding. Treating HSV-1 infected cells with a shedding agonist PMA resulted in elevated virus release compared to mock treated cells. This suggests that enhanced syndecan shedding is capable of enhancing virus release from the cell. Therefore, HSV-1 utilizes HSPG as an attachment receptor, an important factor for virus spread, and a regulator of virus release from infected cell.

Interestingly, in a previous study, PMA and forskolin treatment has been found to induce active Heparanase secretion. PMA is a strong Protein Kinase C (PKC) inducer, while forskolin is

a Protein Kinase A (PKA) inducer. Utilizing PKC and PKA inhibitors; it has been shown that active Heparanase secretion is accelerated by extracellular cues activating PKA and C signaling pathways (Shafat et al., 2006). Therefore, the observed induction of HSV-1 release may also be explained by enhanced active Heparanase secretion. PKC activation has been reported in HSV-1 infection at 8h and 12 h postinfection. HSV-1 activates and recruits PKC to the inner nuclear membrane to phosphorylate lamin B to help modify the nuclear lamina and mediate budding of nucleocapsids at the inner nuclear membrane during virus egress (Park et al., 2006). Therefore, it is possible that HSV-1 mediated PKC activation facilitates further egress of the viral particles at the cellular plasma membrane by inducing active Heparanase secretion.

To summarize, our study has provided new insight into the role of syndecan-1 during HSV-1 infection. Although previously HSPGs were thought to function through their HS chains in virus attachment and entry, our study provides evidence that syndecan-1 core protein is important during virus mediated cell-to-cell fusion. Furthermore, we have shown that syndecan-1 contributes to HSV-1 entry, and cell-to-cell spread. This study also provides information on how HSV-1 exploits Heparanase activity to modulate HSPG on the cell surface to enhance infectious virus release from infected cells, which can engage in further virus spread.

Our model of HSPG modulation during HSV-1 infection is illustrated in Figure 25. This model is based on previous results from our lab, published literature, and the knowledge attained from this study. HSV-1 entry into the host cell induces the protein expression and cell surface expression of syndecans including syndecan-1. This is associated with an increase in HS cell surface expression. The enhancement of syndecan-1 results in more efficient virus induced cell-

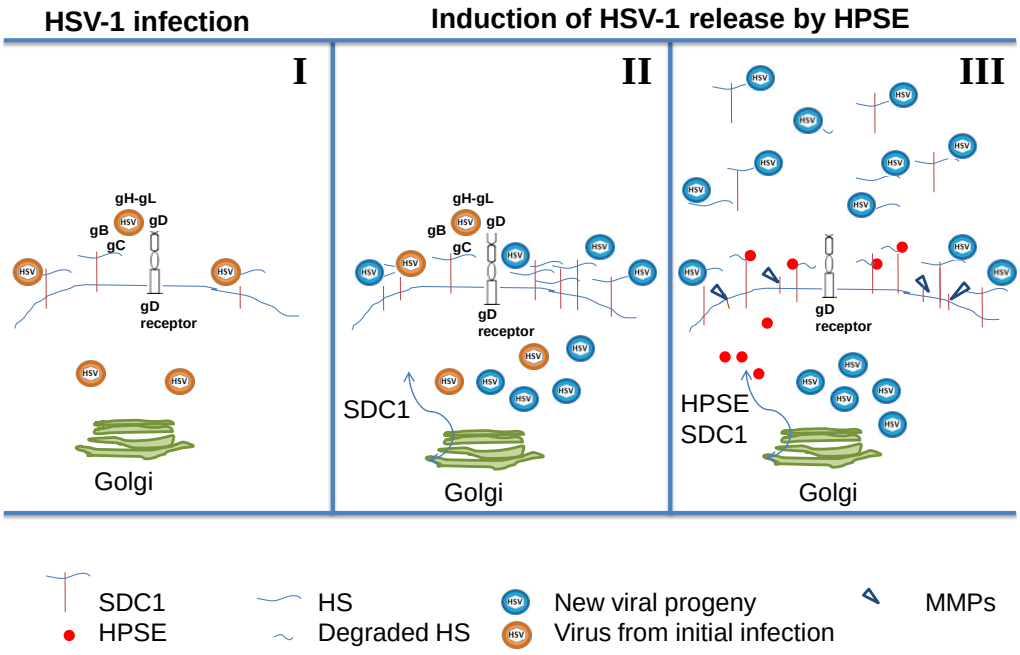


**Figure 25.** Analysis of Heparanase induced herpes simplex virus type-1 (HSV-1) release from infected host cell. This model is based on previous results from our lab, published literature, and the knowledge attained from this study. HSV-1 is believed to modulated Heparan sulfate proteoglycans (HSPG) in order to facilitate efficient viral infection and spread. During this process: (I.) HSV-1 (Orange particles) attaches to HSPG presented here as syndecan-1 (SDC1) through its glycoproteins gB and gC. This is followed by gD interaction with a gD receptor on the cell surface. This trigger viral envelope fusion with the cellular plasma membrane in the presence of gD, gB, gH-gL and a gD receptor. (II.) HSV-1 infection results in an early upregulation of syndecan-1 protein synthesis and cell surface expression as well as Heparan sulfate (HS) cell surface expression (as soon as 2 h post infection). New viral progeny attach to cell surface syndecan-1. The enhancement of syndecan-1 results in more efficient virus induced cell-to-cell fusion, as well as viral cell-to-cell spread. (III.) HSV-1 infection induces the expression of active Heparanase, and the release of active Heparanase from the cell. Active Heparanase release is mediated by PKA and PKC activation by HSV-1 infection. This in turn results in HS degradation, and syndecans shedding. Several metalloproteinases (MMPs) have been shown to promote syndecan-1 shedding. The effects of Heparanase enzyme on HSPG result in more efficient virus release from infected cells to the ECM, enhancing virus spread, and transmission.

to-cell fusion, as well as viral cell-to-cell spread. Following successful virus replication and spread, HSV-1 induces Heparanase expression and active Heparanase release from the cell. Active Heparanase release is mediated by PKA and PKC activation by HSV-1 infection. This in turn results in HS degradation, and syndecans shedding. Several metalloproteinases (MMPs) have been shown to promote syndecan-1 shedding (Fitzgerald et al., 2000). The effects of Heparanase enzyme on HSPG result in more efficient virus release from infected cells to the ECM, enhancing virus spread, and transmission.

Future directions for the role of syndecan-1 during HSV-1 infection include characterization of the mechanism by which syndecan-1 contributes to HSV-1 entry, virus mediated cell-to-cell fusion and cell-to-cell spread. Syndecan-1 has the ability to bind to various molecules, and has been implicated in various biological events. Therefore, future work should include examining signaling pathways that are important for syndecan-1 functions, and known to be activated during HSV-1 infection. One such signaling pathway is the reorganization of actin cytoskeleton through the activation of Rho GTPases. Additionally, our study suggests possible interaction between the ectodomain and the cytoplasmic domain of syndecan-1 core protein with at least one of HSV-1 fusion glycoproteins. Thus, it is of critical importance to understand this interaction between syndecan-1 and HSV-1 fusion glycoproteins because this may help reveal the mechanism of action of syndecan-1 during HSV-1 infection, and possibly identify more players in the infection process.

Future directions for the role of Heparanase during HSV-1 infection include an examination of the proposed model of Heparanase induced HSV-1 release from infected host cell. Specifically, evaluation of the temporal expression of syndecan-1 and Heparanase throughout the infection would greatly enhance our understanding of the contribution of these proteins during the infection. Moreover, syndecan-1 and HSV-1 glycoproteins share post-translational processing in the ER and Golgi. It would be interesting to examine whether syndecan-1 and HSV-1 glycoproteins interact in these intracellular organelles during the infection. This requires a temporal evaluation of syndecan-1 and HSV-1 glycoporteins colocalization using markers for the ER and Golgi compartments. Furthermore, our model suggests that HSV-1 induces active Heparanase release from the cell through PKC activation as another mechanism by which HSV-1 exploits Heparanase to induce its release from the cell. Additionally, our model suggests that Heparanase induces HSV-1 release through HS chains degradation via enhancing syndecan-1 shedding. This implies that HSV-1 infection, by inducing active Heparanase expression, induces syndecan-1 shedding. Therefore, evaluating syndecan-1 shedding in HSV-1 infected cells and the critical MMPs in this process would greatly support our results. Furthermore, it would be of great importance to evaluate whether HSPG contribution to HSV-1 egress by Heparanase is specific to this virus, or a shared mechanism between many viruses that use HSPGs as attachment receptors.

# CITED LITERATURE


Abboud-Jarrous, G., Atzmon, R., Peretz, T., Palermo, C., Gadea, B.B., Joyce, J.A., Vlodavsky, I.: Cathepsin L is responsible for processing and activation of proheparanase through multiple cleavages of a linker segment. J Biol Chem 283;18167-18176: 2008.

Akhtar, J., Shukla, D.: Viral entry mechanisms: cellular and viral mediators of herpes simplex virus entry. FEBS J 276;7228-7236: 2009.

Araki-Sasaki, K., Ohashi, Y., Sasabe, T., Hayashi, K., Watanabe, H., Tano, Y., Handa, H.: An SV40-immortalized human corneal epithelial cell and its characterization. Invest Ophthalmol Vis Sci 36;614-621: 1995.

Arii, J., Goto, H., Suenaga, T., Oyama, M., Kozuka-Hata, H., Imai, T., Minowa, A., Akashi, H., Arase, H., Kawaoka, Y., Kawaguchi, Y.: Non-muscle myosin IIA is a functional entry receptor for herpes simplex virus-1. Nature 467;859-862: 2010.

Arvatz, G., Shafat, I., Levy-Adam, F., Ilan, N., Vlodavsky, I.: The heparanase system and tumor metastasis: is heparanase the seed and soil? Cancer Metastasis Rev 30253-268: 2011.

Atanasiu, D., Saw, W.T., Cohen, G.H., Eisenberg, R.J.: Cascade of events governing cell-cell fusion induced by herpes simplex virus glycoproteins gD, gH/gL, and gB. J Virol 84;12292-12299: 2010.

Atanasiu, D., Whitbeck, J.C., Cairns, T.M., Reilly, B., Cohen, G.H., Eisenberg, R.J.: Bimolecular complementation reveals that glycoproteins gB and gH/gL of herpes simplex virus interact with each other during cell fusion. Proc Natl Acad Sci USA 104;18718-18723: 2007.

Avitabile, E., Forghieri, C., Campadelli-Fiume, G.: Complexes between herpes simplex virus glycoproteins gD, gB, and gH detected in cells by complementation of split enhanced green fluorescent protein. J Virol, 81;11532-11537: 2007.

Avitabile, E., Lombardi, G., Campadelli-Fiume, G.: Herpes simplex virus glycoprotein K, but not its syncytial allele, inhibits cell-cell fusion mediated by the four fusogenic glycoproteins, gD, gB, gH, and gL. J Virol 77;6836-6844: 2003.

Bacsa, S., Karasneh, G., Dosa, S., Liu, J., Valyi-Nagy, T., Shukla, D.: Syndecan-1 and syndecan-2 play key roles in herpes simplex virus type 1 infection. J Gen Virol 92;733–743: 2011.

Barash, U., Cohen-Kaplan, V., Dowek, I., Sanderson, R.D., Ilan, N., Vlodavsky, I.: Proteoglycans in health and disease: new concepts for heparanase function in tumor progression and metastasis. FEBS J 277;3890-3903: 2010.

Barth, H., Schafer, C., Adah, M.I., Zhang, F., Linhardt, R.J., Toyoda, H., Kinoshita-Toyoda, A., Toida, T., Van Kuppevelt, T.H., Depla, E., Von Weizsacker, F., Blum, H.E., Baumert,

T.F.: Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J Biol Chem 278;41003-41012: 2003.

Beauvais, D.M., Ell, B.J., McWhorter, A.R., Rapraeger, A.C.: Syndecan-1 regulates alphavbeta3 and alphavbeta5 integrin activation during angiogenesis and is blocked by synstatin, a novel peptide inhibitor. J Exp Med 206;691–705: 2009.

Beauvais, D.M., Rapraeger, A.C.: Syndecans in tumor cell adhesion and signaling. Reprod Biol Endocrinol 2;3: 2004.

Bernfield, M., Götte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., Lincecum, J., Zako, M.: Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem 68;729–777:1999.

Bobardt, M.D., Saphire, A.C., Hung, H.C., Yu, X., Van der Schueren, B., Zhang**,** Z**.**, David**,** G**.**, Gallay, P.A.: Syndecan captures, protects, and transmits HIV to T lymphocytes. Immunity 18;27–39: 2003.

Campadelli-Fiume, G., Amasio, M., Avitabile, E., Cerretani, A., Forghieri, C., Gianni, T., Menotti, L.: The multipartite system that mediates entry of herpes simplex virus into the cell. Rev Med Virol 17;313-326: 2007.

Campadelli-Fiume, G., Cocchi, F., Menotti, L., Lopez, M.: The novel receptors that mediate the entry of herpes simplex viruses and animal alphaherpesviruses into cells. Rev Med Virol 10;305-319: 2000.

Campadelli-Fiume, G.: The egress of alphaherpesviruses from the cell. p. 151–163. *In* A. Arvin, G. Campadelli-Fiume, E. Mocarski, P. S. Moore, B. Roizman, R. Whitley, and K. Yamanishi (ed.), Human herpesviruses: biology, therapy, and immunoprophylaxis Cambridge University Press, Cambridge, United Kingdom: 2007.

Carey, D.J., Bendt, K.M., Stahl, R.C.: The cytoplasmic domain of syndecan-1 is required for cytoskeleton association but not detergent insolubility. Identification of essential cytoplasmic domain residues. J Biol Chem 271;15253-15260: 1996.

Carey, D.J., Stahl, R.C., Cizmeci-Smith, G., Asundi, V.K. Syndecan-1 expressed in Schwann cells causes morphological transformation and cytoskeletal reorganization and associates with actin during cell spreading. J Cell Biol 124;161–170: 1994.

Carfí, A., Willis, S.H., Whitbeck, J.C., Krummenacher, C., Cohen, G.H., Eisenberg, R.J., Wiley, D.C.: Herpes simplex virus glycoprotein D bound to the human receptor HveA. Mol Cell 8;169-179: 2001.

Chen, Y., Maguire, T., Hileman, R.E., Fromm, J.R., Esko, J.D., Linhardt, R.J., Marks, R.M.: Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat Med 3;866–871: 1997.

Chowdary, T.K., Cairns, T.M., Atanasiu, D., Cohen, G.H., Eisenberg, R.J., Heldwein, E.E.: Crystal structure of the conserved herpesvirus fusion regulator complex gH-gL. Nat Struct Mol Biol, 17;882-888: 2010.

Couchman, J.R.: Syndecans: proteoglycan regulators of cell-surface microdomains? Nat Rev Mol Cell Biol 4;926–937: 2003.

Cocchi, F., Fusco, D., Menotti, L., Gianni, T., Eisenberg, R.J., Cohen, G.H., Campadelli-Fiume, G.: The soluble ectodomain of herpes simplex virus gD contains a membrane-proximal pro-fusion domain and suffices to mediate virus entry. Proc Natl Acad Sci USA 101;7445-7450: 2004.

Connolly, S.A., Jackson, J.O., Jardetzky, T.S., Longnecker, R.: Fusing structure and function: a structural view of the herpesvirus entry machinery. Nat Rev Microbiol 9;369-381: 2011.

Couchman, J.R.: Transmembrane signaling proteoglycans. Annu Rev Cell Dev Biol 26;89–114: 2010.

David, A.T., Baghian, A., Foster, T.P., Chouljenko, V.N., Kousoulas, K.G.: The herpes simplex virus type 1 (HSV-1) glycoprotein K(gK) is essential for viral corneal spread and neuroinvasiveness. Curr Eye Res 33;455-467: 2008.

Davis-Poynter, N., Bell, S., Minson, T., Browne, H.: Analysis of the contributions of herpes simplex virus type 1 membrane proteins to the induction of cell-cell fusion. J Virol 68;7586-7590: 1994.

Day, P.M., Gambhira, R., Roden, R.B., Lowy, D.R., Schiller, J.T.: Mechanisms of human papillomavirus type 16 neutralization by l2 cross-neutralizing and l1 type-specific antibodies. J Virol 82;4638-4646: 2008.

Desai, P., Person, S. Incorporation of the green fluorescent protein into the herpes simplex virus type 1 Capsid. J Virol 72;7563–7568: 1998.

Diefenbach, R.J., Miranda-Saksena, M., Douglas, M.W., Cunningham, A.L.: Transport and egress of herpes simplex virus in neurons. Rev Med Virol 18;35-51: 2008.

Dong, S., Wu, X.Z.: Heparanase and hepatocellular carcinoma: promoter or inhibitor? World J Gastroenterol 16;306-311: 2010.

Eizuru, Y.: Development of new antivirals for herpesviruses. Antivir Chem Chemother. 14;299-308: 2003.

El-Assal, O.N., Yamanoi, A., Ono, T., Kohno, H., Nagasue, N.: The clinicopathological significance of heparanase and basic fibroblast growth factor expressions in hepatocellular carcinoma. Clin Cancer Res 7;1299-1305: 2001.

Elkin, M., Ilan, N., Ishai-Michaeli, R., Friedmann, Y., Papo, O., Pecker, I., Vlodavsky, I.: Heparanase as mediator of angiogenesis: mode of action. FASEB J 15;1661-1663: 2001.

Esko, J.D., Rostand, K.S., Weinke, J.L.: Tumor formation dependent on proteoglycan biosynthesis. Science 241;1092–1096: 1988.

Even, D.L., Henley, A.M., Geraghty, R.J.: The requirements for herpes simplex virus type 1 cell-cell spread via nectin-1 parallel those for virus entry. Virus Res 119;195-207: 2006.

Fairbanks, M.B., Mildner, A.M., Leone, J.W., Cavey, G.S., Mathews, W.R., Drong, R.F., Slightom, J.L., Bienkowski, M.J., Smith, C.W., Bannow, C.A., Heinrikson, R.L.: Processing of the human heparanase precursor and evidence that the active enzyme is a heterodimer. J Biol Chem 274;29587-29590: 1999.

Farhatullah, S., Kaza, S., Athmanathan, S., Garg, P., Reddy, S.B., Sharma, S.: Diagnosis of herpes simplex virus-1 keratitis using Giemsa stain, immunofluorescence assay, and polymerase chain reaction assay on corneal scrapings. Br J Ophthalmol 88;142-144: 2004.

Farooq, A.V., Shukla, D.: Corneal latency and transmission of herpes simplex virus-1. Future Virol 6;101–108: 2011.

Fears, C.Y., Woods, A.: The role of syndecans in disease and wound healing. Matrix Biol 25;443-456: 2006.

Filla, M.S., David, G., Weinreb, R.N., Kaufman, P.L., Peters, D.M.: Distribution of syndecans 1-4 within the anterior segment of the human eye: expression of a variant syndecan-3 and matrix-associated syndecan-2. Exp Eye Res 79;61–74: 2004.

Fitzgerald, M.L., Wang, Z., Park, P.W., Murphy, G., Bernfield, M.: Shedding of syndecan-1 and -4 ectodomains is regulated by multiple signaling pathways and mediated by a TIMP-3-sensitive metalloproteinase. J Cell Biol 148;811-824: 2000.

Freissler, E., Meyer auf der Heyde, A., David, G., Meyer, T.F., Dehio, C.: Syndecan-1 and syndecan-4 can mediate the invasion of OpaHSPG-expressing Neisseria gonorrhoeae into epithelial cells. Cell Microbiol 2;69-82: 2000.

Fusco, D., Forghieri, C., Campadelli-Fiume, G.: The pro-fusion domain of herpes simplex virus glycoprotein D (gD) interacts with the gD N terminus and is displaced by soluble forms of viral receptors. Proc Natl Acad Sci USA 102;9323-9328: 2005.

Fux, L., Ilan, N., Sanderson, R.D., Vlodavsky, I.: Heparanase: busy at the cell surface. Trends Biochem Sci 34;511-519: 2009.

Galdiero, S., Vitiello, M., D'Isanto, M., Falanga, A., Cantisani, M., Browne, H., Pedone, C., Galdiero, M.: The identification and characterization of fusogenic domains in herpes virus glycoprotein B molecules. Chembiochem 9;758-767: 2008.

Gallagher, J.T., Lyon, M., Steward, W.P.: Structure and function of heparan sulphate proteoglycans. Biochem J 236;313-325: 1986.

Geraghty, R.J., Jogger, C.R., Spear, P.G.: Cellular expression of alphaherpesvirus gD interferes with entry of homologous and heterologous alphaherpesviruses by blocking access to a shared gD receptor. Virology 268;147–158: 2000.

Geraghty, R.J., Krummenacher, C., Cohen, G.H., Eisenberg, R.J., Spear, P.G.: Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. Science 280;1618-1620: 1998.

Gerber, S.I., Belval, B.J., Herold, B.C.: Differences in the role of glycoprotein C of HSV-1 and HSV-2 in viral binding may contribute to serotype differences in cell tropism. Virology 214;29-39: 1995.

Gianni, T., Amasio, M., Campadelli-Fiume, G.: Herpes simplex virus gD forms distinct complexes with fusion executors gB and gH/gL in part through the C-terminal profusion domain. J Biol Chem 284;17370-17382: 2009.

Gianni, T., Gatta, V., Campadelli-Fiume, G.: {alpha}V{beta}3-integrin routes herpes simplex virus to an entry pathway dependent on cholesterol-rich lipid rafts and dynamin2. Proc Natl Acad Sci U S A 107;22260–22265: 2010.

Gingis-Velitski, S., Zetser, A., Flugelman, M.Y., Vlodavsky, I., Ilan, N.: Heparanase induces endothelial cell migration via protein kinase B/Akt activation. J Biol Chem 279;23536-23541: 2004.b

Gingis-Velitski, S., Zetser, A., Kaplan, V., Ben-Zaken, O., Cohen, E., Levy-Adam, F., Bashenko, Y., Flugelman, M.Y., Vlodavsky, I., Ilan, N.: Heparanase uptake is mediated by cell membrane heparan sulfate proteoglycans. J Biol Chem 279;44084-44092: 2004.

Giroglou, T., Florin, L., Schäfer, F., Streeck, R.E., Sapp, M.: Human papillomavirus infection requires cell surface heparan sulfate. J Virol 75;1565-1570: 2001.

Gruenheid, S., Gatzke, L., Meadows, H., Tufaro, F.: Herpes simplex virus infection and propagation in a mouse L cell mutant lacking heparan sulfate proteoglycans. J Virol 67;93-100: 1993.

Handel, T.M., Johnson, Z., Crown, S.E., Lau, E.K., Proudfoot, A.E.: Regulation of protein function by glycosaminoglycans-as exemplified by chemokines. Annu Rev Biochem 74;385-410: 2005.

Handler, C.G., Eisenberg, R.J., Cohen, G.H.: Oligomeric structure of glycoproteins in herpes simplex virus type 1. J Virol 70;6067-6070: 1996.

Hannah, B.P., Cairns, T.M., Bender, F.C., Whitbeck, J.C., Lou, H., Eisenberg, R.J., Cohen, G.H.: Herpes simplex virus glycoprotein B associates with target membranes via its fusion loops. J Virol 83;6825-6836: 2009.

Harris, B.Z., Lim, W.A.: Mechanism and role of PDZ domains in signaling complex assembly. J Cell Sci 114(Pt 18);3219-3231: 2001.

Hayashida, A., Amano, S., Park, P.W.: Syndecan-1 promotes Staphylococcus aureus corneal infection by counteracting neutrophil-mediated host defense. J Biol Chem 286;3288-3297: 2011.

Hayashida, K., Johnston, D.R., Goldberger, O., Park, P.W.: Syndecan-1 expression in epithelial cells is induced by transforming growth factor beta through a PKA-dependent pathway. J Biol Chem 281;24365-24374: 2006.

Hayashida, K., Parks, W.C., Park, P.W.: Syndecan-1 shedding facilitates the resolution of neutrophilic inflammation by removing sequestered CXC chemokines. Blood 114;3033-3043: 2009.

Hayashida, K., Stahl, P.D., Park, P.W.: Syndecan-1 ectodomain shedding is regulated by the small GTPase Rab5. J Biol Chem 283;35435-35444: 2008.

Haynes, A. 3rd, Ruda, F., Oliver, J., Hamood, A.N., Griswold, J.A., Park, P.W., Rumbaugh, K.P.: Syndecan 1 shedding contributes to Pseudomonas aeruginosa sepsis. Infect Immun 73;7914-7921: 2005.

Heldwein, E.E., Krummenacher, C.: Entry of herpesviruses into mammalian cells. Cell Mol Life Sci, 65;1653-1668: 2008.

Heldwein, E.E., Lou, H., Bender, F.C., Cohen, G.H., Eisenberg, R.J., Harrison, S.C.: Crystal structure of glycoprotein B from herpes simplex virus 1. Science 313;217-220: 2006.

Herold, B.C., Spear, P.G.: Neomycin inhibits glycoprotein C (gC)-dependent binding of herpes simplex virus type 1 to cells and also inhibits postbinding events in entry. Virology 203;166-171: 1994.b

Herold, B.C., Visalli, R.J., Susmarski, N., Brandt, C.R., Spear, P.G.: Glycoprotein Cindependent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. J Gen Virol 75;1211-1222: 1994.

Herpetic Eye Disease Study Group: Acyclovir for the prevention of recurrent herpes simplex virus eye disease. N Engl J Med 339;300-306: 1998.

Hu, C., Ahmed, M., Melia, T.J., Söllner, T.H., Mayer, T., Rothman, J.E.: Fusion of cells by flipped SNAREs. Science 300;1745–1749. 2003.

Inatani, M., Irie, F., Plump, A.S., Tessier-Lavigne, M., Yamaguchi, Y.: Mammalian brain morphogenesis and midline axon guidance require heparan sulfate. Science 302;1044-1046: 2003.

Ishikawa, T., Kramer, R.H.: Sdc1 negatively modulates carcinoma cell motility and invasion. Exp Cell Res 316;951-965: 2010.

Jackson, J.O., Longnecker, R.: Reevaluating herpes simplex virus hemifusion. J Virol 84;11814-11821: 2010.

Jenssen, H.: Anti herpes simplex virus activity of lactoferrin/lactoferricin -- an example of antiviral activity of antimicrobial protein/peptide. Cell Mol Life Sci 62;3002-3013: 2005.

Jenssen, H., Sandvik, K., Andersen, J.H., Hancock, R.E., Gutteberg, T.J.: Inhibition of HSV cell-to-cell spread by lactoferrin and lactoferricin. Antiviral Res 79;192-198: 2008.

Johnson, D.C., Webb, M., Wisner, T.W., Brunetti, C.: Herpes simplex virus gE/gI sorts nascent virions to epithelial cell junctions, promoting virus spread. J Virol 75;821-833: 2001.

Kato, M., Wang, H., Bernfield, M., Gallagher, J.T., Turnbull, J.E.: Cell surface syndecan-1 on distinct cell types differs in fine structure and ligand binding of its heparan sulfate chains. J Biol Chem 269;18881-18890: 1994.

Krummenacher, C., Nicola, A.V., Whitbeck, J.C., Lou, H., Hou, W., Lambris, J.D., Geraghty, R.J., Spear, P.G., Cohen, G.H., Eisenberg, R.J.: Herpes simplex virus glycoprotein D can bind to poliovirus receptor-related protein 1 or herpesvirus entry mediator, two structurally unrelated mediators of virus entry. J Virol, 72;7064-7074: 1998.

Krummenacher, C., Supekar, V.M., Whitbeck, J.C., Lazear, E., Connolly, S.A., Eisenberg, R.J., Cohen, G.H., Wiley, D.C., Carfí, A.: Structure of unliganded HSV gD reveals a mechanism for receptor-mediated activation of virus entry. EMBO J 24;4144-4153: 2005.

Kuschert, G.S., Coulin, F., Power, C.A., Proudfoot, A.E., Hubbard, R.E., Hoogewerf, A.J., Wells, T.N.: Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses. Biochemistry 38;12959-12968: 1999.

Kwon, H., Bai, Q., Baek, H.J., Felmet, K., Burton, E.A., Goins, W.F., Cohen, J.B., Glorioso, J.C.: Soluble V domain of Nectin-1/HveC enables entry of herpes simplex virus type 1 (HSV-1) into HSV-resistant cells by binding to viral glycoprotein D. J Virol 80;138-148: 2006.

Lambaerts, K., Wilcox-Adelman, S.A., Zimmermann, P.: The signaling mechanisms of syndecan heparan sulfate proteoglycans. Curr Opin Cell Biol 21;662-669: 2009.

Laquerre, S., Argnani, R., Anderson, D.B., Zucchini, S., Manservigi, R., Glorioso, J.C.: Heparan sulfate proteoglycan binding by herpes simplex virus type 1 glycoproteins B and C, which differ in their contributions to virus attachment, penetration, and cell-to-cell spread. J Virol 72;6119-6130: 1998.

Liesegang TJ.: Biology and molecular aspects of herpes simplex and varicella-zoster virus infections. Ophthalmology 99;781-799: 1992.

Lindahl, U., Kusche-Gullberg, M., Kjellén, L.: Regulated diversity of heparan sulfate. J Biol Chem 273;24979-24982: 1998.

Lin, E., Spear, P.G.: Random linker-insertion mutagenesis to identify functional domains of herpes simplex virus type 1 glycoprotein B. Proc Natl Acad Sci USA 104;13140-13145: 2007.

Lopes, C.C., Dietrich, C.P., Nader, H.B.: Specific structural features of syndecans and heparan sulfate chains are needed for cell signaling. Braz J Med Biol Res 39;157-167: 2006.

Lopez, M., Cocchi, F., Menotti, L., Avitabile, E., Dubreuil, P., Campadelli-Fiume, G.: Nectin2alpha (PRR2alpha or HveB) and nectin2delta are low-efficiency mediators for entry of herpes simplex virus mutants carrying the Leu25Pro substitution in glycoprotein D. J Virol 74;1267-1274: 2000.

Maeda, T., Alexander, C.M., Friedl, A.: Induction of syndecan-1 expression in stromal fibroblasts promotes proliferation of human breast cancer cells. Cancer Res 64;612-621: 2004.

Ma, P., Beck, S.L., Raab, R.W., McKown, R.L., Coffman, G.L., Utani, A., Chirico, W.J., Rapraeger, A.C., Laurie, G.W.: Heparanase deglycanation of syndecan-1 is required for binding of the epithelial-restricted prosecretory mitogen lacritin. J Cell Biol 174;1097-1106: 2006.

Manoj, S., Jogger, C.R., Myscofski, D., Yoon, M., Spear, P.G.: Mutations in herpes simplex virus glycoprotein D that prevent cell entry via nectins and alter cell tropism. Proc Natl Acad Sci USA 101;12414-12421: 2004.

Manon-Jensen, T., Itoh, Y., Couchman, J.R.: Proteoglycans in health and disease: the multiple roles of syndecan shedding. FEBS J 277;3876-3889: 2010.

McDermott, S.P., Ranheim, E.A., Leatherberry, V.S., Khwaja, S.S., Klos, K.S., Alexander, C.M.: Juvenile syndecan-1 null mice are protected from carcinogen-induced tumor development. Oncogene 26;1407-1416: 2007.

McGeoch, D.J., Rixon, F.J., Davison, A.J.: Topics in herpesvirus genomics and evolution. Virus Res 117;90-104: 2006.

McQuade, K.J., Beauvais, D.M., Burbach, B.J., Rapraeger, A.C.: Syndecan-1 regulates alphavbeta5 integrin activity in B82L fibroblasts. J Cell Sci 119;2445–2456: 2006.

McQuade, K.J., Rapraeger, A.C.: Syndecan-1 transmembrane and extracellular domains have unique and distinct roles in cell spreading. J Biol Chem 278;46607-46615: 2003.

Mettenleiter, T.C.: Budding events in herpesvirus morphogenesis. Virus Res 106;167-180: 2004.

Mettenleiter, T.C.: Intriguing interplay between viral proteins during herpesvirus assembly or: the herpesvirus assembly puzzle. Vet Microbiol 113;163-169: 2006.r

Mettenleiter, T.C., Klupp, B.G., Granzow, H.: Herpesvirus assembly: a tale of two membranes. Curr Opin Microbiol 9;423-429: 2006.

Mettenleiter, T.C.: Pathogenesis of neurotropic herpesviruses: role of viral glycoproteins in neuroinvasion and transneuronal spread. Virus Res 92;197-206: 2003.

Montgomery, R.I., Warner, M.S., Lum, B.J., Spear, P.G.: Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell 87;427-436: 1996.

Muggeridge, M.I.: Characterization of cell-cell fusion mediated by herpes simplex virus 2 glycoproteins gB, gD, gH and gL in transfected cells. J Gen Virol 81(Pt 8);2017-2027: 2000.

Multhaupt, H.A., Yoneda, A., Whiteford, J.R., Oh, E.S., Lee, W., Couchman, J.R.: Syndecan signaling: when, where and why? J Physiol Pharmacol 60 Suppl 4;31–38: 2009.

Muto, T., Miyoshi, K., Munesue, S., Nakada, H., Okayama, M., Matsuo, T., Noma, T.: Differential expression of syndecan isoforms during mouse incisor amelogenesis. J Med Invest 54;331–339: 2007.

Nagel, C.H., Döhner, K., Fathollahy, M., Strive, T., Borst, E.M., Messerle, M., Sodeik, B.: Nuclear egress and envelopment of herpes simplex virus capsids analyzed with dual-color fluorescence HSV1(17+). J Virol 82;3109-3124: 2008.

Nahmias, A.J., Kibrick, S.: Inhibitory effect of heparin on herpes simplex virus. J Bacteriol 87;1060-1066: 1964.

O'Donnell, C.D., Kovacs, M., Akhtar, J., Valyi-Nagy, T., Shukla, D.: Expanding the role of 3-O sulfated heparan sulfate in herpes simplex virus type-1 entry. Virology 397;389-398: 2010.

O'Donnell, C.D., Shukla, D.: A novel function of heparan sulfate in the regulation of cell-cell fusion. J Biol Chem 284;29654–29665: 2009.

O'Donnell, C.D., Shukla, D.: The Importance of Heparan Sulfate in Herpesvirus Infection. Virol Sin 23;383–393: 2008.

Ogren, S., Lindahl, U.: Cleavage of macromolecular heparin by an enzyme from mouse mastocytoma. J Biol Chem 250;2690-2697: 1975.

Oh, J.H., Lee, H.S., Park, S.H., Ryu, H.S., Min, C.K.: Syndecan-1 overexpression promotes tumor growth and angiogenesis in an endometrial cancer xenograft model. Int J Gynecol Cancer 20;751–756: 2010.

Oh, M.J., Akhtar, J., Desai, P., Shukla, D.: A role for heparan sulfate in viral surfing. Biochem Biophys Res Commun 391;176–181: 2010.

Ojala, P.M., Sodeik, B., Ebersold, M.W., Kutay, U., Helenius, A.: Herpes simplex virus type 1 entry into host cells: reconstitution of capsid binding and uncoating at the nuclear pore complex in vitro. Mol Cell Biol 20;4922-4931: 2000.

Park, R., Baines, J.D.: Herpes simplex virus type 1 infection induces activation and recruitment of protein kinase C to the nuclear membrane and increased phosphorylation of lamin B. J Virol 80;494-504: 2006.

Payne, C.K., Jones, S.A., Chen, C., Zhuang, X.: Internalization and trafficking of cell surface proteoglycans and proteoglycan-binding ligands. Traffic 8;389-401: 2007.

Pertel, P.E., Fridberg, A., Parish, M.L., Spear, P.G.: Cell fusion induced by herpes simplex virus glycoproteins gB, gD, and gH-gL requires a gD receptor but not necessarily heparan sulfate. Virology 279;313–324: 2001.

Pertel, P.E., Spear, P.G.: Partial resistance to gD-mediated interference conferred by mutations affecting herpes simplex virus type 1 gC and gK. J Virol 71;8024-8028: 1997.

Purushothaman, A., Uyama, T., Kobayashi, F., Yamada, S., Sugahara, K., Rapraeger, A.C., Sanderson, R.D.: Heparanase-enhanced shedding of syndecan-1 by myeloma cells promotes endothelial invasion and angiogenesis. Blood 115;2449-2457: 2010.

Richards, R.M. Lowy, D.R., Schiller, J.T., Day, P.M.: Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. Proc Natl Acad Sci USA 103;1522-1527: 2006.

Roller, R.J., Herold, B.C.: Characterization of a BHK(TK-) cell clone resistant to postattachment entry by herpes simplex virus types 1 and 2. J Virol 71;5805-5813: 1997.

Scanlan, P.M., Tiwari, V., Bommireddy, S., Shukla, D.: Cellular expression of gH confers resistance to herpes simplex virus type-1 entry. Virology 312;14–24: 2003.

Sacks, S.L., Griffiths, P.D., Corey, L., Cohen, C., Cunningham, A., Dusheiko, G.M., Self, S., Spruance, S., Stanberry, L.R., Wald, A., Whitley, R.J.: HSV shedding. Antiviral Res 63 Suppl 1;S19-26: 2004.

Satoh, T., Arii, J., Suenaga, T., Wang, J., Kogure, A., Uehori, J., Arase, N., Shiratori, I., Tanaka, S., Kawaguchi, Y., Spear, P.G., Lanier, L.L., Arase, H.: PILRalpha is a herpes simplex virus-1 entry coreceptor that associates with glycoprotein B. Cell 132;935-944: 2008.

Schofield, K.P., Gallagher, J.T., David, G.: Expression of proteoglycan core proteins in human bone marrow stroma. Biochem J 343;663-668: 1999.

Shafti-Keramat, S., Handisurya, A., Kriehuber, E., Meneguzzi, G., Slupetzky, K.: Different heparan sulfate proteoglycans serve as cellular receptors for human papillomaviruses. J Virol 77;13125–13135: 2003.

Shafat, I., Vlodavsky, I., Ilan, N.: Characterization of mechanisms involved in secretion of active heparanase. J Biol Chem 281;23804-23811: 2006.

Shah, A., Farooq, A.V., Tiwari, V., Kim, M.J., Shukla, D.: HSV-1 infection of human corneal epithelial cells: receptor-mediated entry and trends of reinfection. Mol Vis 16;2476–2486: 2010.

Shimada, K., Nakamura, M., De Velasco, M.A., Tanaka, M., Ouji, Y., Miyake, M., Fujimoto, K., Hirao, K., Konishi, N.: Role of syndecan-1 (CD138) in cell survival of human urothelial carcinoma. Cancer Sci 101;155-160: 2010.

Shoji, H., Azuma, K., Nishimura, Y., Fujimoto, H., Sugita, Y., Eizuru, Y.: Acute viral encephalitis: the recent progress. Intern Med 41;420-428: 2002.

Shukla, D., Liu, J., Blaiklock, P., Shworak, N.W., Bai, X., Esko, J.D., Cohen, G.H., Eisenberg, R.J., Rosenberg, R.D., Spear, P.G.: A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell 99;13-22: 1999.

Shukla, D., Spear, P.G.: Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. J Clin Invest 108;503-510: 2001.

Shukla, S.Y., Singh, Y.K., Shukla, D.: Role of nectin-1, HVEM, and PILR-alpha in HSV-2 entry into human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 50;2878-2887: 2009.

Spear, P.G.: Herpes simplex virus: receptors and ligands for cell entry. Cell Microbiol 6;401-410: 2004.

Spear, P.G., Longnecker, R.: Herpesvirus entry: an update. J Virol 77;10179-10185: 2003.

Stannard, L.M., Fuller, A.O., Spear, P.G.: Herpes simplex virus glycoproteins associated with different morphological entities projecting from the virion envelope. J Gen Virol 68(Pt 3);715-725: 1987.

Subramanian, R.P., Geraghty, R.J.: Herpes simplex virus type 1 mediates fusion through a hemifusion intermediate by sequential activity of glycoproteins D, H, L, and B. Proc Natl Acad Sci USA 104;2903-2908: 2007.

Suenaga, T., Satoh, T., Somboonthum, P., Kawaguchi, Y., Mori, Y., Arase, H.: Myelin-associated glycoprotein mediates membrane fusion and entry of neurotropic herpesviruses. Proc Natl Acad Sci USA 107;866-871: 2010.

Su, G., Blaine, S.A., Qiao, D., Friedl, A.: Shedding of syndecan-1 by stromal fibroblasts sitmulates human breast cancer cell proliferation via FGF2 activation. J Biol Chem 282;14906-14915: 2007.

Sun, H., Berquin, I.M., Owens, R.T., O'Flaherty, J.T., Edwards, I.J.: Peroxisome proliferator-activated receptor gamma-mediated up-regulation of syndecan-1 by n-3 fatty acids promotes apoptosis of human breast cancer cells. Cancer Res 68;2912-2919: 2008.

Teng, Y.H., Aquino, R.S., Park, P.W.: Molecular functions of syndecan-1 in disease. Matrix Biol 31;3-16: 2012.

Tiwari, V., Clement, C., Duncan, M.B., Chen, J., Liu, J., Shukla, D.: A role for 3-O-sulfated heparan sulfate in cell fusion induced by herpes simplex virus type 1.J Gen Virol 85(Pt 4);805-809: 2004.

Tiwari, V., O'donnell, C., Copeland, R.J., Scarlett, T., Liu, J., Shukla, D.: Soluble 3-Osulfated heparan sulfate can trigger herpes simplex virus type 1 entry into resistant Chinese hamster ovary (CHO-K1) cells. J Gen Virol 88;1075-1079: 2007.

Tkachenko, E., Rhodes, J.M., Simons, M.: Syndecans: new kids on the signaling block. Circ Res 96;488–500: 2005.

Trybala, E., Liljeqvist, J.A., Svennerholm, B., Bergström, T.: Herpes simplex virus types 1 and 2 differ in their interaction with heparan sulfate. J Virol 74;9106-9114: 2000.

Tumova, S., Woods, A., Couchman, J.R.: Heparan sulfate proteoglycans on the cell surface: versatile coordinators of cellular functions. Int J Biochem Cell Biol 32;269-288: 2000.

Turner, A., Bruun, B., Minson, T., Browne, H.: Glycoproteins gB, gD, and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system. J Virol 72;873-875: 1998.

Vaday, G.G., Lider, O.: Extracellular matrix moieties, cytokines, and enzymes: dynamic effects on immune cell behavior and inflammation. J Leukoc Biol 67;149-159: 2000.

Warner, M.S., Geraghty, R.J., Martinez, W.M., Montgomery, R.I., Whitbeck, J.C., Xu, R., Eisenberg, R.J., Cohen, G.H., Spear, P.G.: A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus. Virology, 246;179-189: 1998.

Whitbeck, J.C., Connolly, S.A., Willis, S.H., Hou, W., Krummenacher, C., Ponce de Leon, M., Lou, H., Baribaud, I., Eisenberg, R.J., Cohen, G.H.: Localization of the gD-binding region of the human herpes simplex virus receptor, HveA. J Virol 75;171-180: 2001.

Whitbeck, J.C., Muggeridge, M.I., Rux, A.H., Hou, W., Krummenacher, C., Lou, H., van, Geelen. A., Eisenberg, R.J., Cohen, G.H.: The major neutralizing antigenic site on herpes simplex virus glycoprotein D overlaps a receptor-binding domain. J Virol, 73;9879-9890: 1999.

Wittrup, A., Zhang, S.H., ten Dam, G.B., van Kuppevelt, T.H., Bengtson, P., Johansson, M., Welch, J., Mörgelin, M., Belting, M.: ScFv antibody-induced translocation of cell-surface heparan sulfate proteoglycan to endocytic vesicles: evidence for heparan sulfate epitope specificity and role of both syndecan and glypican. J Biol Chem 284;32959-32967: 2009.

WuDunn, D., Spear, P.G.: Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J Virol 63;52-58: 1989.

Xian, X., Gopal, S., Couchman, J.R.: Syndecans as receptors and organizers of the extracellular matrix. Cell Tissue Res 339;31–46: 2010.

Yang, Y., Macleod, V., Miao, H.Q., Theus, A., Zhan, F., Shaughnessy, J.D. Jr, Sawyer, J., Li, J.P., Zcharia, E., Vlodavsky, I., Sanderson, R.D.: Heparanase enhances syndecan-1 shedding: a novel mechanism for stimulation of tumor growth and metastasis. J Biol Chem 282;13326-13333: 2007.

Yoon, M., Zago, A., Shukla, D., Spear, P.G.: Mutations in the N termini of herpes simplex virus type 1 and 2 gDs alter functional interactions with the entry/fusion receptors HVEM, nectin-2, and 3-O-sulfated heparan sulfate but not with nectin-1. J Virol 77;9221-9231: 2003.

Zago, A., Jogger, C.R., Spear, P.G.: Use of herpes simplex virus and pseudorabies virus chimeric glycoprotein D molecules to identify regions critical for membrane fusion. Proc Natl Acad Sci USA 101;17498-17503: 2004.

Zetser, A, Bashenko, Y., Edovitsky, E., Levy-Adam, F., Vlodavsky, I., Ilan, N.: Heparanase induces vascular endothelial growth factor expression: correlation with p38 phosphorylation levels and Src activation. Cancer Res 66;1455-1463: 2006.

Zetser, A., Levy-Adam, F., Kaplan, V., Gingis-Velitski, S., Bashenko, Y., Schubert, S., Flugelman, M.Y., Vlodavsky, I., Ilan, N.: Processing and activation of latent heparanase occurs in lysosomes. J Cell Sci 117(Pt 11);2249-2258: 2004.

Zhang, Y.F., Tang, X.D., Gao, J.H., Fang, D.C., Yang, S.M.: Heparanase: a universal immunotherapeutic target in human cancers. Drug Discov Today 16;412-417: 2011.

Zheng, M., Deshpande, S., Lee, S., Ferrara, N., Rouse, B.T.: Contribution of vascular endothelial growth factor in the neovascularization process during the pathogenesis of herpetic stromal keratitis. J Virol 75;9828–9835: 2001.

Zhe, R., Mei-Ying, Z., Kitazato, K., Kobayash, N., Qin-Chang, Z., Pei-Zhuo, Z., Zhi-Rong, Y., Yi-Fei, W.: Effect of siRNA on HSV-1 plaque formation and relative expression levels of UL39 mRNA. Arch Virol 153;1401–1406: 2008.

Zhou, Y., Guo, Y.J., Liu, X., Mei, Y.W.: Cell cycle inhibitor enhances the resolution of HSV-1-induced proinflammatory response in murine microglial cells. Neurol Res 31;910–916: 2009.

Zimmermann, P., Zhang, Z., Degeest, G., Mortier, E., Leenaerts, I., Coomans, C., Schulz, J., N'Kuli, F., Courtoy, P.J., David, G.: Syndecan recycling [corrected] is controlled by syntenin-PIP2 interaction and Arf6. Dev Cell 9;377-388: 2005.

Zong, F., Fthenou, E., Castro, J., Péterfia, B., Kovalszky, I., Szilák, L., Tzanakakis, G., Dobra, K.: Effect of syndecan-1 overexpression on mesenchymal tumour cell proliferation with focus on different functional domains. Cell Prolif 43;29–40: 2010.

# VITA

Ghadah A Karasneh
1905 W Taylor St
LEIRI Bldg. Room 227
Chicago, IL 60612
Email: gkaras2@uic.edu

## GRADUATE EDUCATION

Aug. 2007 - May 2012
School: Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, IL USA
Degree: Ph.D., Microbiology and Immunology
Thesis title: Modulation and Contribution of Heparan Sulfate Proteoglycans During Herpes Simplex Virus Type-1 Infection.

Supervisor: Dr. Deepak Shukla, PhD.

GPA: 3.12

## WORK EXPERIENCE

Dec. 2006 – Jul. 2007

University of Illinois at Chicago, Department of Infectious Diseases

Volunteer research specialist

## UNDERGRADUATE EDUCATION

Fall 2000-Spring 2004
School: Jordan University of Science and Technology. Irbid, Jordan
Degree: B.S., Medical laboratory sciences
GPA: 85.4 % (Excellent)

## TEACHING EXPERIENCE

Served as a mentor for multiple high school students from the Illinois Math and Science Academy (IMSA) for their Student Research and Inquiry (SIR) program. Mentored and supervised several undergraduate and medical students who have performed research in the lab.

## RESEARCH SKILLS

Western blot; flow cytometry; siRNA assays; cloning; ELISA; PCR; viral culture; viral assays; viral purification; tissue/cell culture (mammalian, primary); protein, RNA, and DNA isolation;

dot blotting; immunocytochemistry; microscopy (light, immunofluorescent, confocal, inverted light); work with DNA/RNA; transfection; transformation; cell-cell fusion assays; viability assays; work with anti-viral peptide

**LANGUAGE SKILLS**
Expert language skills in English and Arabic; basic language skills in French.

**COMPUTER SKILLS**
Windows (98, ME, XP, Vista, 7), MS Office (Excel, Word, PowerPoint, Frontpage), Adobe(Acrobat, Photoshop, Illustrator), SigmaPlot, Leica Confocal Software, MetaMorph. GraphPad Prism software.

**AWARDS AND HONORS**
Ranked the first on her fellow student undergraduates.
Listed on university honor list for academic excellence and achievements for spring semester of 2000-2001, Fall semester of 2003-2004, and Spring semester of 2003-2004.

**PUBLICATIONS**
Only original peer-reviewed international articles:

**Karasneh, G.A.**, and Shukla, D. (2012). An Important Role For Heparanase in Herpes Simplex Virus Type-1 Release from Infected Cells. (manuscript in preparation).

Ali, M.M., **Karasneh, G.A**[*]., Jarding, M.J., Tiwari, V., Shukla, D. (2012). A 3-O Sulfated Heparan Sulfate Binding Peptide Preferentially Targets Herpes Simplex Virus Type-2 Infected Cells. *J Virol.* (manuscript accepted by Journal of Virology, in press)

*** Co-first author**

**Karasneh, G.A.**, Shukla, D. (2011) Herpes Simplex Virus Infects Most Cell Types in Vitro: Clues to its Success. *Virol J.* 8,481.

*** Designated as Highly Accessed paper relative to age**

**Karasneh, G.A.**, Ali, M., Shukla, D. (2011). An Important Role for Syndecan-1 in Herpes Simplex Virus Type-1 Induced Cell-to-Cell Fusion and Virus Spread. *PLoS One.* 6(9),e25252.

Bacsa, S., **Karasneh, G.**, Dosa, S., Liu, J., Valyi-Nagy, T., Shukla, D. (2011) Syndecan-1 and Syndecan-2 Play Key Roles in Herpes Simplex Virus Type-1 Infection. *J Gen Virol.* 92(Pt 4),733-743.

*** Designated as Hot paper in the Society for General Microbiology (SGM), and was featured in MICRO BIOLOGY TODAY magazine.**

**ORAL/POSTER PRESENTATIONS**

**Karasneh, G.A.,** Bacsa, S., Shukla, D. The Role of Syndecan-1 in Herpes Simplex Virus Type 1. *Vision Sciences Research Group (VSRG): Frontiers in Vision Science (2011),* Chicago, IL USA. (oral presentation)

**Karasneh, G.A.** and Shukla, D. The Role of Syndecan-1 in Herpes Simplex Virus Type 1 Infection. *Chicago Area Virology Association Symposium (2011),* Chicago, IL USA. (oral presentation).

Shukla, D., **Karasneh, G.A.** , Bacsa, S., Valyi-Nagy, T. Up-regulation Of Syndecan Expression May Be A Viral-induced Mechanism For HSV-1 Shedding. *The Association for Research in Vision and Ophthalmology (ARVO) Annual meeting (2011),* Fort Lauderdale, FL USA.

Farooq, A.V., Kim, M.J., Shah, A., Ali, M., **Karasneh, G.A.,** Shukla, D. Determining A Role For Syndecan-1 In Herpes Simplex Virus-1 Corneal Infection. *The Association for Research in Vision and Ophthalmology (ARVO) Annual meeting (2011),* Fort Lauderdale, FL.

**Karasneh, G.A.** and Shukla, D. A Role for the Syndecans in HSV-1 Induced Cell-Cell Fusion. *35th International Herpes Workshop (2010),* Salt Lake City, UT USA.

**Karasneh, G.A.** and Shukla, D. A Role for the Syndecans in HSV-1 Induced Cell-Cell Fusion. *University of Illinois at Chicago College of Medicine Research Forum 2010,* Chicago, IL USA

Farooq, A.V., **Karasneh, G.A.,** Shah, A., Valyi-Nagy, T., Shukla, D. Trends in HSV-1 Infection of Ex-Vivo Corneal Tissue and Correlation to Heparan Sulfate Expression. *The Association for Research in Vision and Ophthalmology (ARVO) Annual meeting (2010),* Fort Lauderdale, FL.

**Karasneh, G.A.**, Bacsa, S., Shukla, D. An Important Role of Syndecans in HSV-1 Infection *34th International Herpes Workshop,* Ithaca, NY (2009). Abstract 4.34.

Patel, L., Veeramasuneni, L., **Karasneh, G.A.,** Shukla, D. Pathogenic Entry of Herpes Simplex Virus: Syndecan Coreceptor Expression. *Illinois Mathematics and Science Academy IMSaloquium Student Investigation Showcase (2009),* Aurora, IL USA.