A HUMAN NEURONAL MODEL FOR HERPES SIMPLEX VIRUS LATENCY AND REACTIVATION

by

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ABSTRACT

A human neuronal model for herpes simplex virus latency and reactivation

A defining characteristic of alphaherpesviruses is the establishment of lifelong latency in host sensory ganglia with occasional reactivation causing recurrent lytic infections. Much remains unknown regarding the cellular and viral mechanisms involved in HSV exit from latency. We hypothesize that VP16 recruits chromatin-remodeling enzymes to immediate early gene promoters on compact-latent chromatin as a necessary step for reactivation. In order to test this hypothesis, a robust *in vitro* assay in which HSV latency can be established in neurons was required. In this dissertation, I explored the use of a human sensory neuron cell line as a novel *in vitro* model of HSV-1 latency and reactivation. HD10.6 cells were derived from embryonic human dorsal root ganglia and immortalized by a tetracycline-regulated *v-myc* oncogene. HD10.6 cells mature to express a sensory neuron-associated phenotype when treated with doxycycline which suppresses proliferation mediated by the v-*myc* oncogene. Infection at a low MOI in the presence of acyclovir results in a quiescent infection resembling latency in matured cells. HD10.6 cells provide a novel context in which to study the host and viral mechanisms of HSV-1 latency establishment, maintenance, and reactivation.

I dedicate this Dissertation to my best friend and husband Shane Thellman. You have never wavered in your encouragement of my going “back to school” to pursue a second doctoral degree. Your faith in my potential has always been empowering. For all this, and more, I will forever be grateful for our unexpected dinner date almost 14 years ago.

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LIST OF SYMBOLS AND ABBREVIATIONS

CNTF	ciliary neurotrophic factor
CPE	cytopathic effect
DG1	HSV-1 VP26-GFP (KOS background)
DRG	dorsal root ganglia
GDNF	glial–derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HATs	histone acetyltransferases
HCF-1	host cell factor 1
HD10.6	human dorsal root ganglia clone 10.6
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
IE, E, L	immediate early, early, late viral genes
LATs	latency associated transcripts
LUHMES	Lund human mesencephalic
MOI	multiplicity of infection
NGF	nerve growth factor
NT-3	neurotrophin-3
Oct-1	octamer binding transcription factor 1
SNAP	sensory neuron–associated phenotype
TG	trigeminal ganglia
VZV	varicella zoster virus

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INTRODUCTION

VP16 and Chromatin Regulation in Reactivation

Alphaherpesviruses infect a variety of species, from reptiles to mammals. In humans, three significant disease-causing alphaherpesviruses exist. Herpes simplex virus 1 (HSV-1) commonly causes orolabial lesions, HSV-2 causes genital lesions, and varicella zoster virus (VZV) causes chicken pox and shingles. Alphaherpesviruses maintain life-long infections by persisting as quiescent DNA episomes in neurons (a state referred to as latency), with periodic reactivation and production of virus particles that ensure viral transmission. The contrasting viral strategies (lytic infection and latency), combined with viral immune evasion mechanisms, have made vaccine development challenging (Koelle and Corey 2003; Johnston, et al. 2011; Coleman and Shukla 2013). Whereas approved prophylactic and therapeutic vaccines have been in use for VZV (Giovanni, et al. 2016; Prymula, et al. 2016; Yoshikawa, et al. 2016), to date, there are no effective vaccines for either HSV-1 or HSV-2 (Corey, et al. 1999; Koelle and Corey 2003; Belshe, et al. 2012; Coleman and Shukla 2013; Awasthi, et al. 2017).

Successful therapeutic intervention for alphaherpesvirus infection has long been achieved by targeting viral replication. Antiviral drugs such as acyclovir and its derivatives reduce viral shedding and dampen clinical symptoms (Elion, et al. 1977). Unfortunately these antiviral therapies do not prevent recurrence, and asymptomatic shedding occurs frequently (Koelle and Wald 2000). In addition, antiviral resistance has emerged, mostly frequently in immunocompromised patients (Grey, et al. 2003; Morfin and Thouvenot 2003; Stranska, et al. 2005; Frobert, et al. 2014). The clinical need, therefore, is for novel therapeutics that target viral reactivation mechanisms, preventing recurrence altogether.

Unfortunately, much remains unknown regarding the cellular and viral mechanisms involved in HSV exit from latency. To address this scientific gap in knowledge, the contrasting viral strategies can be compared in terms of viral genome regulation. The chromatin state of the double-stranded DNA genome of HSV is context-dependent. In the virion capsid, the genome is packaged without nucleosomes (naked). Upon entry into an epithelial cell for productive infection, the naked DNA undergoes dynamic regulation to favor a relatively histone-free state for robust viral gene expression (Conn and Schang 2013; Lee, et al. 2016). Lytic gene expression is a highly ordered cascade of events, triggered by the coactivator and tegument protein, virion protein 16 (VP16).

The host's attempt to silence foreign DNA with nucleosomes is thwarted by viral mechanisms. For example, immediate early (IE) gene products assist in removal of heterochromatin marks in favor of viral gene expression and ultimately replication. This is in stark contrast to the latent state of viral DNA, in which HSV genomes in the nucleus of sensory neurons accumulate repressive histone marks at lytic genes and persist as episomes (Wang, et al. 2005; Cliffe, et al. 2009; Kwiatkowski, et al. 2009). Viral proteins are not made during maintenance of latency, and relative to lytic infection, viral transcription is repressed with the exception of noncoding RNAs called latency-associated transcripts (LATs). The primary LAT is spliced into a 2.0 kb stable intron (major LAT) and accumulates in the nuclei of neurons during latency (Thomas, et al. 2002; Bloom 2016). **Figure 1** depicts a general schematic of the current understanding of the different chromatin states of HSV in a virion, a lytically infected epithelial cell, and a latently infected neuron.

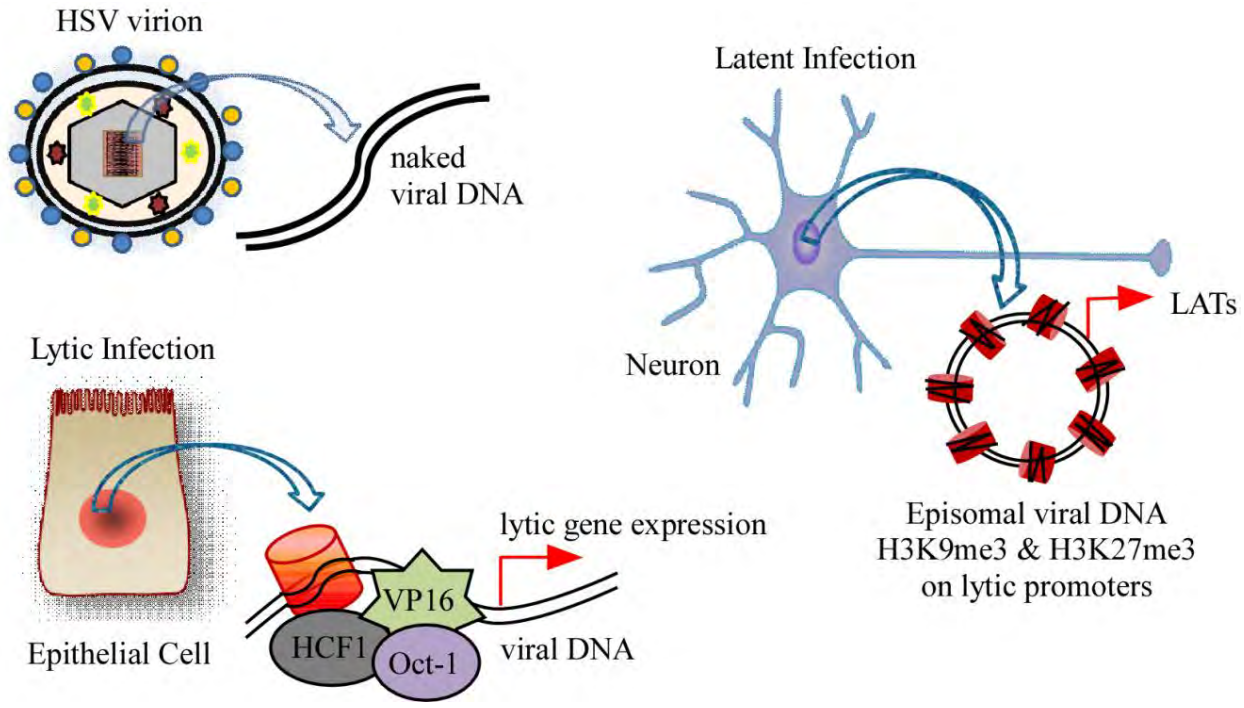

Figure 1. The state of HSV genomes is context-dependent. In the virion, the double stranded DNA of HSV is linear and nucleosome-free (naked). In an epithelial cell, lytic infection results in an HSV genome that is actively transcribed. Viral gene products promote heterochromatin removal, prevent silencing, and favor the ordered cascade of lytic gene expression. In a latently infected neuron, viral DNA is episomal, with heterochromatin marks on lytic genes favoring viral gene repression, with the exception of expression of noncoding RNAs called latency-associated transcripts (LATs). Abbreviations: host cell factor (HCF1), octamer binding protein 1 (Oct-1), viral protein 16 (VP16), histone (H) 3 modifications [trimethylation (me3) at lysine (K) 9 and 27].

The dynamic state of histone deposition and nucleosome remodeling on HSV genomes during lytic infection has long been an area of interest. The interaction between histones within nucleosomes, as well as between nucleosomes and DNA, regulates access to DNA for transcriptional activity. Early studies revealed that histones are loaded and removed rapidly on HSV-1 early (E) and late (L) promoters during lytic infection (Knipe, et al. 2013). Specifically, differential assembly of histone variants H3.3 and H3.1 into HSV-1 nucleosomes during lytic infection may be induced by the virus to facilitate chromatin dynamics to circumvent silencing (Conn, et al. 2013; Conn and Schang 2013). The viral proteins ICP0 and VP16 have also been implicated in this critical chromatin regulation. ICP0, an IE gene product, is a multifunction E3 ubiquitin ligase. ICP0 prevents removal of histone acetylation on viral chromatin by disrupting histone deacetylase 1 (HDAC1) binding in the CoREST repressor complex (Alwine, et al. 1974; Gu and Roizman 2007; Zhou, et al. 2010; Zhou, et al. 2013). ICP0 also promotes degradation of nuclear domain 10 (ND10) components that are believed to be involved in epigenetic silencing (Glass and Everett 2013).

Past studies in our lab aimed to elucidate the mechanism by which VP16 regulates chromatin in favor of robust viral transcription during lytic infection (Kutluay and Triezenberg 2009a; b). VP16 (a L gene product) is brought into the cell as a tegument protein upon *de novo* infection, and aside from recruiting the general transcription machinery necessary for IE gene expression, it also recruits chromatin-modifying enzymes (CMEs). VP16 recruits CMEs which promote histone modifications associated with active transcription, such as the histone acetyltransferases (HATs) p300/CBP and PCAF/GCN5 as well as the ATPase remodelers BRG1/BRM (Herrera and Triezenberg 2004; Kutluay, et al. 2009). Surprisingly, these CMEs were found to be dispensable for IE gene expression during lytic infection (Kutluay, et al. 2009).

These findings, leave the question, when might VP16 recruitment of these chromatin remodeling enzymes be important for the virus, unanswered.

Although once discounted (Steiner, et al. 1990; Sears, et al. 1991; Ecob-Prince, et al. 1993), it is now accepted that *de novo* synthesis of VP16 is required for reactivation (Kim, et al. 2012). Based on this requirement (Thompson, et al. 2009) and the fact that lytic gene promoters are heterochromatinized during latency (Cliffe, et al. 2009), we hypothesize that VP16 recruits CMEs to IE gene promoters as a necessary step to exit latency. A current model of reactivation includes a two-stage program in which neuronal stress induces animation (phase I) of the viral genome, resulting in uniquely transient lytic gene expression, followed by phase II, which more closely resembles the organized cascade of lytic *de novo* infection (Cliffe and Wilson 2017). We further surmise that VP16 recruits HATs and ATPase remodelers to IE gene promoters on compact-latent chromatin as a necessary step for phase II of the reactivation process (**Figure 2**).

In order to demonstrate this VP16-dependent recruitment of CMEs to IE gene promoters, we would compare occupancy (using chromatin immunoprecipitation (ChIP) and qPCR) from reactivating neurons from latent infections established with HSV-1 wild-type and VP16 activation domain mutant strains. We would expect that HSV-1 strains with mutations in the activation domain of VP16 would fail to recruit CMEs to IE gene promoters during reactivation, as was the case in lytic infections in epithelial cell types (Herrera and Triezenberg 2004). To demonstrate which CMEs are required for viral exit from latency, we would modulate different HATs and chromatin remodelers by shRNA knock-down or pharmacological inhibition during

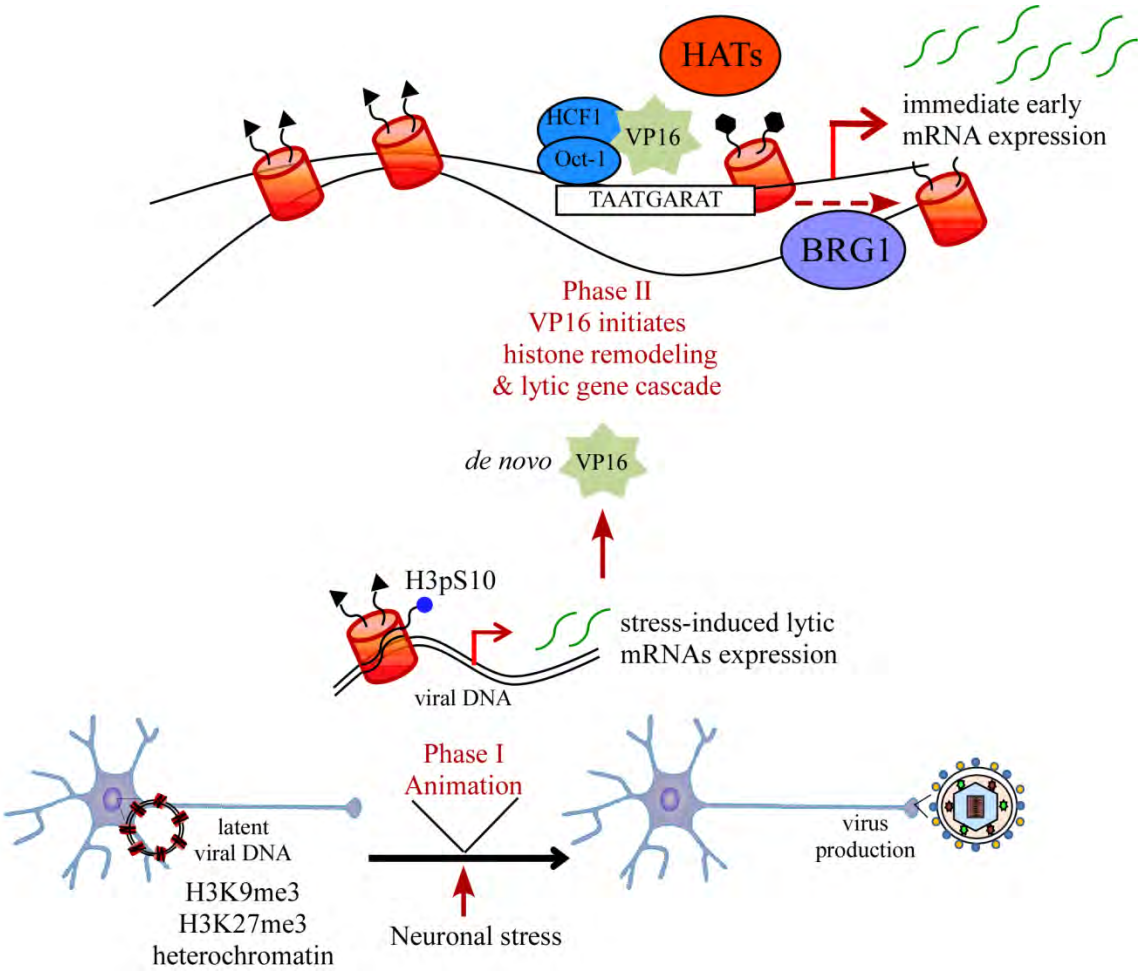

Figure 2. Reversal of viral episomal silencing leads to reactivation. Latent viral genomes are maintained as heterochromatin carrying histone 3 (H3) lysine (K) tri-methylation (me3) marks at lytic gene promoters (H3K9me3, H3K27me3). Phase I of reactivation begins when neuronal stress triggers animation of latent HSV-1 genome with a phospho-switch on H3 at serine (S) 10 (H3pS10) at key lytic promoters. As a result, VP16 is synthesized *de novo* and, during phase II of reactivation, recruits histone acetyltransferases (HATs) and ATPase chromatin remodeling enzymes (BRG1) to immediate early gene promoters (TATGARAT sequences) for initiation of the lytic gene cascade by acetylating (activating) histone tails and repositioning nucleosomes.

reactivation. We could then assess viral exit from latency through IE gene expression analysis and virus production. These molecular approaches require a robust *in vitro* assay in which HSV latency can be established specifically in neurons and reactivation can be quantified.

Many labs use dissociated embryonic rat or adult mouse ganglia for *in vitro* latency infections. We argue that ideally, the neurons would be of human origin due to protein structure differences in rodent host cell factors known to associate with VP16 (Cleary, et al. 1993). Due to the availability of cells, the only human studies of HSV latency have been done postmortem on ganglia samples. Ganglia taken from autopsy samples have been used to quantify HSV copy numbers (Cai, et al. 2002), characterize viral RNA expression (Croen, et al. 1988; Gordon, et al. 1988; Steiner, et al. 1988; Stevens, et al. 1988), and identify neurons or inflammatory cells involved in latency (Held, et al. 2011; Flowerdew, et al. 2013). A human model in which HSV latency can be studied at the molecular level did not exist. My thesis advisory committee member, Patrik Brundin, informed me about the existence of an immortalized human dorsal root ganglia cell line (HD10.6 cells) (Raymon, et al. 1999). These cells were immortalized with a tetracycline-regulated v-*myc* oncogene in a manner very similar to how the LUHMES (Lund human mesencephalic) cell line used in Parkinson's disease research was derived (Lotharius, et al. 2005).

In this dissertation, I explored the use of this human sensory neuron cell line as a novel *in vitro* model of HSV-1 latency and reactivation. As it was my intention to explore cellular mechanisms responsible for chromatin regulation during reactivation, I surmised that HD10.6 cells would provide a robust *in vitro* latency model in which host and viral proteins could be modulated, and the latent viral genome analyzed. As part of this dissertation, I reviewed the currently available cell culture models used to study HSV latency and highlight the advantages

that our human neuronal model brings to the field. Experimentally, I demonstrated that HD10.6 cells mature to have a sensory neuron-associated phenotype (SNAP) and are a novel context in which to study HSV-1 latency and reactivation. The validation of HSV-1 latency in SNAP cells has provided an exciting starting point for further characterization as this is the only *in vitro* human HSV-1 latency model demonstrated in sensory neurons. Moving forward, viral and host mechanisms for establishing latency as well as those necessary for reactivation can be explored for HSV-1 as well as other alphaherpesviruses.

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CHAPTER 1. The *in vitro* Modeling of Herpes Simplex Virus Latency

Clinical and Experimental Outlook

The significant global prevalence of herpes simplex virus (HSV) is largely due to the fact that a defining characteristic of the herpesviruses is lifelong infection. Recent models estimate that in 2012, the global prevalence for HSV 1 (HSV-1) in people aged 0-49 years was 3.7 billion (67%) (Looker, et al. 2015b), and the prevalence for HSV-2 in people aged 15-49 years was over 400 million (11.3%) (Looker, et al. 2015a). Alphaherpesviruses employ two contrasting infection strategies to ensure both transmission of virus and long-term infection. Replication and viral shedding is referred to as lytic or productive infection, whereas latent infection refers to a quiescent state where viral genomic material persists in cells of the host, poised for reactivation.

HSV-2 is most commonly spread by sexual contact (Mertz, et al. 1992), whereas HSV-1 is frequently acquired through oral secretions as an upper respiratory infection during early childhood (Xu, et al. 2007), although the epidemiology is changing such that many countries are seeing increasing sexually transmitted infections of HSV-1 (Bernstein, et al. 2013). Most often, reactivation of latent HSV leads to recurring ulcerative blisters at the mucosal surfaces near the primary site of infection (orolabial or genital herpes) (Gupta, et al. 2007), but other painful lesions can occur as well (Sanders and Garcia 2014). Reactivation resulting in ocular lesions (herpetic keratitis) can lead to blindness (Burcea, et al. 2015; Tsatsos, et al. 2016). In rare cases, HSV can cause encephalitis (Whitley 2015), most notably in neonates born to mothers shedding either genital HSV-1 or HSV-2 (Jones, et al. 2014; James and Kimberlin 2015) or in adults due to reactivation of HSV-1 (Steiner, et al. 2007; Choi, et al. 2014; Jaques, et al. 2016).

The lytic infection cycle of HSV has been well studied, and as such, is the primary target of most antiviral therapeutic drugs (Kukhanova, et al. 2014). Acyclovir (acycloguanosine) was

first designed as an antiherpetic drug in 1977 and remains the primary therapeutic approach today (Elion, et al. 1977). Acyclovir and its derivatives inhibit viral replication, reducing the duration and severity of clinical signs. Unfortunately, these therapies do not prevent recurrence arising from reactivation of latent virus (Koelle and Wald 2000). In addition, viral strains resistant to acyclovir occur in 5-7% of the immunocompromised patient population (Grey, et al. 2003; Morfin and Thouvenot 2003; Stranska, et al. 2005; Frobert, et al. 2014).

Research efforts for prophylactic vaccine development have been ongoing since the 1920's, yet despite numerous clinical trials of potential vaccines (Koelle and Corey 2003; Coleman and Shukla 2013; Awasthi, et al. 2017), none have been effective at preventing infection (Corey, et al. 1999; Belshe, et al. 2012). Even with advancements in vaccine technology, the immune evasion mechanisms utilized by HSV-1 and HSV-2 has proven too complicated to produce long-term benefits (Koelle and Corey 2003; Johnston, et al. 2011; Coleman and Shukla 2013). With such high prevalence of disease, development of a therapeutic vaccine is also being explored (Dutton, et al. 2016; Kaufmann and Flechtner 2016), but to date none have been effective at both alleviating clinical symptoms and reducing viral shedding (Chentoufi, et al. 2012; Coleman and Shukla 2013).

Therefore, new treatments that prevent the painful, sometimes life-threatening recurrence of HSV infections could benefit a significant portion of the human population. Unfortunately, the molecular mechanisms underlying viral reactivation are poorly understood, arising in part from a lack of robust experimental models in which the critical molecules involved in HSV reactivation can be studied. For many years, *in vivo* models using small animals (especially mice, rabbits, and guinea pigs) have been used to characterize latency in an anatomically and immunologically relevant context. Animal models are expensive and time-consuming. *In vivo* infections take over

a month to establish latency. Moreover, latent virus is typically found in less than 20% of neurons (Sawtell, et al. 1998; Sawtell 2005), and only a small fraction of those latently-infected neurons will reactivate following experimental induction. As a result, molecular studies of viral reactivation mechanisms using *in vivo* models have been limited (Bloom 2016).

With recent advancements in neuronal culturing techniques, however, *in vitro* cell culture models have emerged as powerful tools to study the molecular and genetic mechanisms of HSV latency and reactivation. Cell culture models offer several research advantages, including consistency, reproducibility, and cost-effectiveness. The expression or activity of viral and cellular proteins can be more readily modulated in cell culture by genetic approaches (including targeted gene editing), RNA interference, or pharmacological agents. In addition, confounding contributions from inflammatory or support cells are removed, providing an opportunity to study viral mechanisms at the single cell level. The purpose of this review is to examine the different HSV latency cell-culture models currently available, highlighting the specific contributions and advantages of each model.

HSV Life Cycle: Lytic and Latent

HSV is highly complex, with genomes that encode essential enzymes for gene regulation, DNA replication, processing, and packaging. In a lytic infection of the epithelium, virion protein 16 (VP16) is delivered as a tegument protein and drives viral gene expression by complexing with host cell proteins Oct-1 and HCF1. This VP16-induced complex recruits general transcription machinery to viral immediate early (IE) gene promoters, triggering lytic gene expression (Campbell, et al. 1984; Dalrymple, et al. 1985; Gerster and Roeder 1988; Kristie and Sharp 1990; Hagmann, et al. 1995). Lytic gene expression is highly ordered, with the IE gene products setting off a cascade of viral early and late gene expression allowing DNA synthesis

and nucleocapsid assembly to take place in the nucleus (Honess and Roizman 1974; Wysocka and Herr 2003). Virion assembly and maturation continues in the cytoplasm.

In contrast, latent infections are established under conditions in which the mechanisms supporting the efficient, organized lytic life cycle are not adequately supported. Virus particles released from lytically-infected epithelial tissue enter the innervating sensory neuron axons. Capsids carrying viral genomes are released into the cytoplasm and traverse to the neuronal soma in ganglia driven by microtubule-based molecular motors; cytoplasmic dynein provides the motor force for retrograde movements towards the soma (Scherer, et al. 2016). Capsids deliver viral genomes to neuronal nuclei where a non-productive infection (latency) occurs. One current hypothesis considers the architecture of the innervating neuron to be a key component in the process (Hafezi, et al. 2012). The extensive trafficking of capsids through long axons, results in the inefficient transport of tegument proteins (such as VP16) to the nucleus and subsequently in insufficient transcriptional activation of IE gene expression (Luxton, et al. 2005; Hafezi, et al. 2012). Without robust expression of immediate early proteins, histone deposition across viral genomes is more prominent (Conn and Schang 2013; Lee, et al. 2016), favoring genome repression by epigenetic regulation.

Operationally, latency is described as “the persistence of a viral genome within tissue where, at any given time, there is a population of cells that lack detectable infectious virus, viral proteins, or viral lytic transcripts that are dormant but have the capability of being reactivated” (Bloom 2016). During latency, the viral genome is stably maintained in the neuronal nucleus, where it persists as an episome (Deshmane and Fraser 1989) carrying marks of heterochromatin at lytic genes (Cliffe, et al. 2009; Bloom, et al. 2010; Kim, et al. 2012). Viral genomes are therefore transcriptionally repressed during latency with the exception of expression of non-

coding RNAs known as latency associated transcripts (LATs). The major LAT (LAT) is a stable 2.0 kb spliced intron that accumulates in neurons and is believed to be important in maintaining HSV latency, although the mechanisms of LAT action remain under investigation (Wagner and Bloom 1997; Nicoll, et al. 2016). Physiological triggers in the host result in cellular stresses that re-animate latent virus in neurons; genomes are actively transcribed, replicate, and produce viral particles *de novo* that are transported back to the mucosal surface to cause a lesion (reactivation). The terminology used to describe HSV latency has been refined over the years, as recently reviewed by Sawtell and Thompson. They propose a partitioning of the process of reactivation from latency into a series of events including pre-initiation (comprising reversible changes in signaling or viral chromatin), exit from latency (detectable expression of viral proteins), and reactivation (presence of infectious virus from cells where none had been present), and argue that consistent and common use of the latency lexicon will help to illuminate aspects of these mechanisms that might emerge from various experimental models (Sawtell and Thompson 2016).

Recapitulating Latent Infection

The longstanding challenge in recapitulating HSV latency in cell culture involves simplifying a complex process with multiple steps (establishment, maintenance, and reactivation) (Preston and Efstathiou 2007) and multiple anatomical compartments (mucosa and ganglia). Consequently, most of the two-dimensional cell culture systems are permissive to lytic infection and require experimental conditions that favor non-productive infection, such as treatment with acyclovir, using low multiplicities of infection, or using replication-defective mutants. These experimental conditions create certain limitations in various cell culture models discussed in this

review. However, modification of molecular and genetic events specific to viral latency and/or reactivation is still more accessible in cell culture studies compared to *in vivo* studies.

Defining the specific site of HSV latency, one aspect necessary in building a relevant model, has been historically challenging. The current consensus is that HSV is most frequently found in sensory neurons that innervate the primary site of infection (Cohrs, et al. 2016). For example, latent HSV-1 can be found in the trigeminal ganglia (TG) that innervate the lips, gingiva, and eyes, and latent HSV-2 can be found in the dorsal root ganglia (DRG) that innervate the skin and muscle of the back as well as the mucosa of the genitalia (Steiner and Benninger 2013). This consensus, however, is most likely an oversimplification; other ganglia, including sympathetic neurons from vestibular, geniculate, spiral and sacral ganglia have been documented as sites of HSV latency (Warren, et al. 1978; Furuta, et al. 1993; Ohashi, et al. 2011; Lee, et al. 2015). Interestingly, an argument for non-neuronal sites of latency re-emerged in 2015, when evidence of HSV latency in corneas of patients with herpetic keratitis was reported (Higaki, et al. 2015). Nonetheless, many in the field agree that neurons are the preferred cell type in which to recapitulate latent infection in cell culture.

Latency Cell Culture Models

Unfortunately, reliable and reproducible sources of neurons for cell culture investigation of HSV latency are hard to come by. For ethical reasons, human neuronal tissue is not readily available for primary culture. Here we examine the different HSV latency cell culture models that are currently available, which we segregate into three categories (i) non-neuronal cell lines, (ii) non-human primary neurons, and (iii) human-neuronal cell lines. Each model provides unique opportunities to study different aspects of HSV latency and reactivation. In our opinion, when the findings from these models are taken into consideration collectively, progress in

addressing critical gaps in understanding the molecular and genetic components of latency and reactivation can be made.

Non-neuronal Cell Lines

Quiescent states that resemble HSV latency can be established in certain non-neuronal cell lines. Initially, HSV-1 quiescent infections were established in fibroblasts using a combination of elevated temperatures and replication inhibitors (O'Neill 1977; Scheck, et al. 1986; Shiraki and Rapp 1986). Quiescence with HSV-2 was established using elevated temperatures and a very low multiplicity of infection (Russell and Preston 1986). In another example, a non-replicating, non-toxic infection state was established in normal human diploid fibroblasts upon infection with HSV-1 mutants impaired for IE gene expression (Harris and Preston 1991). More recently, one report demonstrated quiescent wild-type HSV-1 infection of primary (serum starved) human diploid fibroblasts without the use of chemical replication inhibitors, by inducing heat shock (HS) proteins through elevated pre-incubation temperatures (McMahon and Walsh 2008). Using this fibroblast model and wildtype virus, entry into a quiescent state was characterized by expression of some IE proteins (ICP4, ICP22), but a failure to process ICP22, and inefficient production of ICP0; all of which resulted in low-level viral replication (McMahon and Walsh 2008). Because fibroblasts readily proliferate and generate ample material for experiments, the pathways necessary for the establishment of latency can be studied in a human cell type in a rapid and robust manner.

Another interesting model uses rat pheochromocytoma (PC12) cells which are derived from a type of endocrine tumor from the adrenal gland that has neural crest origins (Greene and Tischler 1976). Although not technically considered neurons, they can be differentiated into “neuron-like” cells when treated with nerve growth factor (NGF) or dexamethasone. Neuronally

differentiated PC12 (ND-PC12) cells have been used to study HSV-1 quiescent infection where virus can be reactivated with cellular stressors such as heat shock (43°C for 3 hours) or treatment with forskolin (Danaher, et al. 1999; Danaher, et al. 2005; Hogk, et al. 2013). One specific study used a series of viral mutants with various deletions in the VP16 transactivation domain to evaluate the importance of specific sub-regions for IE gene expression and stress-induced reactivation (Danaher, et al. 2013). This approach is not feasible *in vivo* due to the poor replication of mutant strains. In ND-PC12 cells, however, equivalent viral genome copy numbers of mutant and wild-type viruses established quiescence. After reactivation was induced, neurons demonstrated unique regulation of IE gene activation and the VP16 transactivation domain was found to be critical for reactivation (Danaher, et al. 2013).

Both of these systems are simple and efficient; these cell lines proliferate readily and provide opportunities to use biochemical or molecular assays to study quiescent infection of wild-type HSV-1. In addition, there is no need for the dissection of animal tissue for primary culture, thus saving time and reducing animal use. Diploid fibroblast models allow quiescent infection to be studied specifically in human cells, which provides a natural host-cell context. Unfortunately, fibroblasts are not the cell type in which latency and reactivation naturally occur (Jurak, et al. 2014). Whereas ND-PC12 cells offer a “neuronal-like” state, they are not human and only appear morphologically similar to neurons.

Host cell proteins that are critical in HSV gene regulation differ both in expression levels and cellular localization in neurons compared to non-neuronal cell types. For example, the POU domain transcription factor Oct-1 is robustly expressed in epithelial cells, and as previously mentioned a critical gene regulatory protein that complexes with VP16 and HCF1 (Wysocka and Herr 2003). In sensory neurons, however, other POU domain proteins such as Brn-2 and Brn-3A

are more robustly expressed (Lakin, et al. 1995) and these may bind to IE promoters with a higher affinity than Oct-1 (Hagmann, et al. 1995; Turner, et al. 1996). In addition, HCF1 is an important cell cycle regulatory protein that in most cells localizes to the nucleus (Mahajan, et al. 2002; Zargar and Tyagi 2012). In sensory neurons, however, HCF1 localizes in the cytoplasm (Kolb and Kristie 2008). This may play a role in favoring a non-productive infection in neurons if HCF1 differentially binds VP16 in distal axons, impeding its trafficking to the nucleus (Kim, et al. 2012). Lastly, HSV does not infect cells lacking HCF1 and Oct-1 well (Nogueira, et al. 2004). This supports the notion that mechanisms of HSV latency and reactivation should also be investigated in neurons, which presumably expresses the host cellular machinery of the natural site for viral latency.

Non-human Primary Neurons

The first latent infection of cultured neurons was reported by Wilcox and Johnson in the late 1980s, in which primary sympathetic neuronal tissue from prenatal rats was infected with either HSV-1 or HSV-2 in the presence of acyclovir (Wilcox and Johnson 1987; 1988). Cultures that were initially shown to be quiescent (no viral antigens detected with immunohistochemistry) could be reactivated by NGF deprivation (Wilcox and Johnson 1988).

Some years later, the use of cultured rodent neurons re-emerged as a way to model latency *in vitro*. One group dissociated neonatal rat DRG and infected with lytic-gene reporter viruses developed on the backbone of a replication-defective mutant virus to better characterize primary infection and establishment of latency in neurons (Arthur, et al. 2001). Specifically, this group demonstrated that from 3 days post infection, lytic phase expression was shut down whereas LAT promoter expression was highly variable, suggesting that certain neuronal subtypes may be more permissive for LAT expression during latency (Arthur, et al. 2001). The use of

replication-defective virus strains limits the ability to study complete reactivation in this model, since productive infection is the final hallmark reactivation. Still, early parts of the process of reactivation can be studied in this model; for example, NGF deprivation or treatment with trichostatin-A (a histone deacetylase inhibitor) resulted in activation of lytic promoters (Arthur, et al. 2001), giving an early indication for the role of epigenetic regulation in the maintenance of HSV latency.

Mohr and Wilson established latency using wild-type HSV-1 in a manner similar to the Wilcox model, in which superior cervical ganglia from prenatal rats were infected in the presence of acyclovir (Kobayashi, et al. 2012b). After treatment with an anti-mitotic agent, dissociated sympathetic neurons provide a near-homogenous population of cells that express one of the neurotrophic tyrosine receptor kinases, TrkA, which is expressed in some nociceptive sensory neurons. In this model, viral genomes were maintained at an average copy number of 25 per neuron, with LAT expression persisting in neuronal nuclei; lytic mRNAs, proteins, and infectious virus were undetectable. Reactivation was induced by disruption of the NGF-TrkA pathway, either by direct NGF deprivation or by modulation of downstream signaling through PI3-kinase, AKT, and mTOR using shRNA knockdown or pharmacological inhibition (Camarena, et al. 2010; Kobayashi, et al. 2012a), which indicated that these signaling molecules were critical for maintaining viral genome in a latent state (Camarena, et al. 2010). Moreover, by growing primary neurons in compartmentalized culture systems such that media for axons are separated from media for neuronal cell bodies, spatial-specific parameters of HSV latency in neurons can be investigated (Hafezi, et al. 2012); latent HSV genomes in dissociated rodent ganglia soma responded to localized changes in mTOR signaling in axons (Kobayashi, et al. 2012a). The transition from latent to lytic transcription (reactivation) was carefully profiled using

this model and revealed two distinct waves of viral mRNA accumulation during the exit from latency (Kim, et al. 2012).

These models have enabled the molecular analysis of mechanisms necessary in maintaining HSV-1 in a quiescent state specifically in neurons, the cell type of latency in the natural host. However, the permissiveness of rodent ganglia tissue to HSV lytic infection in culture means that either acyclovir treatment or replication-defective mutants are required to establish a non-productive infection. One critique of using acyclovir is that the guanine analog may lead to stalled viral replication forks and thus establish an aberrant latent state in which viral DNA is incompetent for subsequent replication and reactivation.

Some hypothesize that adult-rodent ganglia are less permissive to lytic infection than immature (embryonic or neonatal) ganglia and therefore do not require acyclovir. Margolis and Bertke infected dissociated adult-mouse TG with HSV-1 and HSV-2 and demonstrated establishment of latency without the use of acyclovir (Bertke, et al. 2011). This adult sensory-neuron model also revealed a virus type (HSV-1 verses HSV-2) preference for establishing latency in specific nociceptive-neuron subtypes (A5+ verses KH10+ respectively) that may be driven by the differences in virus-type LAT expression (Margolis, et al. 2007; Bertke, et al. 2013). Specifically, HSV-2 LAT contains a cis-acting regulatory element near the transcription start site that promotes productive infection in A5+ neurons and a second element in exon 1 that inhibits productive infection in KH10+ neurons, whereas HSV-1 does not contain such regulatory sequences and subsequently productive infection is not promoted in A+ neurons (Bertke, et al. 2013).

A recent report illustrates the value of using non-human neurons to probe the roles of specific molecular pathways in regulating latency and reactivation. In this case, latent infection

was established in dissociated sympathetic (superior cervical ganglia) and sensory neurons (TG) from adult mice using acyclovir (Cliffe, et al. 2015). The use of a number of pharmacological inhibitors revealed that during stress-induced reactivation, the c-Jun N-terminal kinase (JNK) activated the DLK/JIP-3 proteins which mediate methylation and phosphorylation of specific amino acids of histone H3 in nucleosomes of latent viral genomes, and subsequently triggered early viral gene expression by initiating changes in chromatin structure that favor gene activation (Cliffe, et al. 2015).

Latency can rapidly be established in dissociated primary ganglia (1 week compared to 1 month *in vivo*) and results in an increased number of latently infected neurons, making the model more robust for molecular analysis. As such, these models are valuable for systematically dissecting the molecular details of latency establishment and maintenance, as well as the reactivation processes. In addition, a variety of ganglia types can be harvested and compared more easily than *in vivo*, which would require different infection sites. Comparing the efficiency of HSV latency in immature versus adult ganglia or sympathetic versus sensory ganglia, for example, may provide insight into specific neuronal molecular requirements for latency establishment, maintenance, or reactivation.

Still, rodent neurobiology is different than human neurobiology. The functional neuronal composition within anatomically distinct ganglia, for example, differs between species (Lawson and Waddell 1991; Lawson 1992; Josephson, et al. 2001). Therefore, specific molecular characteristics identified as important in rodent infection will need to be validated in human infections. One human autopsy study of TG was unable to identify a positive correlation of HSV-1 LAT expression in subtypes of nociceptive neurons (Flowerdew, et al. 2013), contradicting the notion (derived using animal models) of a viral preference for a specific subtype in latency

establishment (Bertke, et al. 2013). In addition, species differences exist in critical host molecules known to associate with viral proteins, with one example being Oct-1 (Suzuki, et al. 1993). As a result of these subtle differences in protein structure, VP16 has a lower affinity for rodent Oct-1 than human (Cleary, et al. 1993). As a component of the VP16-induced complex, Oct-1 is critical for driving IE gene expression but this species-specific difference in protein affinity might confound findings involving viral molecular mechanisms and/or infection kinetics in rodents.

Human-neuronal Cell Lines

Because access to primary human neuronal tissue is limited for ethical reasons, human neuronal-like cell lines are an option to consider. Human neuroblastoma cell lines, such as SH-SY5Y cells, have been explored as infection models for HSV infection. The SH-SY5Y cell line was sub-cloned from cells isolated from a bone marrow biopsy of a child with neuroblastoma, and is therefore believed to be of a neural crest origin. In culture, proliferating SH-SY5Y cells can be differentiated from epithelial-like into a homogenous population of cells with a branched-neuronal phenotype (Encinas, et al. 2000; Constantinescu, et al. 2007). Differentiated SH-SY5Y cells have a higher efficiency of HSV-1 uptake compared to undifferentiated (proliferating) SH-SY5Y cells (Gimenez-Cassina, et al. 2006). Typically SH-SY5Y cells have been used in lytic infection studies or herpesviral vector studies (Gimenez-Cassina, et al. 2006; De Chiara, et al. 2010; Christensen, et al. 2011). The prospect that SH-SY5Y cells might be useful as a human neuronal HSV latency model has not yet been fulfilled (Shipley, et al. 2016). Recently, one group demonstrated “dormant” HSV-2 infection in undifferentiated SH-SY5Y by using acyclovir (Sun, et al. 2010), but as undifferentiated cells, SH-SY5Y lack a neuronal-like phenotype and a non-productive infection was not experimentally confirmed.

Varicella zoster virus (VZV) is another human alphaherpesvirus that shares similar infection strategies as the simplex viruses. Without access to a neuronal cell line of human origin, the VZV field has taken advantage of the advances in stem cell technology. Recently, a few infection models have been described based on human neurons differentiated from induced pluripotent stem cells (iPSCs) (Lee, et al. 2012; Yu, et al. 2013), neuronal stem cells (Pugazhenthi, et al. 2011) and embryonic stem cells (Dukhovny, et al. 2012; Markus, et al. 2015). Infection models using neurons derived from iPSCs are still in early stages, and as such major advances in HSV latency and reactivation specifically have not been made. In one study, approximately 80% of the cells derived after differentiation of iPSCs were neuron-specific, but only 15% of the total cell population co-expressed sensory neuron markers (Lee, et al. 2012); using this model, undifferentiated iPSCs, neural precursor cells, and sensory neurons all supported lytic HSV-1 infection.

The process of neuronal differentiation results in a heterogeneous cell population, whereby establishing latency may be challenging if permissive cells contribute to background virus production. In order to have a more pure neuronal population in which to establish a latent, non-productive VZV infection, Gilden and colleagues began using iCells (Cellular Dynamics International) which are a mixture of post-mitotic neural subtypes derived from human iPSCs (Yu, et al. 2013). To date, an HSV model has not been demonstrated in this relatively pure iPSC-derived cell line, but considering iPSC-derived neurons are permissive to HSV-1 (Lee, et al. 2012; D'Aiuto, et al. 2015; D'Aiuto, et al. 2017) infection, iCells may provide a promising human neuronal model in which to study HSV latency and reactivation.

An alternative approach to differentiating human neurons in culture arises from establishing cell lines derived directly from human neuronal tissue. For example, the HD10.6 cell

line was derived from human DRG and proliferates via a tetracycline-regulated v-*myc* oncogene (Raymon, et al. 1999). In the presence of doxycycline, cellular proliferation is suppressed, and cells mature to have a sensory neuron-associated phenotype (SNAP cells) (Raymon, et al. 1999; Thellman, et al. 2017). These cells were immortalized using the same technique as LUHMES cells, which were isolated from human mesencephalon and are widely used in neurodegenerative disease studies (Lotharius, et al. 2002; Lotharius, et al. 2005). This is the first available human cell line derived specifically from sensory neurons. The cell line can be rapidly expanded and eliminates the requirement for animal usage.

We obtained HD10.6 cells in order to develop a human *in vitro* latency model in which to study HSV reactivation. Modeled after dissociated rodent ganglia models (Kobayashi, et al. 2012b), a single treatment of acyclovir enabled a non-productive, quiescent infection in a small population of matured HD10.6 cells (SNAP cells) at relatively low viral genome copy numbers per cell (Thellman, et al. 2017). HSV-1 maintained the capacity to reactivate in this model, but NGF deprivation induced only a modestly increased probability of reactivation relative to spontaneous reactivation (Thellman, et al. 2017). SNAP cells therefore provide the opportunity to determine whether the neutrophin-signaling network responsible for maintaining latency in rodent ganglia is recapitulated in human neurons. In addition, the mechanism of LAT in HSV latency has never been studied directly in human sensory neurons and at present, both LAT and lytic RNAs were repressed in this model. SNAP cells can be matured and infected in compartmented chambers, and like dissociated rodent ganglia (Campenot 1977; Curanovic, et al. 2009), support axonal-only quiescent infections (unpublished data).

Human neuronal cell lines such as iCells or HD10.6 cells offer an exciting platform to study the relevant molecular mechanisms of HSV latency in the natural host cell type. One

caveat to consider, however, is that compared to *in vivo* rodent models, natural latent infection in human TG results in fewer neurons infected with HSV at lower genome copy numbers per cell (Cai, et al. 2002). Moreover, even in the face of external stressors, reactivation occurs infrequently in humans. Therefore, human-neuronal cell culture models may not prove to be as robust as rodent models and more advanced molecular techniques may be required.

Concluding Remarks and Future Directions

Advances in neuronal culturing techniques and the availability of high quality neuronal supplements have made it relatively feasible to study HSV latency and reactivation in dissociated ganglia from rodents. Technical approaches to culturing neurons, for example in fluidic chambers, have allowed for sophisticated experimental design in which viral and host mechanism can be dissected based on spatial-specific parameters more closely resembling natural infection. We have highlighted a variety of *in vitro* cell culture models that have been used to dissect critical viral and host mechanisms involved in the HSV latency cycle.

Studying latency mechanisms in neurons is ideal, but harvesting animal tissues is costly and tedious. Moreover, the field is aware of the caveats of using non-human models for human viral infections. Until recently, however, culturing human neurons for latent infection was not possible. Differentiation techniques have grown more fine-tuned, such that relatively pure populations of neurons can be derived from human iPSCs and are commercially available (iCells). An HSV latency model has yet to be demonstrated in iPSC-derived neurons and although human, iPSC-derived neurons are not sensory-neuron specific. The HD10.6 cell line was derived from human dorsal root ganglia, and therefore more closely resembles sensory neurons.

It is intriguing to consider how latency mechanisms defined in the various cell culture models derived from different species will compare. Using these systems as complementary models to each other, various aspects of the molecular mechanisms of latency can be carefully dissected. Ultimately, the simplicity, reproducibility, and cost-effectiveness of these *in vitro* models will shed light on possible novel drug targets. Of course, with the simplification of cell culture comes the loss of critical contributing factors from the immunological or supporting environment in which a latently infected cell resides. The cancer field has demonstrated that contributions from surrounding non-tumor stroma and inflammatory cells (the microenvironment) can play significant roles in tumor progression, metastatic potential, and response to treatment. We predict that co-culturing of cells and organoid techniques will emerge as feasible tools for analysis of HSV latency and more accurately depict the physiology of latency while maintaining the benefits of an *in vitro* system.

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CHAPTER 2. HD10.6 Cells Provide a Novel Context to Study HSV-1 Latency

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Abstract

A defining characteristic of alphaherpesviruses is the establishment of lifelong latency in host sensory ganglia with occasional reactivation causing recurrent lytic infections. As an alternative to rodent models, we explored the use of an immortalized cell line derived from human dorsal root ganglia. HD10.6 cells proliferate by virtue of a transduced tetracycline-regulated *v-myc* oncogene. In the presence of doxycycline, HD10.6 cells mature to exhibit neuronal morphology and express sensory neuron-associated markers such as neurotrophin receptors TrkA, TrkB, TrkC, and RET and the sensory neurofilament peripherin. Infection of mature HD10.6 neurons by HSV-1 results in a delayed but productive infection. However, infection at a low MOI in the presence of acyclovir results in a quiescent infection resembling latency in which viral genomes are retained in a low number of neurons, viral gene expression is minimal, and infectious virus is not released. At least some of the quiescent viral genomes retain the capacity to reactivate, resulting in viral DNA replication and release of infectious virus. Reactivation can be induced by depletion of nerve growth factor; other commonly used reactivation stimuli had no significant effect.

Importance: Infections by HSV cause painful cold sores or genital lesions in many people; less often, they affect the eye or even the brain. After the initial infection, the virus remains inactive (latent) in nerve cells that sense the region where that infection occurred. To

learn how virus maintains and reactivates from latency, studies are done in neurons taken from rodents or in whole animals to preserve the full context of infection. However, some cellular mechanisms involved in HSV infection are different in rodents than in humans. We describe the use of a human cell line that has the properties of a sensory neuron. HSV infection in these cultured cells shows the properties expected for a latent infection, including reactivation to produce newly infectious virus. Thus, we now have a cell culture model for latency that is derived from the normal host for this virus.

Introduction

Herpesviruses are unusual among viruses in that they employ two infection strategies, lytic (replicative or productive) infection and latency (quiescent) infection. Alphaherpesviruses typically replicate in epithelial cells and establish latency in the peripheral nervous system within the sensory neurons of the ganglia that innervate mucosa of the primary infection site (Kramer and Enquist 2013), such as the trigeminal ganglia (TG) and dorsal root ganglia (DRG). Operationally, latency is defined as "the persistence of a viral genome within tissue where, at any given time, there is a population of cells that lack detectable infectious virus, viral proteins, or viral lytic transcripts that are dormant but have the capability of being reactivated"(Bloom 2016). During latency by herpes simplex viruses (HSV), the viral genome persists as a transcriptionally repressed episome (in contrast to the actively transcribed state during lytic replication), with the notable exception of the latency-associated transcript (LAT) which is processed into regulatory RNAs important in maintaining HSV latency (Deshmane and Fraser 1989; Wagner and Bloom 1997; Bloom, et al. 2010; Kim, et al. 2012; Nicoll, et al. 2016).

In vivo animal models for HSV can recapitulate the general infection cycle of the virus while maintaining the complex anatomical and immunological context for lytic and latent infection strategies. Mouse models, with their amendable genetics, have proven valuable in studying acute infection and latency of HSV-1 (Proenca, et al. 2008; Nicoll, et al. 2012; Nicoll and Efstathiou 2013; Shivkumar, et al. 2013). Rabbit and guinea pig models have been used to better understand recurrent lesions and to assess efficacy of antiviral therapies (Bloom 2016).

As a complement to *in vivo* models, cell culture models offer several general advantages including consistency, reproducibility, and cost-effectiveness. Genetic or chemical manipulation of gene expression or protein activities in tissue and cell culture can yield insights into the molecular mechanisms underlying latency establishment, maintenance, and reactivation (Danaher, et al. 1999; Efstathiou and Preston 2005; Camarena, et al. 2010). Notably, rodent ganglia culture models have been employed to study HSV latency and reactivation (Wilcox and Johnson 1987; 1988; Camarena, et al. 2010; Bertke, et al. 2011; Kobayashi, et al. 2012). But because rodents are not the natural host in which HSV has coevolved (Davison 2011), molecular mechanisms in those animals or cells may be subtly different than in humans. For example, molecular differences in rodent protein Oct-1 diminish its affinity for viral protein 16 (VP16) relative to human Oct-1 (Cleary, et al. 1993); this interaction is critical for immediate early (IE) gene expression and may contribute to latency establishment (Gerster and Roeder 1988; Kristie and Sharp 1990; Hagmann, et al. 1995).

Several approaches have been advanced for studying HSV infection in human neuron-like cells in culture, including the use of human neuroblastoma cell lines (De Chiara, et al. 2010; Christensen, et al. 2011; Shipley, et al. 2016) and a differentiated human embryonal carcinoma cell line (Hsu and Everett 2001; Weir 2001). In addition, human neurons derived

from induced pluripotent stem cells (iPSCs) (Lee, et al. 2012), neuronal stem cells (Pugazhenthi, et al. 2011) and embryonic stem cells (ESCs) (Dukhovny, et al. 2012; Markus, et al. 2015) have also been explored as infection models for human alphaherpesvirus. While these human cells support HSV productive infection (Hsu and Everett 2001; Weir 2001; Christensen, et al. 2011; Dukhovny, et al. 2012; Lee, et al. 2012), reliable models to study the establishment of latency and/or reactivation have not been achieved using these human cells.

Neuronal diversity may play a role in HSV tropism for establishing latency and therefore characterization of neuronal culture models is warranted. Sensory neurons are broadly classified based on function and neurotrophin receptor expression (Muragaki, et al. 1995; Phillips and Armanini 1996) as proprioceptive (TrkC-expressing [TrkC+]), mechanoreceptive (TrkB+ and/or TrkC+), and nociceptive (TrkA+) neurons (Marmigere and Ernfors 2007). Even among the small-diameter nociceptive neurons, in which HSV latency predominantly occurs (Flowerdew, et al. 2013), functionally distinct subpopulations exist including TrkA+ peptidergic neurons and Ret+ non-peptidergic neurons that have lost TrkA signaling (Molliver, et al. 1997; Marmigere and Ernfors 2007; Liu and Ma 2011). Furthermore, the composition of sensory neuronal subtypes can vary depending on the anatomical location of the ganglia (Marmigere and Ernfors 2007), the developmental age (Josephson, et al. 2001), and the species (Lawson 1992; Josephson, et al. 2001; Fang, et al. 2005; Flowerdew, et al. 2013).

In rodent TG models, HSV-1 has shown a preference for establishing latency in A5-immunoreactive nociceptive neurons (which express the receptor TrkA and/or calcitonin gene related peptide [CGRP]), whereas herpes simplex virus 2 (HSV-2) has shown a preference for KH10-immunoreactive nociceptive neurons (which express the Ret receptor and respond to

glial-cell-derived neurotrophic factor [GDNF]) (Yang, et al. 2000; Margolis, et al. 2007; Bertke, et al. 2011; Bertke, et al. 2013). Neuronal diversity may also play a role in reactivation from latency. Disruption of TrkA signaling, by blocking or withdrawing the receptor's neurotrophin nerve growth factor (NGF) or by inhibiting downstream PI3K/AKT/mTOR signaling, induces reactivation in non-human models (Wilcox and Johnson 1988; Hill, et al. 1997; Camarena, et al. 2010). It is unclear, however, whether these patterns of HSV latency establishment or reactivation will hold true for human sensory neurons. In one human autopsy study of TGs, HSV-1 LAT by in situ hybridization (ISH) co-localized with Ret+ or CGRP+ neurons at a frequency that mirrored the population make up of these nociceptive neurons, but no positive correlation was found for TrkA+ neurons (Flowerdew, et al. 2013).

The goal of this project is to establish a human neuronal cell culture model of HSV infection in which the molecular mechanisms involved in latency maintenance and reactivation (such as stress response, transcription factors, chromatin modifying enzymes, etc.) can be examined. For this purpose, we evaluate the use of an immortalized human DRG cell line designated HD10.6, first described in 1999 by H. Raymon and colleagues (Raymon, et al. 1999). To our knowledge, this is the only established human sensory neuron cell line that is currently available, but the cell line has not been utilized since it was initially isolated and described.

HD10.6 cells proliferate in a committed sensory neuronal progenitor state by means of a tetracycline (off)-regulated *v-myc* oncogene (Raymon, et al. 1999). In the presence of doxycycline, HD10.6 cells mature to exhibit a sensory neuron-associated phenotype based on positive staining for neuronal cytoskeletal markers, expression of neuron-associated transcription factors, firing of action potentials, and capsaicin sensitivity (Raymon, et al. 1999).

By infecting matured HD10.6 cells in the presence of acyclovir, we describe a human cell culture model that exhibits experimental hallmarks of HSV-1 latency (Wilcox and Johnson 1987; Bertke, et al. 2011; Kobayashi, et al. 2012; Wilson and Mohr 2012; Markus, et al. 2015) including the presence of non-productive viral genomes that retain the potential to reactivate and produce infectious virus. This model will enable the genetic and biochemical analysis of the molecular mechanisms of alphaherpesvirus latency and reactivation in human sensory neurons.

Results

HD10.6 Cells Mature into Sensory Neurons

Since the HD10.6 cell line was initially derived about twenty years ago, conditions and media for neuronal cell culture have improved significantly. Therefore, we modified the growth conditions for the expansion and the maturation of HD10.6 cells using commercially available products (see materials and methods for details). We first used ATCC's cell line authentication service to perform short tandem repeat (STR) profiling of HD10.6 cells (data not shown). The profile was confirmed as human male, with no match to any existing profile in the ATCC or DSMZ STR databases. We then confirmed that HD10.6 cells consistently mature to adopt a sensory neuron-associated phenotype as originally reported (Raymon, et al. 1999). HD10.6 cells were seeded in proliferation media on cell culture plates or glass cover slips coated with 2 % Matrigel and 10 µg/mL poly-D-lysine (PDL).

Tetracycline-regulated v-*myc* transgene expression was suppressed by switching to complete maturation medium containing 1 µg/mL doxycycline 24 hours after plating. The suppression of proliferation and addition of neurotrophins support maturation of the cell line. Proliferating HD10.6 cells appear relatively flat with two to three short projections, but after

treatment with doxycycline, HD10.6 cells take on a neuronal morphology that includes oval cell bodies and multiple long axon-like projections (**Figure 3**).

To characterize the neuronal phenotype of matured HD10.6 cells, we tested the expression of several neuronal-specific proteins. Using indirect immunofluorescence, we detected a strong signal for the neuron-specific filament, β-III tubulin, as early as day 1 and throughout maturation (day 7 and day 14), whereas only faint nuclear expression of the glial cell intermediate filament protein, glial fibrillary acidic protein (GFAP), was detected (**Figure 4a**). Using immunoblots, we confirmed that maturing HD10.6 cells induce protein expression of β-III tubulin at levels comparable to those seen in differentiating human neuroblastoma cells (SH-SY5Y), but maturing HD10.6 cells do not express GFAP to levels seen in primary mouse astrocytes (**Figure 5a**). We concluded that HD10.6 cells express filament proteins characteristic of neurons rather than neuronal-support cells.

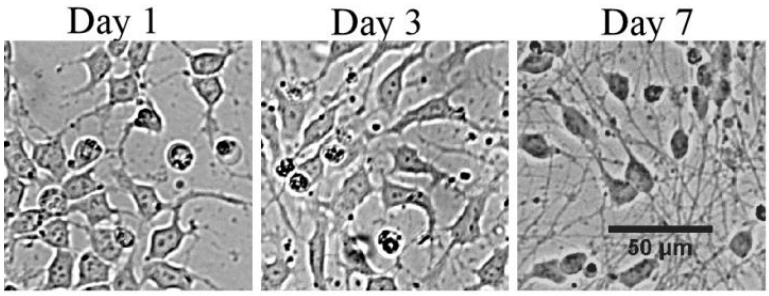

Figure 3. Doxycycline drives morphology change in HD10.6 cells. Bright field microscopy images of HD10.6 cells maturing on Matrigel and poly-D-lysine. Proliferation medium was changed to maturation medium containing 1 µg/mL doxycycline 1 d after plating. Images were taken 1, 3, or 7 d after plating. Scale bar is 50 µm.

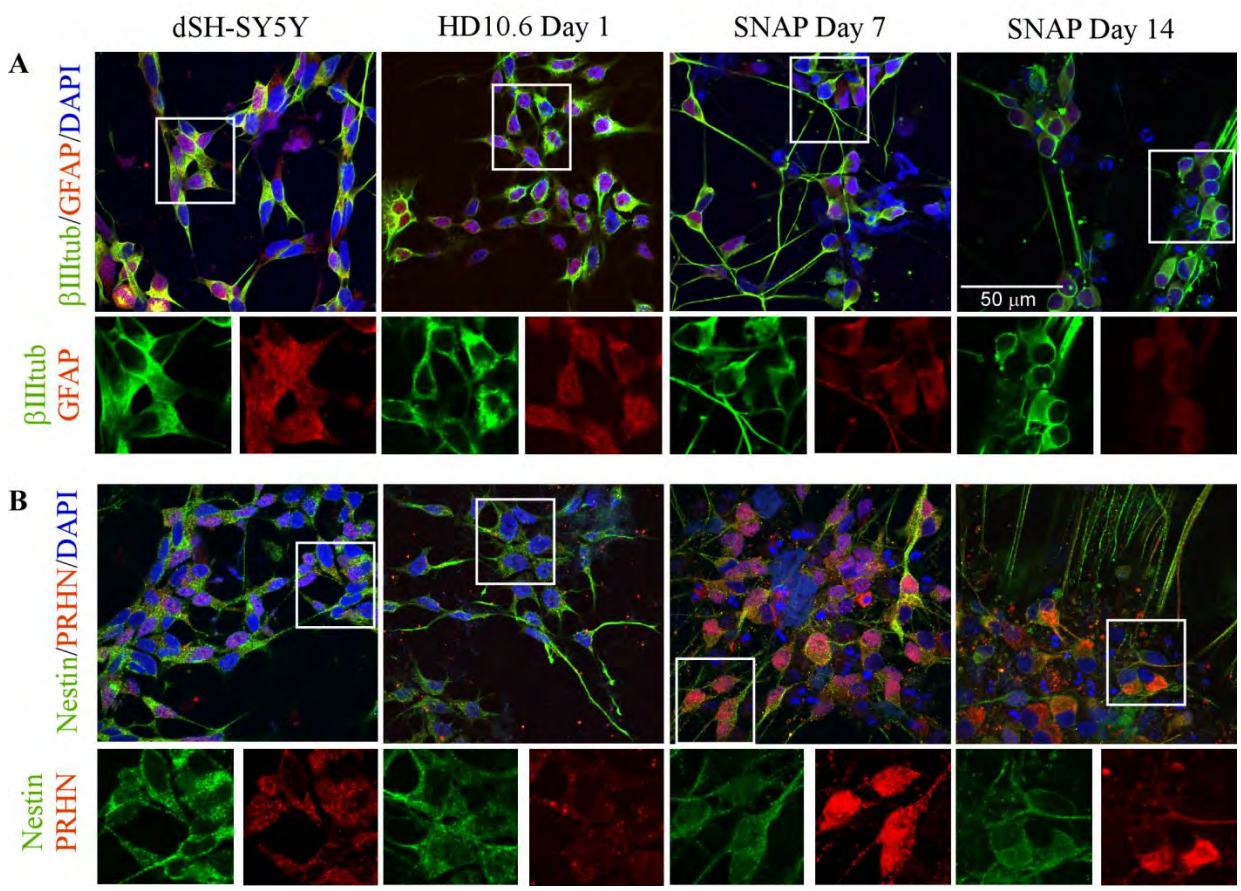

Figure 4. HD10.6 cells express sensory neuron markers. Human SH-SY5Y neuroblastoma cells were differentiated with retinoic acid (dSH-SY5Y) as a positive control for neuronal protein expression. Maturing HD10.6 cells (day 1, 7, or 14) were stained for neuronal lineage markers using immunofluorescent methods. (**A**) The top panel shows merged microscopy images for neuronal filament β-III tubulin (βIIItub; green), glial fibrillary protein (GFAP; red), and DAPI (blue). Below each merged image are images of separate green and red channels (for βIIItub and GFAP, respectively) which have been enlarged from the area denoted by the white box. (**B**) The top panel shows merged microscopy images for neuronal progenitor marker nestin (green), peripherin (PRHN; red), and DAPI (blue). Below each merged image are separate green and red channels (for nestin and PRHN, respectively) which have been enlarged from the area denoted by the white box.

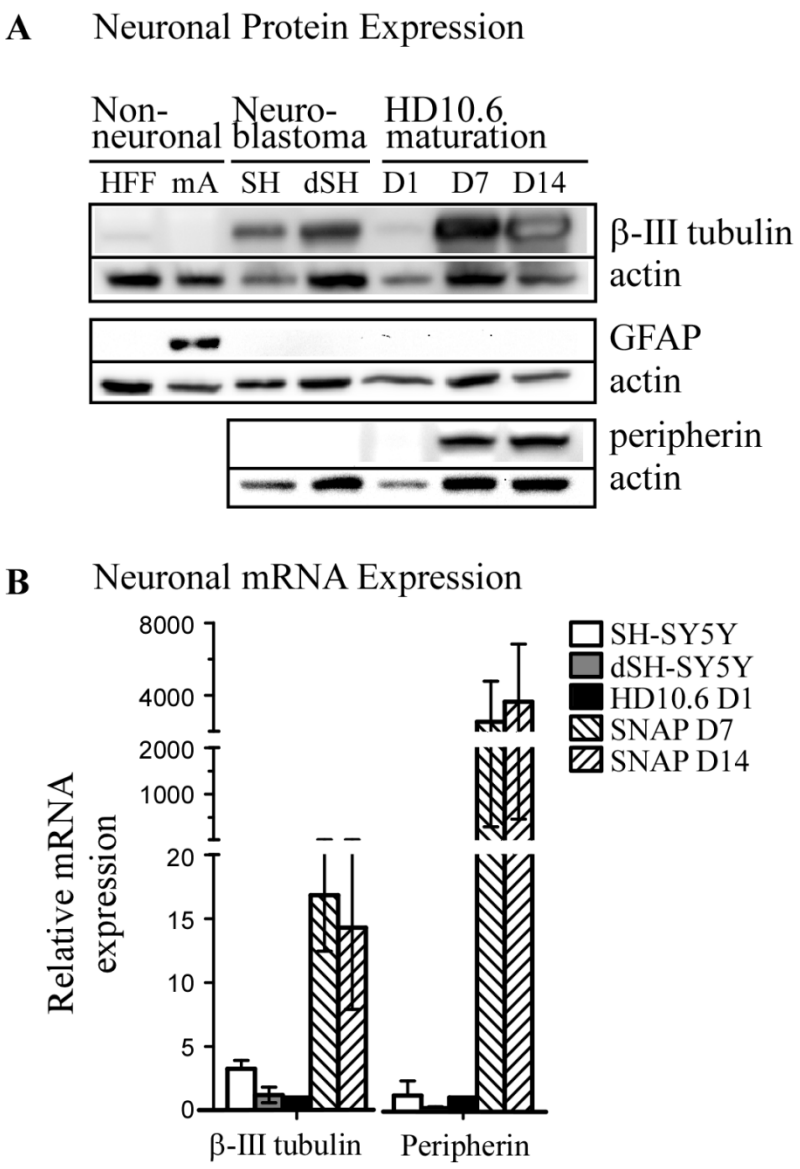

Figure 5. Doxycycline induces sensory neuron filament expression in HD10.6 cells. (**A**) Expression of neuronal lineage markers β-III tubulin and peripherin, glial fibrillary protein GFAP, and actin (as a loading control) was assessed by immunoblot analysis. Samples include immortalized human foreskin fibroblasts (HFF), mouse primary astrocytes (mA), human SH-SY5Y neuroblastoma cells (SH), retinoic acid-differentiated neuroblastoma cells (dSH), and maturing HD10.6 cells collected on day 1 (D1), day 7 (D7), or day 14 (D14). (**B**) mRNA expression for β-III tubulin and peripherin was assayed by quantitative reverse-transcription PCR for SH-SY5Y, dSH-SY5Y, maturing HD10.6 cells on D1, D7, or D14; the graph reports the fold change in mRNA expression relative to HD10.6 D1 (mean and standard deviation from 3 experiments).

We then asked whether HD10.6 cells possess neuronal progenitor qualities or are committed to a neuronal-specific lineage. We detected low levels of the neuronal progenitor marker nestin in maturing HD10.6 cells, similar to that of differentiated SH-SY5Y cells (**Figure 4b**), suggesting that HD10.6 cells are fate-restricted to a neuronal lineage. We confirmed peripheral sensory neuron specificity by detecting a robust induction of peripherin (PRHN), a sensory neuron-specific filament, by both immunofluorescence and immunoblot analysis (**Figure 4b and Figure 5a**). The conclusions based on protein expression levels were reinforced by measurements of mRNA, where by quantitative reverse transcription PCR (RT-qPCR) we observed that β-III tubulin and peripherin mRNA expression was robustly induced throughout HD10.6 maturation as compared to differentiated SH-SY5Y cells which are already neuronal but not sensory neuron-specific (**Figure 5b**).

These data demonstrate that HD10.6 cells are committed to a neuronal specific pathway even before doxycycline treatment inhibits v-*myc* expression and proliferation ceases. For this reason, we use the term "maturing" rather than "differentiating," because the latter suggests a process in which different lineages can be derived from a pluripotent cell. We conclude that under the growth conditions used here, HD10.6 cells are committed to a sensory neuron-associated phenotype (SNAP).

SNAP Cells Express Multiple Neurotrophin Receptors

Ganglia are not homogenous populations of neurons. Most neurons within sensory ganglia have a small-diameter nociceptive phenotype, with a smaller proportion having a larger-diameter mechanoreceptive or proprioceptive phenotype (Marmigere and Ernfors 2007). In adults, nociceptive neurons are thought to be polymodal, and distinct subpopulations exist (Liu and Ma 2011). These distinct subtypes may play a role in neuronal permissiveness to HSV

latency (Bertke, et al. 2011; Bertke, et al. 2013). We therefore asked whether maturation of HD10.6 cells in culture resulted in subpopulations of sensory neurons. Using immunofluorescence to examine cells at day 1, 7, or 14 of maturation, we observed expression of each of the neurotrophin receptors TrkA, TrkB, TrkC, and Ret in nearly all of the cells (**Figure 6**). Immunoblot analysis revealed that TrkA receptor expression was induced during maturation following doxycycline treatment (**Figure 7a**). Gene expression of the four neurotrophin receptors was also assayed by qRT-PCR throughout maturation. TrkA, TrkB, and Ret were robustly induced in SNAP cells, whereas TrkC receptor transcription was only transiently induced at day 7 (**Figure 7b**). We conclude that under our neuronal culture protocol, HD10.6 and SNAP cells express receptors for multiple neurotrophins and do not represent a distinct subpopulation of sensory neurons.

Establishing HSV-1 Latency in SNAP Cells

We next asked whether we could latently infect SNAP cells with HSV-1. HD10.6 cells were seeded and then matured according to the protocol described above. On day 7 of maturation, SNAP cells were infected with one of several HSV-1 strains at relatively low multiplicities of infection (MOI) [0.1 or 1.0 plaque-forming units (pfu) per cell]. HD10.6 and SNAP cells are permissive to lytic infection as will be seen in lytic control experiments throughout the next sections. In order to inhibit viral genome replication, a single treatment of the antiviral drug acyclovir (100 μM) was added to the culture medium one hour post-infection (hpi) when the inoculum was removed. After 3– 4 days post infection (dpi), media containing the initial acyclovir treatment was changed and no additional acyclovir treatments were used. We refer to the time period while acyclovir is present in culture as establishment whereas the time period after withdrawing acyclovir (5 dpi and beyond) is referred to as maintenance. We

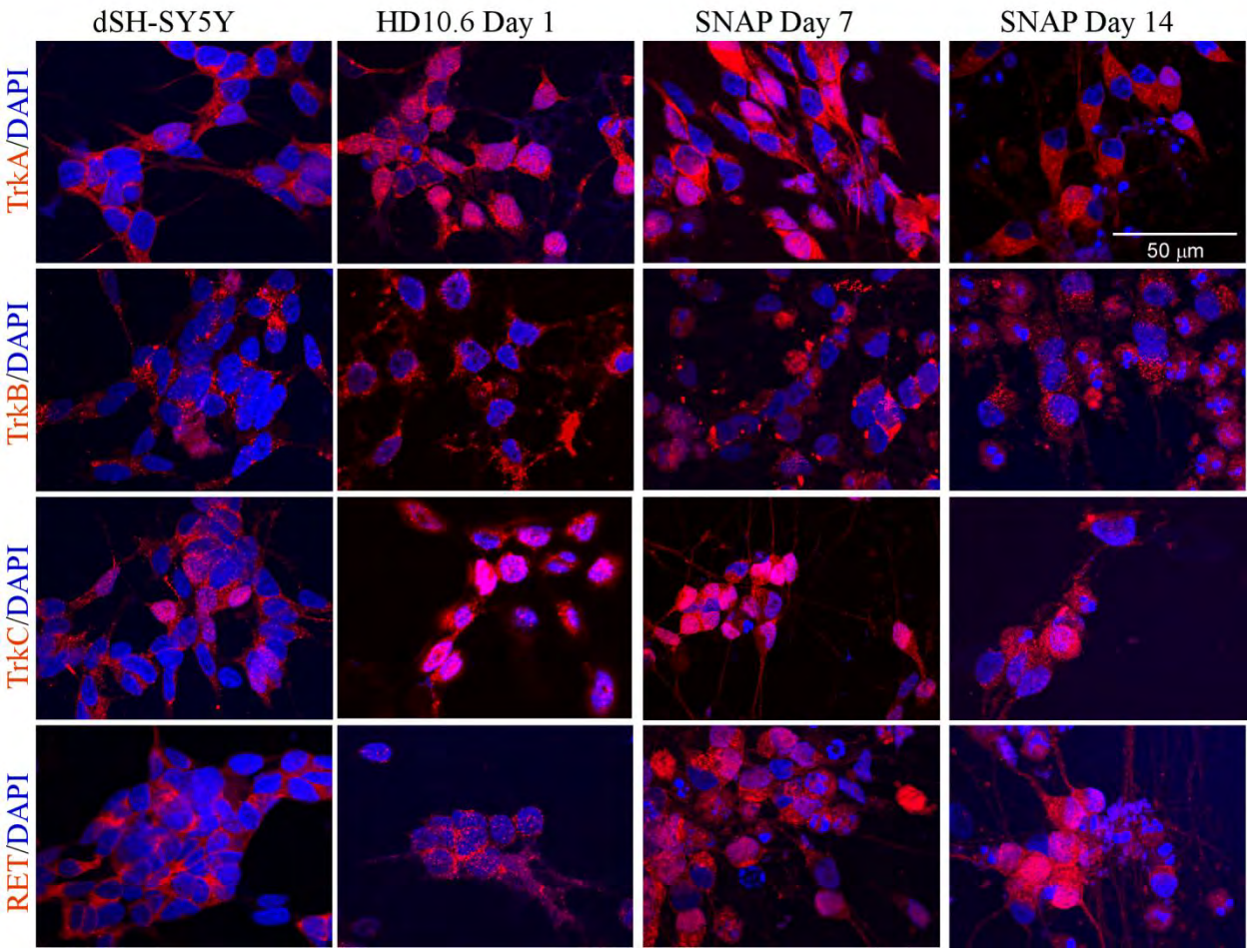

Figure 6. SNAP cells express neurotrophin receptors TrkA, TrkB, TrkC, and RET.
Differentiated neuroblastoma cells (dSH-SY5Y) and maturing HD10.6 cells (Day 1, SNAP Day7, SNAP Day 7) were stained for neurotrophin receptors TrkA, TrkB, TrkC, and RET using immunofluorescence. Each row shows expression of a single neurotrophin receptor (red) merged with DAPI (blue).

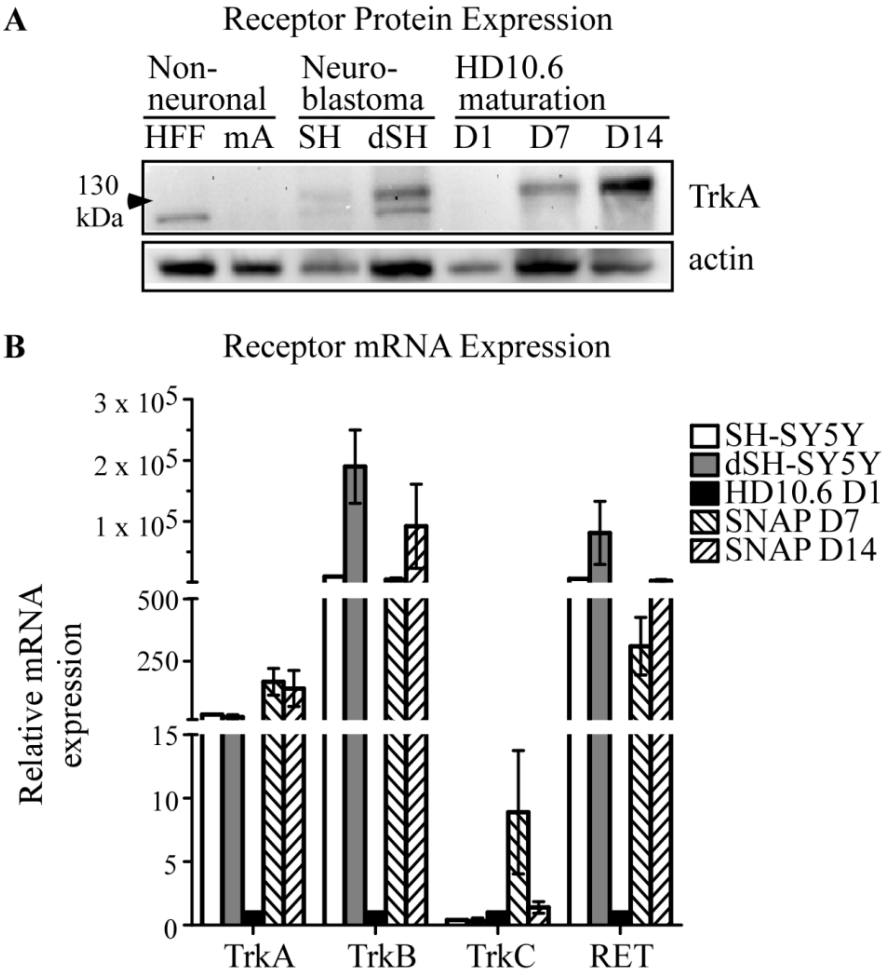

Figure 7. Doxycycline induces neurotrophin receptor expression. (**A**) Expression of TrkA receptor and actin (as a loading control) was assessed by immunoblot analysis. Samples include immortalized human foreskin fibroblasts (HFF), mouse primary astrocytes (mA), human SH-SY5Y neuroblastoma cells (SH), retinoic acid-differentiated neuroblastoma cells (dSH), and maturing HD10.6 cells collected on day 1 (D1), day 7 (D7), or day 14 (D14). (**B**) mRNA expression of TrkA, TrkB, TrkC and RET was assayed by quantitative reverse-transcription PCR for SH-SY5Y, dSH-SY5Y, maturing HD10.6 cells on day 1 (D1), D7, or D14; the graph reports the fold change in mRNA expression relative to HD10.6 D1 (mean and standard deviation from 3 experiments).

used four experimental hallmarks of latency to determine whether SNAP cells support latent HSV-1 infection where (i) viral genomes are present in neurons but in (ii) a non-productive infection, (iii) the viral genomes are transcriptionally repressed with the exception of LAT, and (iv) the viral genome maintains the potential to reactivate and produce infectious virus.

A Low Number of SNAP Cells Harbor a Low Number of HSV-1 Genome

We first asked whether we could detect viral genomes present in SNAP cells after discontinuing acyclovir (during maintenance). We hypothesized that, as seen in natural infection and other HSV latency assays, the distribution of viral genomes per neuron would vary (Maggioncalda, et al. 1996; Sawtell 1998). SNAP cells were infected in the presence of acyclovir as described above. To confirm that cultures were non-productive, media was first screened for infectious virus release by performing plaque assays. We used fluorescent *in situ* hybridization (FISH) to quantify the number of cells harboring viral genomes 5 dpi. Representative FISH images are shown in **Figure 8**.

A total of 200 cells were counted for each infection condition and binned according to the number of HSV-1 FISH signals per cell (**Table 1**). At a low multiplicity of infection (MOI 0.1), using either the lab-attenuated strain KOS or the neurotrophic strain 17 syn+, about 3 % of SNAP cells harbored viral genomes. At a higher multiplicity of infection (MOI 1.0) with strain 17 syn+, 18 % of SNAP cells contained viral genomes, with 14.5 % displaying a single viral FISH signal per cell and 3.5 % displaying two FISH signals per cell. We conclude that following (acyclovir-treated) HSV-1 infection of SNAP cells, viral genomes are present in a small percentage (3– 18 %) of SNAP cells and at a relatively low copy number of genomes compared to lytic (no acyclovir) infection.

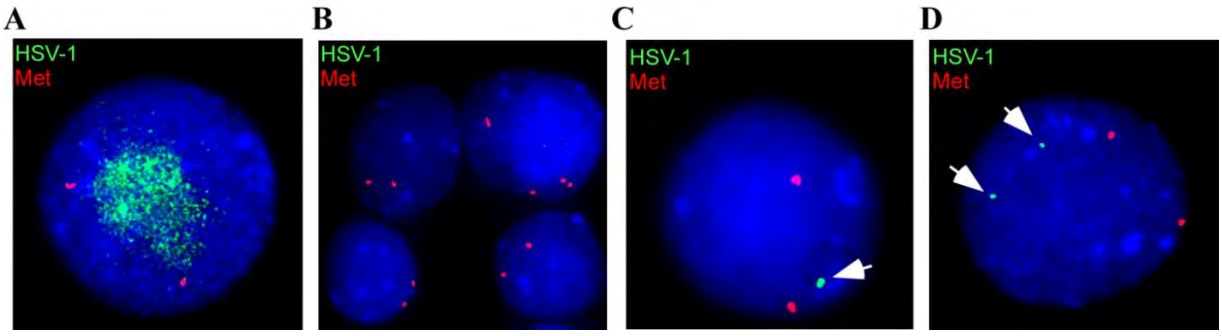

Figure 8. SNAP cells contain HSV-1 genomes in low copy numbers in few cells. Viral genomes residing in SNAP cells were detected by fluorescent in situ hybridization (FISH) 5 DPI with HSV-1 strain 17 syn+. SNAP cells were infected at MOI 0.1 in the absence of acyclovir (lytic conditions) (**A**), or in the presence of a single acyclovir treatment at a MOI 0.1 (**C**) or a MOI 1.0 (**D**). Mock infected SNAP cells are shown (**B**). The HSV-1 DNA signals are colorized green and are highlighted by white arrowheads. The human cellular gene, MET, is colorized red and was used as a positive control for hybridization conditions.

Table 1. Distribution of HSV-1 genomes in SNAP cells (%)

		KOS		17 syn +		
No. of signals per cell[a]	Mock	Latent MOI 0.1	Latent MOI 1.0	Latent MOI 0.1	Latent MOI 1.0	Lytic
0	100	97	94	96.5	82	29.5
1	–	3	6	3	14.5	17
2	–	–	–	–	3.5	13.5
3	–	–	–	0.5	–	9
4	–	–	–	–	–	5.5
5	–	–	–	–	–	2
6+	–	–	–	–	–	23.5

[a] 200 cells counted per infection condition

HSV-1 is Non-productive in SNAP Cells Infected in the Presence of Acyclovir

To test whether viral genomes replicate or remain stably present following infection in the presence of acyclovir, we used quantitative PCR (qPCR) to quantify viral genomes at several time points. SNAP cells were infected with viral reporter strains derived from either KOS (strain DG1, expressing a VP16-GFP fusion protein) or 17 syn+ (VP26-GFP) at relatively low multiplicities of infection (MOI 0.1 and MOI 1.0). Acyclovir-treated infected cells were harvested for DNA extraction at 1 dpi (early), 4 dpi (establishment), or at 8 dpi (maintenance). Lytic infections (no acyclovir treatment) were collected at 4 dpi. Viral genomes were detected at all time points, but replication was evident only in lytic infections **(Figure 9a).** We conclude that following a single treatment of acyclovir, viral genomes persist in a non-replicative state.

To further validate that infection is non-productive, we quantified the release of infectious virus in samples of culture media collected at the same time points used for viral DNA quantification. Using plaque assays on Vero cells, no infectious virus was detected in culture media from latently infected cells 8 dpi (**Figure 9b**), with the exception of a few outliers. We conclude that these culture conditions result in a quiescent, non-productive infection by maintenance. It should be noted that at a multiplicity of infection above MOI 2, we see break-through lytic infection (or a failure to go latent) more frequently, right after acyclovir withdrawal.

Viral Genomes are Transcriptionally Repressed in SNAP Cells

Many studies have shown that HSV genomes are transcriptionally repressed during latency with the exception of the production of LAT, a regulatory RNA important in the establishment and maintenance of latency in several experimental models (Mehta, et al. 1995; Held, et al. 2011; Deleage, et al. 2016; Nicoll, et al. 2016). We asked whether viral genomes in

Figure 9

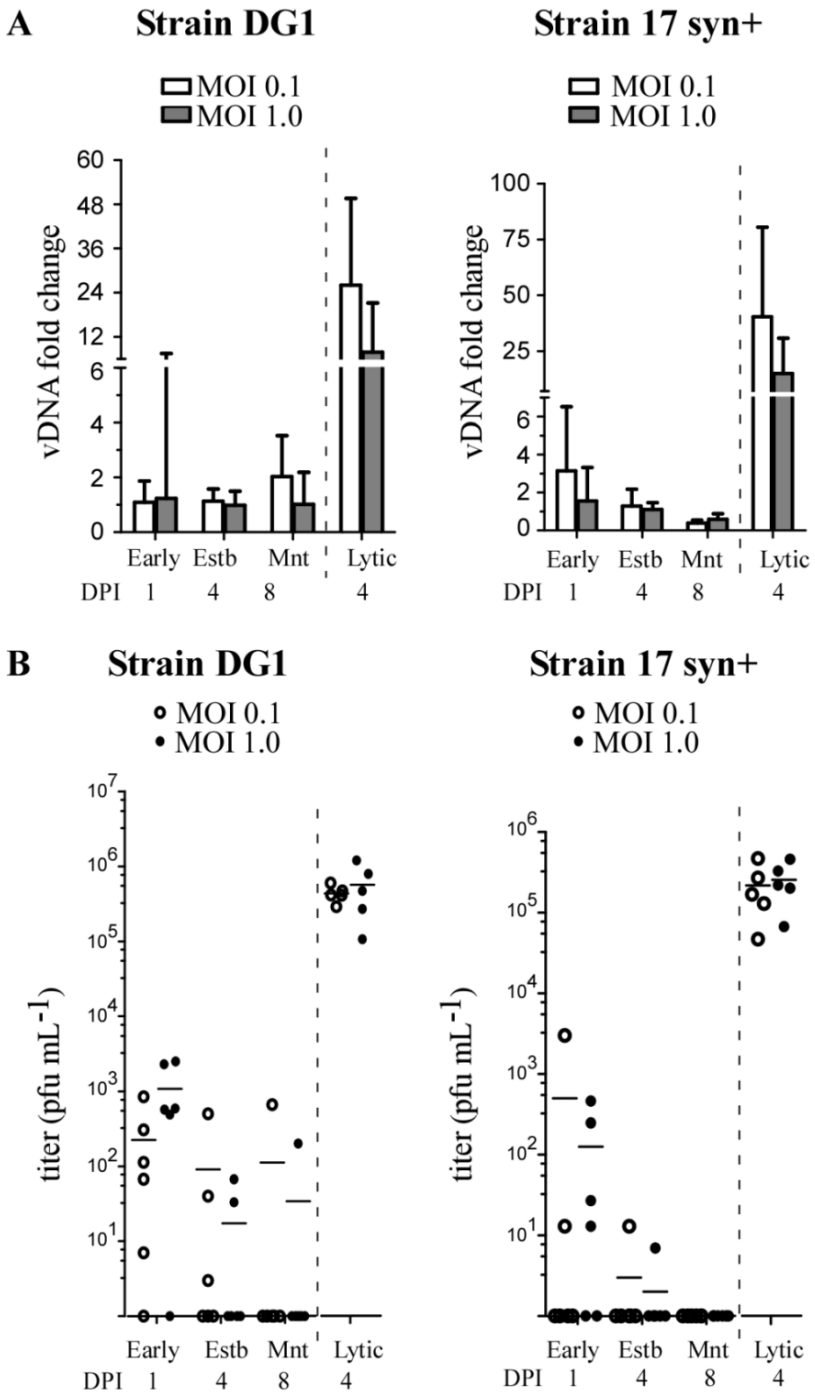
A
Strain DG1
Strain 17 syn+
MOI 0.1
MOI 1.0
vDNA fold change
Early Estb Mnt Lytic
DPI 1 4 8 4
B
Strain DG1
Strain 17 syn+
MOI 0.1
MOI 1.0
titer (pfu mL-1)
Early Estb Mnt Lytic
DPI 1 4 8 4

Figure 9. HSV-1 is non-productive in SNAP cells under acyclovir treatment. Day 7 SNAP cells were infected in the presence of acyclovir for four days with HSV-1 reporter strains DG1 or 17syn+ VP26-GFP at an MOI 0.1(white bars/circles) or MOI 1.0 (dark bars/dots). Cells were harvested for (**A**) DNA extraction and (B) media was collected as inoculum for plaque assays at 1 DPI (Early), 4 DPI [establishment (Estb)], and 8 DPI [maintenance (Mnt)]. Lytic control infections were not treated with acyclovir and (a) DNA or (**B**) media was harvested 4 DPI (Lytic). (**A**) Viral DNA extracted from infected cells was quantified by qPCR and is represented as fold change in viral DNA relative to establishment (Estb) for each infection condition. The mean and upper 95% confidence intervals are plotted. (**B**) Viral titers in the cell culture media were determined by plaquing on Vero cells. Biological duplicates from three separate experiments are shown for each viral latent infection (n=6), with the mean value represented by a horizontal line (n=5 for lytic infections). Dashed vertical line separates lytic infection conditions from the latent time course.

SNAP cells infected under acyclovir treatment were transcriptionally repressed, and predicted that we would detect lytic gene repression accompanied by low level LAT expression. SNAP cells were latently infected with HSV strain 17 syn+ VP26-GFP (MOI 0.1) and harvested for both RNA and DNA extraction at 4 dpi (establishment) and at two maintenance times points, 8 dpi and 13 dpi. Lytic infections (no acyclovir) were collected at 4 dpi. We quantified viral and cellular transcripts and genomes using RT-qPCR and qPCR, respectively. At 8 and 13 dpi, relative transcript numbers (per viral genome) for lytic genes ICP0 and TK, as well as LAT, were one-hundredth the level seen under lytic conditions (**Figure 10**). We conclude that under these conditions the viral genome is transcriptionally repressed for both lytic mRNA and for the LAT transcript.

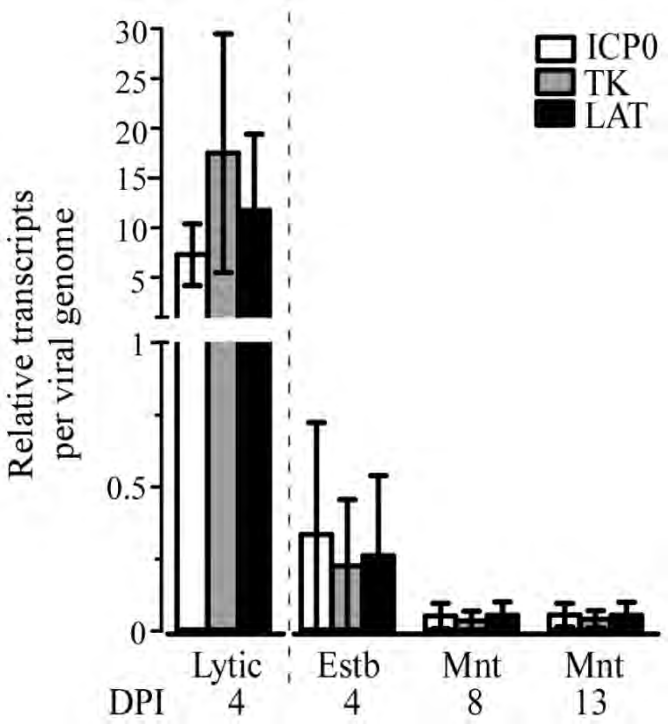

Figure 10. Viral mRNA expression is repressed in latently infected SNAP cells. Day 7 SNAP cells were infected in the presence of acyclovir for four days with HSV-1 strain 17 syn+ VP26-GFP at a low MOI (0.1 pfu/cell). Cells were harvested for DNA and RNA extraction at establishment 4 DPI (Estb) or two maintenance time points 8 DPI and 13 DPI (Mnt). Lytic infections were not treated with acyclovir and cells were harvested for DNA and RNA extraction at 4 DPI (Lytic). Quantities of viral DNA and viral transcripts ICP0, TK, and LAT were normalized to 18S ribosomal DNA or RNA. The mean relative value for transcripts per viral genome is shown with error bars indicating standard deviation from three separate experiments. Dashed vertical line separates lytic infection conditions from the latent time course.

Viral Genomes Maintain the Capacity to Reactivate

A final hallmark of latency is that virus retains the potential to exit from latency, replicate, and produce infectious virus, a process termed reactivation. We saw occasional spontaneous reactivation (i.e., reactivation of virus without intentional induction stimulus) in our control groups which confirmed that viral genomes maintained the capacity to reactivate. However, this spontaneous reactivation confounded the selection of an optimal induction stimulus. We explored a number of reactivation techniques used by others in established *in vitro* and *in vivo* HSV-1 latency models, many of which did not show an obvious increase in reactivation frequency over spontaneous levels (discussed in Appendix C).

We used various physiological factors including hyperthermia (39.5 °C for 24 hours), heat shock (43 ˚C for 1 - 4 hours) (Sawtell and Thompson 1992), or axonal disruption by axotomy to mimic explant (Birmanns, et al. 1993). We validated hyperthermia and heat shock stress by using immunoblots to monitor induced expression of HSP70 (**Appendix C, Figure 19**). We probed inflammatory pathway factors by treating with dexamethasone (Kook, et al. 2015), by treating with media from LPS-activated microglial cells, or by direct ultraviolet (UV) irradiation of latent SNAP cells. We also used pharmacological agents such as trichostatin-A (TSA) and forskolin that have been used by others to induce reactivation in rodent cell culture models (Danaher, et al. 1999; Arthur, et al. 2001). We disrupted TrkA signaling by treating with a PI3-kinase inhibitor (LY294002 or PX866) or by removing NGF from the media (NGF withdrawal only) (Camarena, et al. 2010). We validated both PI3-kinase inhibitors using immunoblots for total AKT and phosphorylated AKT (Appendix C). In our hands, none of these treatments resulted in reactivation frequencies notably above the spontaneous rate.

It was only when we combined NGF withdrawal with anti-NGF sera (NGF-depletion), superinfected with UV-inactivated (UVi) HSV-1 virus, or used a combination of both NGF-depletion followed by superinfection with UVi virus that we saw evidence of induced reactivation. In these experiments, NGF was restored after 48 hours of withdrawal to sustain neuronal cell viability. Reactivation typically was observed 7– 14 days after induction stimulus, as indicated by GFP-positive SNAP cells (**Figure 11b**). Release of infectious virus was confirmed by inoculating confluent Vero cells with medium from SNAP cell cultures (10 days post-induction) and detecting GFP expression or cytopathic effect (CPE) in Vero cells 3–5 days post-inoculation (**Figure 11b inset**). An example of non-induced latent SNAP cells and Vero inoculation in which no GFP or CPE is detected in either cell type is represented in **Figure 11a**.

Although NGF-depletion, superinfection with UVi-virus (UViR), or a combination of both techniques appeared to be most consistent at inducing reactivation, the overall reactivation frequency remained low **(Figure 12a)**. In order to determine whether the probability of a reactivation event differed among the various induction conditions, a logistic mixed-effects model was applied. To fit the model, we used data from 14 separate experiments; an experimental replicate is defined by separate HD10.6 cell platings, infections from separate virus aliquots (same stock), and different induction conditions randomized across sectors of 96-well plates of latently-infected SNAP cells. Using Vero cell inoculation as described above to amplify the GFP-signal from reactivation, we scored GFP-positive wells as “yes” for reactivation or GFP-negative as “no” for reactivation.

The probability of reactivation following NGF-depletion was significantly higher than the probability of spontaneous reactivation **(Figure 12b)**. UViR modestly increased the

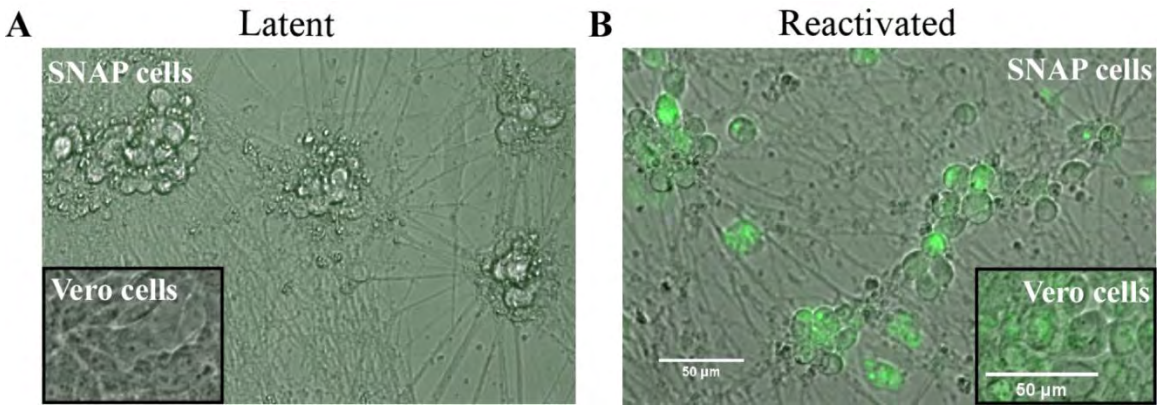

Figure 11. Latently infected SNAP cells maintain the capacity to reactivate. Day 7 SNAP cells were infected in the presence of a single dose of acyclovir with HSV-1 strain 17 syn+ VP26-GFP at a low MOI (0.1 pfu/cell) and were induced to reactivate 7 DPI with nerve growth factor depletion (A) SNAP cells latently infected but not induced to reactivate are shown in a merged FITC and bright field image; the inset shows a merged FITC and bright field image of Vero cells inoculated with media from the non-induced latent SNAP cells. (B) SNAP cells latently infected and reactivated are shown in a merged FITC and bright field image; the inset shows a merged FITC and bright field image of Vero cells inoculated with media from the reactivated SNAP cells. Scale bars are 50 µm.

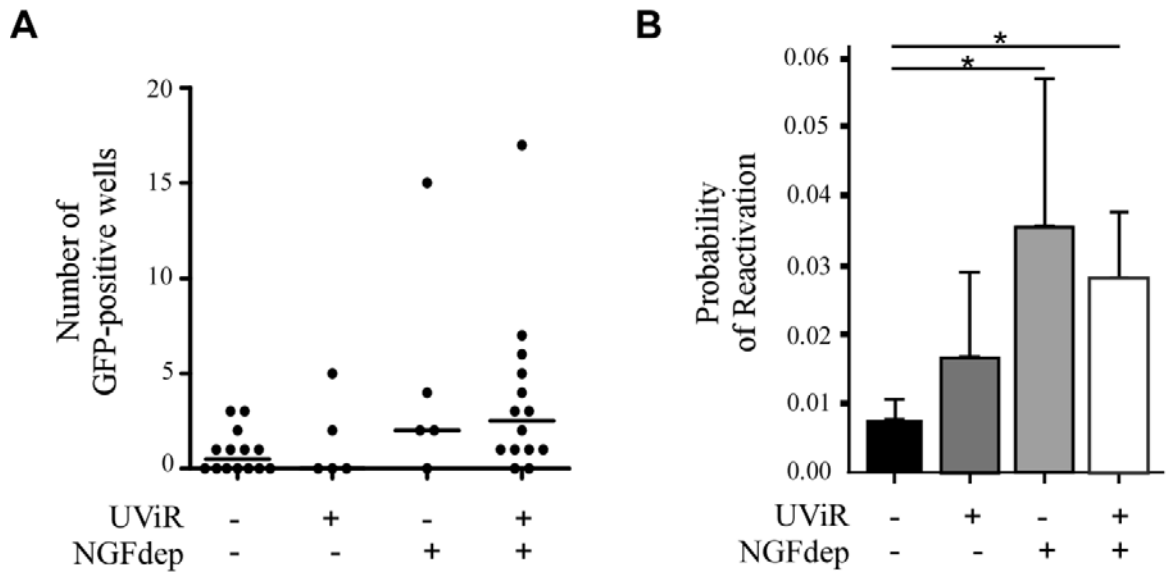

Figure 12. Reactivation frequency is low. Day 7 SNAP cells were infected in the presence of a single dose of acyclovir with HSV-1 strain 17 syn+ VP26-GFP at a low MOI (0.1 pfu/cell) and were induced to reactivate 7 DPI with ultraviolet-irradiated HSV-1 superinfection (UViR), nerve growth factor depletion (NGFdep), or a combination of UViR and NGF-depletion.10 days post induction stimuli, media from latently infected SNAP cells was used to inoculate Vero cells (for 3-5 days). Vero cell infections were scored from 14 separate experiments using a fluorescent plate reader for GFP-expression. GFP-positive wells are plotted per experiment (A), and the median is depicted with a solid horizontal line. (B) Estimated probabilities of reactivation and standard error are plotted from a logistic mixed-effects model that was used to determine whether the probability of a well reactivating differed between induction conditions. (*)Denotes comparison significance where $p < 0.0001$.

probability of reactivation above that seen spontaneously, but the difference was not statistically significant. Moreover, the combined induction (NGF-depletion and UViR) did not increase the probability of reactivation above that observed by NGF-depletion alone. False coverage intervals for each comparison depict significantly different odds of reactivation between non-induced versus NGF-depletion induction, and between non-induced versus combination induction **(Figure 13)**, but not for any other pairwise combination. From this we conclude that viral genomes maintain the capacity to reactivate from latently infected SNAP cells, but do so at a low frequency. Furthermore, these data suggest that NGF signaling is one component important for maintaining HSV-1 latency in this model.

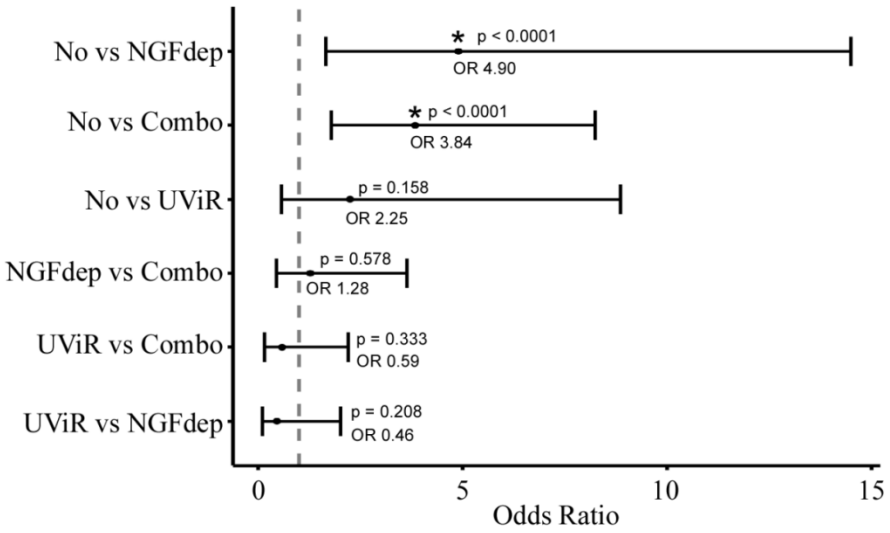

Figure 13. NGF-depletion increases reactivation probability. Plotted are the false coverage intervals for the odds ratio of each reactivation rate comparison in Figure 12b. By plotting the estimated odds ratio with lower and upper 95% false coverage intervals for each comparison, the groups that have a significantly different odds of reactivating (*) are depicted by failing to cross the "no effects" line (dashed vertical line) where the odds ratio is equal to one. Non-induced or spontaneous reactivation (No), NGF-depletion induction only (NGFdep), ultraviolet-irradiated HSV-1 superinfection only (UViR), NGFdep combined with UViR (Combo).

Discussion

The results shown in this report confirm that HD10.6 cells are fate-restricted to a neuronal phenotype, consistent with the conclusion of the original publication describing this cell line (Raymon, et al. 1999). The morphology of matured HD10.6 cells resembles that of neurons, and the induced expression of β-III tubulin and of peripherin support the conclusion of a sensory neuron-associated phenotype. We found that under culture conditions with NGF, NT-3, GDNF, and CNTF supplementation, SNAP cells express multiple neurotrophin receptors including TrkA, TrkB, TrkC, and RET and therefore HD10.6 cells do not mature into a specific subpopulation of sensory neuron. This outcome might arise from the inclusion of multiple neurotrophins in the maturation medium (Reichardt 2006), which was selected during early optimization studies based on overall cell viability counts. Given that HD10.6 cells were isolated from a first-trimester fetus and express multiple receptors, it seems that SNAP cells resemble a prenatal/neonatal neuronal phenotype. Dissociation and *in vitro* culture of adult ganglia also results in elevated expression of multiple neurtrophin receptors in primary sensory neurons relative to *in vivo* expression (Genc, et al. 2005). We have not yet explored whether these cells could be systematically differentiated into a specific subpopulation based on alternative neurotrophic supplementation.

Studies of HSV latency will benefit significantly from a human neuronal culture model in which viral and host cell factors can be experimentally manipulated. Here we demonstrate that a quiescent state resembling latency can be established in human DRG-derived neurons following HSV-1 infection in the presence of acyclovir. Traditionally, the latent state is defined as the absence of infectious virus production despite the presence of viral genomes in neuronal nuclei (Wagner and Bloom 1997; Camarena, et al. 2010), and this model fulfills that definition.

The latent genomes show little or no DNA replication and low levels of viral gene expression (Sedarati, et al. 1993), including both lytic genes and LAT. In addition, these quiescent viral genomes maintain the capacity to reactivate.

The function of LAT, first detected in latently infected murine ganglia (Stevens, et al. 1987) and later in latently infected human (Krause, et al. 1988; Stevens, et al. 1988) and rabbit (Rock, et al. 1987) ganglia, continues to be an area of some uncertainty (Knipe and Cliffe 2008). Some studies using murine models show recombinant viruses lacking various LAT domains can establish latency at normal levels (Javier, et al. 1988; Leib, et al. 1989; Steiner, et al. 1989), whereas others report significant decreases in establishment or reactivation in the absence of LAT (Sawtell and Thompson 1992; Thompson and Sawtell 1997). In a recent report looking at LAT expression in human TGs in relation to local T-cell infiltration, HSV viral genome occupancy was often accompanied by LAT expression, but also by low levels of lytic gene expression (Held, et al. 2011). Evidence for heterogeneity of LAT expression in neurons during latency has been shown in rodent models (Proenca, et al. 2008) and not all detection techniques for LAT expression are sufficiently sensitive (Ramakrishnan, et al. 1996). Given that RT-qPCR detects gene expression from a pool of infected cells and does not depict the heterogeneity of viral gene expression on a cell by cell basis, single-cell PCR or RNA *in situ* hybridization may be useful to address this issue. Alternatively, if LAT does not efficiently accumulate in SNAP cells, this may contribute to the low reactivation frequency seen; exploration of this mechanism would warrant further investigation.

We looked at transcript levels relative to genome copy number over a course of latent infection as an indication of the genome activity. An alternative approach to demonstrate the repressed nature of the latent viral genome would be to characterize the chromatin state at lytic

and LAT genes. However, one limitation of this model is that latency is established in a small population of cells and at low genome copy numbers. This may contribute to the lack of detection of LAT accumulation, but also makes characterization of latent viral genome technically challenging. Nonetheless, HSV-1 LAT-deficient strains could be used to explore the dependency of latency establishment on LAT expression in this model.

Considering that the number of cells in which the virus is latent (Maggioncalda, et al. 1996; Sawtell 1998) and the copy number of viral genomes in individual latently-infected cells (Sawtell 1997) are factors that impact reactivation frequency, the low level of latency established in our model may contribute to the low reactivation frequency. The efficiency of establishment and reactivation may be viral strain-specific based on *in vivo* models whereby KOS and 17 syn+ latently infect comparable numbers of neurons, but KOS does so with significantly fewer viral genomes per cell and has a lower reactivation frequency (Sawtell, et al. 1998). We did not directly compare these strains in our model, but a comparison of establishment efficiency and reactivation frequency for different HSV-1 strains, such as KOS, 17 syn+, and McKrae, may provide insight on this strain-specific phenomenon seen *in vivo*.

Autopsy analysis of human TG, which consist of approximately 27,000 neurons (LaGuardia, et al. 2000), shows that about 3 % of neurons harbor latent viral genomes (Cai, et al. 2002), similar to the frequency revealed by FISH in our *in vitro* infection model. In human autopsy samples the average HSV genome copy number ranged from 2 to 50 copy numbers per neuron (Cai, et al. 2002), whereas in latently-infected mouse TG the viral genome copy number per neuron can exceed 1000 (Sawtell 1997). In our system, the signal from viral genome (as indicated by FISH) rarely exceeded two. That is not to imply that the mechanisms establishing latency are necessarily the same in our model as *in vivo*, but only that the resulting

frequencies and abundances of viral genomes in our model are more closely comparable to natural human infection than to rodent models. We surmise that by increasing the number of viral genomes establishing latency per neuron, the system would have a higher propensity for inducible reactivation as seen in rodent models.

Spontaneous reactivation occurred occasionally in our experiments, but most often the virus remained quiescent, suggesting that reactivation is likely a low-frequency stochastic event. Ideally, inducing reactivation to boost the frequency would better enable a robust analysis of potential reactivation mechanisms (Kobayashi, et al. 2012). Even though NGF-depletion increased the probability of reactivation over background (spontaneous) reactivation, the frequency after induction remained relatively low compared to *in vitro* rodent models, in which (in one report) NGF signaling disruption through PI3-kinase pathway resulted in over 50 % of wells reactivating (Camarena, et al. 2010). We may be failing to observe reactivation in some experiments where low levels of infectious virus are released. We established a threshold of sensitivity for the Vero cells in 96-well format in which infections using 10 pfu/ml were detected 44 % of the time whereas infections using 100 pfu/ml were detected 100 % of the time (data not shown). Given that the wells contain approximately 0.1 ml media volume, release of 10 infectious particles or less may not always be detected. In order to interrogate the reactivation mechanism, a more sensitive method for detecting reactivation frequency would be useful.

Alternatively, the low frequency of induced reactivation may be inherently unique to the property of this cell line. We have not ruled out contributions from unknown genetic changes that may have been acquired during immortalization and acquisition of indefinite proliferation capability. Perhaps the presence of multiple neurotrophin receptor expression

means classical induction techniques which target one signaling branch are not sufficient due to compensating pathways otherwise not present in fully differentiated neuronal subtypes. Although the majority of cells expressed all three Trk receptors and RET as evident by immunofluorescence, we do not know the neurotrophic receptor status of the few SNAP cells (3-18 %) that harbor latent viral genome as detected by FISH. Co-localization studies of viral genome and receptor status is certainly warranted. Lastly, the fact that PI3-kinase inhibition did not appear to induce reactivation above spontaneous levels, but NGF-depletion does (albeit modestly), suggests that the low affinity neurotrophin receptor (p75), which also can bind NGF (Zhou, et al. 1996), may be playing a role in maintaining latency in these cells. At this time, we have not probed SNAP cells for p75 expression.

Nonetheless, this model presents opportunities to examine cellular factors necessary for establishing latency and the pathways involved during reactivation in a human neuronal system. Determining which factors are necessary for HSV reactivation (Bloom, et al. 2010; Camarena, et al. 2010; Cliffe, et al. 2015; Kristie 2015), specifically in human neurons, will help tailor the development of novel therapeutics. Of specific interest to our lab is whether the recruitment of chromatin-modifying enzymes by VP16 during latency is necessary for reactivation. VP16 recruits histone acetyltransferases and ATPase-remodeling enzymes to IE gene promoters during lytic infection in epithelial cell lines, but viral gene expression is not effected by targeted knockdown of these enzymes (Herrera and Triezenberg 2004; Kutluay, et al. 2009). We hypothesize that IE gene activation depends on recruitment of these chromatin-modifying enzymes by VP16 during reactivation. This model also presents opportunities to study other alphaherpesvirus, including HSV-2 and varicella zoster virus (VZV) (Sloutskin, et al. 2014), and potentially other neurovirulent viruses for which experimental models are even

more limited (Koyuncu, et al. 2013). HD10.6 cells can also be matured and infected in compartmented chambers (Appendix B) which opens up the possibility for studying viral transport and trafficking specifically in human sensory neurons (Hafezi, et al. 2012).

Materials and Methods

Cell Culture

Immortalized human dorsal root sensory ganglia cells (HD10.6 cells) were a gift from Celgene (Raymon, et al. 1999). HD10.6 cells were passaged in proliferation media on Nunc flasks coated with fibronectin (Millipore FC010). Proliferation media comprises advanced Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12 Life Technology 12634010) supplemented with glutaMAX (Gibco 35050061), B-27 supplement (Life Technology 17504044), 10 ng/mL prostaglandin E1 (Sigma P5515), and fresh 0.5 ng/mL fibroblast growth factor-basic (bFGF Stemgent 03-0002). For maturation in a dish or plate, cells were seeded on day 0 (D0) at a density of 5 x 10^4 cells/cm^2 in proliferation media on plates coated with 2% Matrigel (Corning 356234) and 10 µg/mL poly-D-lysine (coating done at room temperature for 1 hour). Media was changed to complete maturation media 24 hours after plating on (D1) with half-volume media changes every 3 days thereafter. Complete maturation media comprises NeuralQ Basal Medium (MTI-GlobalStem GSM-9420) supplemented with glutaMAX (Gibco 35050061), GS21 neuronal supplement (MTI-GlobalStem GSM-3100), 1 µg/mL doxycycline (Sigma T-7660), 50 ng/mL 2.5S nerve growth factor (NGF) (Harlan B.5017), and 25 ng/mL each of ciliary neurotrophic factor (CNTF) (PeproTech 450-13), glial cell derived neurotrophic factor (GDNF) (Gibco), and neurotrophin-3 (NT-3) (PeproTech 450-03). Telomerase-transformed human foreskin fibroblasts (HFFs) provided by Wade Bresnahan (Bresnahan, et al. 2000) and Vero cells acquired from ATCC were grown in DMEM supplemented with 10 %

fetal bovine serum (FBS). Human neuroblastoma cells (SH-SY5Y) were grown in a 1:1 ratio of modified Eagle's medium and F12 supplemented with 10% FBS. SH-SY5Y cells were differentiated by adding fresh (10 μM) retinoic acid every other day for 5 days (dSH-SY5Y). All cells were incubated at 37 °C and 5% CO_2.

Cell Line Authentication

We submitted HD10.6 cells to ATCC for short tandem repeat (STR) profiling. Seventeen STR loci plus the gender determining locus, Amelogenin, were amplified using the commercially available PowerPlex® 18D Kit from Promega. The cell line sample was processed using the ABI Prism® 3500xl Genetic Analyzer. Data were analyzed using GeneMapper® ID-X v1.2 software (Applied Biosystems). Appropriate positive and negative controls were run and confirmed for each sample submitted. Cell lines were authenticated using Short Tandem Repeat (STR) analysis as described in 2012 in ANSI Standard (ASN-0002) by the ATCC Standards Development Organization (SDO) and in (Capes-Davis, et al. 2010).

Viral Strains and Infection Techniques

All viruses were propagated on Vero cells. Viral titers were determined by plaque assays on Vero cells grown to confluent monolayers, infected with inoculum for 1 hour at 37 °C, and overlaid with 0.9% Sea Plaque agarose and DMEM supplemented with 5% FBS. At three to four days post infection, cells were stained with neutral red for 2 h at 37 °C to visualize and count plaques. HSV-1 strain 17 syn+ was a gift from Nancy Sawtell. HSV-1 reporter strains 17 syn+ VP26-GFP and 17 syn+ VP26-RFP were gifts from Andrea Bertke. HSV-1 strain KOS was acquired from ATCC. HSV-1 strain DG1 (KOS background) expresses wild-type VP16 fused at its carboxyl terminus to the enhanced green fluorescent protein (EGFP)

(Greenside 2000; Ottosen, et al. 2006). For neuronal infections in a dish or plate, media was removed from day 7 SNAP cells and virus (diluted in NeuralQ Basal Medium) was added for 1 h at 37 °C and 5% CO_2 with gentle rocking every 15 minutes. Multiplicity of infection (MOI) was estimated using a dilution factor quantified from the equation [(cell number × desired MOI) ÷ (virus stock titer × inoculum volume)] where cell number is the number of plated cells multiplied by 1.2 to account for initial proliferation and cell loss during maturation. At 1 hpi the inoculum was removed and complete maturation media with 100 μM acyclovir (Cayman Chemicals #14160) was added for latent infection or without acyclovir for lytic infection. Three to four DPI, fresh media was added without acyclovir for both latent and lytic infections.

Antibodies and Immunofluorescence

For immunofluorescent staining, SNAP cells were grown on glass coverslips coated with 2% Matrigel and 10 μg/mL poly-D-lysine. Cells were fixed with 4% paraformaldehyde (PFA) [16% formaldehyde methanol-free solution by Thermo Scientific 28908 diluted with phosphate-buffered saline (PBS)] for 15 minutes at room temperature without rocking. To minimize peeling, 4% PFA was added directly to cell media for 2 min (400 μL into roughly 2 mL media) prior to aspirating and proper fixation. Fixed cells were carefully washed in PBS three times and either stored at 4°C or permeablized and blocked in a single step [2% bovine serum albumin (BSA) in PBS, 0.5% Triton X-100, and 0.05% Tween 20] for 45 min at RT. Antibodies against TrkA (sc-118), TrkB (sc-20542), TrkC (sc-14025), RET (sc--1290), and peripherin (sc-28539) were purchased from Santa Cruz. Antibodies against β-III tubulin (MAB1195) and nestin (MAB2736) were purchased from R & D Systems. Antibody against GFAP (#04-1062) was purchased from Millipore. All primary antibodies were diluted in TBST (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1 % Tween 20) containing 1% BSA and incubated

either 1 hour at room temperature or overnight 4°C. Coverslips were gently washed three times in PBS. Secondary antibodies conjugated to Alexa Flour 594 or 488 (Jackson) were used at 1:250 in PBS with 1% BSA for 1 hour at room temperature (protected from light). Coverslips were gently washed three times in PBS. Coverslips were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) for 10 minutes followed by washing in distilled water. Coverslips were mounted on slides using FluorSave Reagent (Calbiochem) and allowed to air-dry for 24 hours before imaging. Images were acquired using a Nikon A1plus-RSi scanning confocal microscope with a 60 x oil objective and NIS-Elements software. Images were analyzed and colorized using FIJI software.

Immunoblot Analysis

Cell lysates were collected and analyzed by gel electrophoresis and immunoblot as follows. Cell culture medium was aspirated and cells were collected by using trypsin (TrypLE, Gibco 12604013) for 5 minutes at 37°C. Cells were washed by centrifugation (1000 rpm, 5 min), resuspension in PBS and recentrifugation (1000 rpm, 5 min). The cell pellet was directly lysed in 2X Laemmli Buffer (Bio-Rad #161-0737) and 5 % β-mercaptoethanol (Bio-Rad #161-0710). Lysates were heated to 95°F for 10 min and separated on 4-20 % Tris-glycine gels (Novex). Proteins were transferred to polyvinylidene fluoride membranes and blocked in TBST containing 5% BSA. The blocked membrane was incubated with primary antibody overnight at 4 °C or for 1 hr at room temperature at the dilution suggested by the supplier. The membrane was washed with TBST and incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (HRP) at 2500-fold dilution for 1 hour at room temperature (Cell Signaling, anti-mouse #7076 or anti-rabbit #7074). The membrane was again

washed and the HRP was detected by chemiluminescence. Actin antibodies for immunoblot loading controls were obtained from Millipore (MAB1501).

Gene Expression and Quantitative PCR

For neuronal gene expression studies, HD10.6 cells were matured in 6-well plates, with 3 wells representing biological triplicates for each experimental condition. RNA was collected using Qiagen RNeasy Kit. For viral genome quantification studies, HD10.6 cells were matured and infected in 12-well plates, with 3 wells representing biological triplicates. DNA was collected using Qiagen DNeasy Blood and Tissue Kit. For viral gene expression studies, HD10.6 cells were matured and infected in 24-well plates and a total of 12 wells were combined for each time point (in biological duplicates) by trypsinizing (TrypLE) and re-suspending cells in 500 µL of PBS. The cell suspension was split in half for separate extraction of RNA or DNA using the Qiagen RNeasy Kit or Qiagen DNeasy Blood and Tissue Kit respectively. Purified RNA (500 ng) was reverse-transcribed with High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) and RNAseOut (Invitrogen) according to the manufacturer's protocol. Gene expression was quantified by quantitative reverse transcription PCR (RT-qPCR) using primers specific for the selected viral or human genes (**Table 2**).

Purified DNA was quantified by quantitative PCR (qPCR) with primers specific for the viral ICP4 or ICP0 viral genes or the human 18S ribosomal RNA gene. The qPCR was performed on an ABI 7500 RT-PCR system (Applied Biosystems) using FastStart Universal SYBR Green master mix (Roche). For neuronal gene expression studies, relative RNA levels were analyzed by the $2^{-\Delta\Delta Ct}$ method normalized to HD10.6 cell expression at day 1. For viral genome quantification studies, DNA levels were analyzed by the $2^{-\Delta\Delta Ct}$ method normalized to

establishment time point for a given MOI. For viral gene expression studies, both transcripts and DNA were normalized to 18S (ΔCt) and analyzed by $2^{-(\Delta Ct(transcript)-\Delta Ct(DNA)}$.

Table 2. Quantitative PCR primer sequences

Target [gene] [a]	Primer Bank ID	Sequence [b]
TrkA [NTRK1] (h)	56118209c1	AACCTCACCATCGTGAAGAGT(f) TGAAGGAGAGATTCAGGCGAC(r)
TrkB [NTRK2] (h)	65506645c2	ACCCGAAACAAACTGACGAGT (f) AGCATGTAAATGGATTGCCCA (r)
Trk C [NTRK3] (h)	340745350c3	ACGAGAGGGTGACAATGCTG (f) CCAGTGACTATCCAGTCCACA (r)
[RET] (h)	126273513c1	AAAGTGGCATTGGGCCTCTAC (f) GCAGGGCATGGACGTACAG (r)
β-III Tubulin [TUBB3] (h)	308235961c1	GGCCAAGGGTCACTACACG (f) GCAGTCGCAGTTTTCACACTC(r)
Peripherin [PRPH] (h)	66932907c1	GCCTGGAACTAGAGCGCAAG(f) CCTCGCACGTTAGACTCTGG(r)
[GFAP] (h)	334688843c2	AGGTCCATGTGGAGCTTGAC(f) GCCATTGCCTCATACTGCGT(r)
18S [RNA18S5] (h)		CGGTCCAAGAATTTCACCTC(f) CCGCAGCTAGGAATAATGGA(r)
HSV-1 ICP0 (v)		CTGTCGCCTTACGTGAACAA(f) CCATGTTTCCCGTCTGGTC(r)
HSV-1 ICP4 (v)		GAAGTTGTGGACTGGGAAGG(f) GTTGCCGTTTATTGCGTCTT(r)
HSV-1 TK (v)		TACCCGAGCCGATGACTTAC(f) AAGGCATGCCCATTGTTATC(r)
HSV-1 LAT (v)		CAGACAGCAAAAATCCCCTGAGT(f) GGGACGAGGGAAAACAATAAGG(r)

[a] Human (h) or viral (v) target gene
[b] Forward (f) and reverse (r) primer pairs

Fluorescent *in situ* Hybridization (FISH)

The human control FISH probe for the MET proto-oncogene was prepared from purified BAC clones RP11-163C9, RP11-564A14, and RP11-39K12 (locus 7q31) (BACPAC Resource Center (BPRC), bacpac.chori.org). HSV-1 DNA probes spanning most of the viral genome were synthesized from cosmid templates Cos 14, Cos 28, and Cos 56 (gifts from Patrick Lomonte) (Catez, et al. 2012). Briefly, the individual cosmids were purified from NEB10 bacterial cells using a PrepEase kit (USB) and mixed in equimolar amounts. The BAC DNA and cosmid DNA were labeled with Orange-dUTP and Green-dUTP (Abbott Molecular Inc., Des Plaines, IL), respectively, by nick translation. HD10.6 cells were matured in 100 mm dishes and infected at day 7 as described in virus techniques. At 3 DPI, media containing acyclovir was replaced with fresh media lacking acyclovir. At 5 DPI, supernatant was collected (stored at -80°C) and cells were collected by trypsinizing with TrypLE and then resuspending in neuronal media. Sample slides were prepared from cultured SNAP cells that were incubated in 0.075M KCl at 37°C for 15 min and fixed with methanol:acetic acid (3:1) using standard cytogenetic methods. Glass slides were pretreated with 2X saline/sodium citrate (SSC) at 37°C for 10 min, 0.005 % pepsin / 0.01 M HCl at 37°C for 3 min, and PBS for 5 min. The slides were then placed in 1% formaldehyde for 10 min at room temperature, washed with PBS for 5 min, and dehydrated in an ethanol series (70%, 85%, and 95%) for 2 min each. Samples on slides were denatured in 70% formamide / 2X SSC at 74°C for 3 min, washed in a cold ethanol series (70%, 85%, 95%) for 2 min each, and air-dried. FISH probes were denatured at 73°C for 5 min and kept at 37°C for 10-30 min. Eight microliters of probe was applied onto each slide and mounted with a glass coverslip. The slides were hybridized overnight at 37°C, washed with 2X SSC at 73°C for 2 min, and rinsed briefly in distilled water. Slides were air-

dried, counterstained with VECTASHIELD mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA), and coverslips were applied. Images were acquired at 1000x system magnification with a COOL-1300 SpectraCube camera (Applied Spectral Imaging-ASI, Vista, CA) mounted on an Olympus BX43 microscope. Images were analyzed using FISHView v7.2 software (ASI). 200 interphase nuclei were scored for each sample and binned according to the number of discrete HSV-1 signals per cell.

Statistical analysis

Logistic linear mixed-effects models were fit using R v 3.3.1 (https://cran.r-project.org/) to test for differences in reactivation probabilities. A random intercept was included for each plate to account for within plate correlation. Linear contrasts with a false discovery rate correction were used to test specific hypotheses (Benjamini and Yekutieli 2005). Assumptions were assessed visually using quantile-quantile and residual plots. Significance level was set at alpha = 0.05.

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develop the FISH HSV-1 probe. We thank lab member Glen Alberts for his thoughtful input and technical support and Julie Koeman of the VARI cytogenetics core for performing FISH.

Specific contributions from authors other than Nikki M. Thellman are as follows: Carolyn Botting (Triezenberg Laboratory member), carried out viral infections in SNAP cells for FISH analysis; Julie Koeman (VARI Genomics Core) fixed, stained, and imaged, and quantified cells for FISH; Zachary Madaj (VARI Bioinformatics and Biostatistics Core) ran the mixed effects logistic model and created data graphs.

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CHAPTER 3. Conclusions and Future Directions

In general, cell culture models offer several research advantages including consistency, reproducibility, and cost-effectiveness. However, modeling herpes simplex virus (HSV) infection *in vitro* is not straight-forward due to the complex lifecycle and contrasting infection strategies of the virus. After robust replication and virion production in epithelial cells at the mucosa, HSV is transported via innervating axons to the neuronal nucleus where it is maintained as repressed episomes. Typically, rodent ganglia are dissociated for *in vitro* HSV latency modeling in cell culture (Wilcox and Johnson 1988; Arthur, et al. 2001; Bertke, et al. 2011; Kobayashi, et al. 2012). These rodent *in vitro* HSV infections provide a more rapid and robust analysis of latent infection compared to *in vivo* animal models. Host and viral molecules are more easily modulated *in vitro* by using RNA interference and pharmacological agents (Camarena, et al. 2010). Furthermore, mutant viral strains can be used *in vitro* that are otherwise not feasible in living animal models.

A main goal of the Triezenberg lab is to determine whether VP16 and recruitment of chromatin modifying enzymes are important for HSV-1 reactivation. Ideally, an *in vitro* HSV latency model derived from human neurons should be used to modulate the host chromatin modifying enzymes and determine which are necessary for reactivation. Due to the lack of available human derived neuronal cell lines, an HSV latency model in human neurons did not exist (Wilson and Mohr 2012). This dissertation describes the development and validation of an *in vitro* HSV-1 latency infection model in human sensory neurons.

HD10.6 cells were derived from embryonic human dorsal root ganglia (DRG) but are expandable and proliferate robustly via a tetracycline-regulated v-*myc* oncogene (Raymon, et al. 1999). The treatment of HD10.6 cells with doxycycline suppresses v-*myc* driven proliferation

allowing cells to mature to have a sensory neuron-associated phenotype (SNAP). HSV-1 and HSV-2 establish latency in sensory neurons, most commonly the nociceptive neurons in trigeminal ganglia (TG) and DRG. I therefore hypothesized that doxycycline-matured HD10.6 cells, SNAP cells, would support HSV-1 latency.

Sensory Neuron-Associated Phenotype

Because materials and methods for *in vitro* culturing of neurons have improved in recent years, we significantly changed both the media and matrix used for HD10.6 cells (proliferating and maturing). Despite the thorough characterization of the cells when originally published (Raymon, et al. 1999), we felt it important to confirm that HD10.6 cells are in the most broad sense what we assumed them to be: human sensory neurons. We authenticated cell samples by short tandem repeat (STR) profiling through ATCC, confirming HD10.6 cells are human (male) and that they did not match any profile in the ATCC or DSMZ STR database. We demonstrated that HD10.6 cells express the neuronal filament β-III tubulin even before v-*myc* proliferation is suppressed by doxycycline treatment. We confirmed that β-III tubulin and peripherin, a peripheral sensory neuron filament, are induced throughout maturation. In addition, we found only low protein expression of the neuronal progenitor marker, nestin, and little evidence of a glial cell marker, GFAP. Taken together, we concluded that in our hands HD10.6 cells can mature to have a sensory neuron-associated phenotype as the original publication demonstrated.

Sensory neurons differentiate into subpopulations with specific functions and as such express different neurotrophin receptors. Evidence for HSV-1 and HSV-2 tropism among subpopulations of nociceptive sensory neurons exists in rodent ganglia models (Bertke, et al. 2011). In addition, the most classical stimuli used to induce alphaherpesvirus reactivation from experimental latency models in sympathetic neurons and immature sensory neurons, is a

disruption in the nerve growth factor (NGF) signaling pathway (Wilcox and Johnson 1987; Arthur, et al. 2001; Camarena, et al. 2010). TrkA is the major receptor for NGF and disruption of molecules downstream, like PI3-kinase, AKT, and mTOR, induce reactivation (Camarena, et al. 2010).

We demonstrated that HD10.6 cells and SNAP cells expressed all three major neurotrophic receptors TrkA, TrkB, TrkC, in addition to another nociceptive receptor, RET, which binds glial derived neurotrophic factor (GDNF). We saw a significant induction of TrkA, TrkB, and RET at the transcript level throughout maturation, and only a modest, transient, induction of TrkC. We concluded that SNAP cells are not a distinct subpopulation of sensory neurons, but rather express multiple neurotrophic factor receptors. HD10.6 cells were isolated from a fetus during the first trimester; it could be that SNAP cells maintain the prenatal phenotype, and do not fully differentiate into adult sensory neuron subtypes (Josephson, et al. 2001). Alternatively, our supplementation of specific neurotrophic factors (which includes NGF, GDNF, and NT-3) may be driving the resulting phenotype, as this combination binds to all three Trk receptors and RET at some level (Genc, et al. 2005; Reichardt 2006). This characterization is important because the presence of multiple signaling pathways may play a role in HSV latency establishment and reactivation in these cells.

Neurotrophic Factor Signaling and Latency

Further characterization of the small subset of neurons harboring virus detected in our FISH analysis might provide insight on host cell factors contributing to the establishment of latency. At a multiplicity of infection (MOI) of 1.0 plaque-forming unit (pfu) / cell, only about 18% of latently infected SNAP cells had viral genomes present. Immunofluorescence revealed that about 90% of the cells stained positive for TrkA, TrkB, TrkC, and RET expression. Due to

the high proportion of cells expressing each receptor, we did not do co-labeling studies. However, it is possible that there is a small population of neurons with a unique expression pattern and these cells more permissive to latency establishment. Determining the neurotrophic factor receptor expression pattern of the latently infected SNAP cells may help determine whether unique neuronal characteristics contribute to permissibility to HSV-1 latency. Inquiries like this have been done in human autopsy samples (Flowerdew, et al. 2013) and rodent models (Bertke, et al. 2011). In order to determine whether latency is established in a unique, unidentified subpopulation of neurons, we would co-label latently infected SNAP cells for combinations of neuronal proteins or receptors by immunohistochemistry followed by *in situ* hybridization for HSV-1 DNA.

Another approach would be to attempt to drive specific sensory neuron subpopulations by systematically altering neurotrophin combinations over the course of maturation. Dissociation of ganglia for use in cell culture dramatically altered Trk receptor expression of rat TG, and neurotrophin switch experiments demonstrated an instructive role for neurotrophins on axonal differentiation, rather than just a passive or permissive role as survival factors (Genc, et al. 2005). If different subpopulations of neurons could be derived from HD10.6 cells, then the numbers of cells latently infected as well as HSV-1 copy number per cell could be analyzed by FISH. The comparison of efficiency of HSV-1 latency in each of the neuronal subpopulations may elucidate a role for neurotrophic factor signaling in establishment and maintenance of latency. Further characterization of mature SNAP cells, in terms of neurotrophin receptor signaling, might also improve the efficiency of latency establishment in this model.

Neurotrophic Factor Signaling and Reactivation

The poor induction of reactivation from latently infected SNAP cells makes studying host and viral mechanisms challenging in this system. On the other hand, investigating ways to make HSV-1 reactivation more robust in this model may help elucidate critical host factors important in natural infection and reactivation. In appendix C, I summarized different approaches for reactivation that failed in this model. Although statistically significant, NGF-depletion only modestly increased the probability of reactivation. I hypothesize that due to multiple active neurotrophic signaling pathways, NGF-depletion alone is not sufficient to disrupt the complex signaling network maintaining the viral genome in a latent state. Contrary to rodent models, disruption of PI3-kinase signaling by LY294002 did not induce reactivation above spontaneous levels (Camarena, et al. 2010). However, disrupting TrkA signaling of PI3-kinase/ AKT / mTOR *upstream*, by depleting NGF from media, resulted in an increased probability of reactivation. These confounding data suggest that SNAP cells may also express the low affinity neurotrophin receptor (p75) to which NGF binds, but at a lower affinity than TrkA (Zhou, et al. 1996). The subsequent downstream signaling from p75 in SNAP cells may be important for maintaining HSV-1 latency in this model.

SNAP cells could be probed for expression of the p75 receptor and downstream signaling. The binding of neurotrophins to p75 activates NF-κB, the Jun kinase pathway, and Rho signaling; these three signaling pathways are different than those pathways activated by Trk receptors (Reichardt 2006). A closer look at the Jun kinase pathway in latently infected SNAP cells is also warranted based on recent findings linking this neuronal-specific stress pathway and HSV reactivation from latency in rodent ganglia (Cliffe, et al. 2015). Targeting p75 downstream signaling with inhibitors may induce reactivation at a higher frequency that NGF-depletion alone

as other neurotrophins can bind the receptor. Alternatively, by combining NGF-depletion together with PI3-kinase inhibition, a combination that otherwise seemed redundant, might disrupt separate signaling arms and increase the frequency of induced reactivation (Yoon, et al. 1998).

HD10.6 Cells are Permissive to HSV-1 Lytic Infection

We found that both proliferating HD10.6 cells and doxycycline-treated HD10.6 (SNAP) cells are permissive to lytic HSV-1 infection. In other words, HD10.6 cells undergo a productive infection when inoculated with HSV-1 in which virus replicates and infectious virus is released. I was able to harvest strain 17 syn+ VP26-GFP from proliferating HD10.6 cells at titer levels similar to those harvested from Vero cells. Unlike epithelial cells, HD10.6 cells showed no obvious phenotypic changes (cytopathic effect) to indicate that they were productively infected. On the other hand, SNAP cells demonstrated very subtle retraction in axonal outgrowth. We also noted that the kinetics of lytic infection appeared delayed relative to lytic infection in epithelial type cells (data not shown). This was evident in multiple contexts, but most notably when using lytic controls for latency studies. Typically, in epithelial type cells late-gene expression is robustly evident by 24 hours post infection, but in SNAP cells, late gene expression was most robust at 36 – 48 hours post infection.

Characterization of Lytic Infection

Thorough characterization of lytic infection by HSV-1 in HD10.6 and SNAP cells is warranted. Growth curves and gene expression studies over a 48 h period using both lab-attenuated strains of HSV-1 (KOS) and neurotrophic strains (17 syn+, McKrae) are necessary to define the parameters of lytic infection in SNAP cells. For example, we would determine how early the lytic genes are expressed and whether they are expressed in an organized cascade as

seen in epithelial cells. In addition we would determine when peak viral production and release occurs. Defining the lytic cycle in SNAP cells would provide important parameters to compare with latent infection and would provide better predictions of viral genome activity during the exit of latency for reactivation assays.

The number of latent genomes within individual neurons is, at least in part, regulated by viral genetic factors, and latent genome copy numbers are an important parameter for reactivation frequency (Sawtell, et al. 1998). At this time, it is unclear whether viral genetic factors affect neuronal transport for latency establishment, initial replication within neurons, or even neuronal survival, but acute infection and latency pathogenesis in animal models is viral strain specific (Dix, et al. 1983; Wander, et al. 1987). SNAP cells provide a robust lytic infection model in which to compare acute viral replication and neuronal survival in human neurons. Viral strain-dependent genetic factors could be first analyzed by comparing the acute lytic infection of different HSV-1 strains in SNAP cells. The specific virus-host mechanisms of the most well studied neurovirulence factor γ34.5 are still being elucidated (Wilcox and Longnecker 2016). In addition, clinical isolates could also be compared in this manner (Wander, et al. 1980; Szpara, et al. 2014).

Establishing Latency without Acyclovir

In animal models, primary infections are mimicked by inoculating surfaces that have been excoriated, such as the cornea or whisker pads, which ensures virus replication in epithelial cells and subsequent virus for transport to neurons. In cell culture, inoculum is placed directly on neurons, which leads to a productive infection in neurons in multiple models (Wilson and Mohr 2012). To overcome this permissiveness to lytic infection, acyclovir is used at the time of infection (Wilcox and Johnson 1988; Kobayashi, et al. 2012); this allows viral entry but inhibits

viral replication. Some argue that acyclovir (a guanine analog) may alter or damage viral genomes by incorporating into the DNA and causing stalled replication forks. Reactivation mechanisms deduced from defective or aberrant viral genomes would therefore be inherently flawed.

As a proof of principle, we asked whether latency could be established in SNAP cells without using acyclovir. By maturing SNAP cells in compartmented chambers, cell bodies are separated from axonal termini by a diffusion barrier allowing for inoculum to be used on axons only. The anatomical separation of the cell body from the site of viral entry (i.e. where the axon innervates epithelial cells) favors a non-productive infection due to lengthy capsid transport and poor transport of viral tegument proteins to the nucleus (Hafezi, et al. 2012). Without viral tegument proteins, robust lytic gene infection is not triggered, and viral DNA is silenced by chromatin (Conn and Schang 2013; Lee, et al. 2016).

We found that SNAP cells matured well in compartmented chambers; actually, SNAP cells survived longer (well over 30 days) in compartmented chambers because they peeled less often. We designed a modified Campenot chamber, the Botting chamber, to maximize access to axonal termini while maintaining sufficient cell numbers for quantitative PCR (qPCR) analysis. We have preliminary evidence that quiescence can be established by axonal-only infections using a low MOI (Appendix B). Further experimental latency replicates quantifying viral DNA by qPCR, after using the new sulfuric acid-NoChromix cleaning protocol, are necessary for more robust data before publication. In addition, reactivation has not been demonstrated from chambers at this time; this is a critical hallmark to meet in order to validate infections as “latent”. Nonetheless, successful infection in compartmented chambers also provides opportunity to study viral transport and transmission.

Another approach to establishing latency without acyclovir would be to mimic an antiviral state at the time of infection. Interleukin-6 (IL-6), a cytokine produced by microglia during HSV-1 infection in the CNS, promoted neuronal survival in mouse neuroprogenitor cells (NPCs) lytically infected with HSV-1 (Chucair-Elliott, et al. 2014). In addition, varicella zoster virus (VZV) suppresses the early antiviral innate immune response by inducing SOCS3 expression, resulting in reduction of the type I interferon response through the STAT-3 pathway (Choi, et al. 2015). Using IL-6, or other cytokines such as IL-27 (Heikkila, et al. 2016), at the time of infection may permit HSV-1 viral entry into cells but suppress productive infection and therefore promote the accumulation of viral genomes in the neuronal nucleus.

SNAP Cells Support Latent Infection

Because SNAP cells are permissive to lytic infection, acyclovir was used to inhibit viral replication and favor a quiescent, non-productive infection. In addition to acyclovir, a low MOI was required to promote a non-productive infection in SNAP cells. At MOIs above 2 pfu / cell, break-through lytic infection occurred at a frequency that became a nuisance to experimental design. This is likely due to the delayed replication cycle of HSV-1 in SNAP cells and the short half-life of acyclovir in the media.

The experimental hallmarks used to validate the model developed in this dissertation were derived from the operational definition of latency as "the persistence of a viral genome within tissue where, at any given time, there is a population of cells that lack detectable infectious virus, viral proteins, or viral lytic transcripts that are dormant but have the capability of being reactivated" (Bloom 2016). We demonstrated the presence of non-productive HSV-1 genome in latently infected SNAP cells. We also demonstrated that once quiescent, HSV-1 maintained the capacity to reactivate.

Specifically we found that latency is maintained in a small number of SNAP cells and at a low genome copy number relative to *in vitro* rodent ganglia models. In addition, induction of HSV-1 with NGF-depletion only modestly increased the probability for reactivation. It is unclear if the low efficiency of establishment contributes to a low reactivation frequency, or whether human cells (or perhaps specifically SNAP cells) maintain a more stably repressed viral genome. Relative to lytic infection, transcription of the viral genome was repressed, but we were unable to demonstrate accumulation of LAT.

Exploring LAT Expression and Function

We did not detect a preference of LAT RNA expression over lytic mRNAs (ICP0 and TK). This may be due to sensitivity of detection from low numbers of latently infected cells, or the low genome viral copy per cell. Alternatively, a few cells in the population may be robustly expressing lytic genes, making detection of LAT accumulation in the remaining latent cells appear equivalent. I attempted to characterize the distribution of viral gene expression during latency using RNAscope, a highly sensitive RNA *in situ* hybridization technique. Detection of HSV-1 LAT accumulation has been demonstrated using this technique in rodent ganglia (Bloom 2016). Sample collection and probing techniques are still being optimized. This approach would allow visualization of a heterogeneous population of viral RNA expressing neurons.

Historically, accumulation of the HSV-1 major LAT (the 2.0 kb spliced intron) has been a hallmark in rodent latency models. Whereas LAT mutant viruses are still able to establish latent infection, reactivation efficiency is impaired in animal models (Cliffe and Wilson 2017). In addition, not all neurons that harbor HSV-1 DNA during latency produce detectable levels of LAT (Gressens and Martin 1994; Mehta, et al. 1995; Bloom 2016). However, determining whether LAT accumulation is necessary to establish latency in human neurons specifically has

never been explored due to the lack of available models. Using HSV-1 strains with various LAT mutations in the HD10.6 cell model developed here could be used to explore whether LAT is necessary for establishment of latency (Ng, et al. 2004). Alternatively, if LAT is necessary for reactivation from latency, perhaps exogenous expression of LAT would enhance induced reactivation from latently infected SNAP cells.

The LAT locus contains transcriptionally complex regions; the primary transcript itself encodes at least seven microRNAs (miRNAs) (Bloom 2016). Determining the function of this complex gene has not been straight-forward, but as a result of decades of investigation, a variety of HSV-1 (and HSV-2) LAT mutant viruses are available. Determining the different functions of LAT in human neurons could be systematically explored using these mutants in HD10.6 cells. HSV-1 LAT exon 1 deletion mutants have distinct effects on virulence in mice compared to rabbits (Perng, et al. 2001; Bertke, et al. 2009) and the reactivation phenotypes of LAT mutants varies across species (Bertke, et al. 2009). In addition, as a result of this model, detection and characterization of LAT encoded miRNAs in latently infected human neurons is now possible (Du, et al. 2015).

Characterization of Latent Viral Chromatin

The chromatin state of latent HSV-1 genomes could also be explored in SNAP cells. Chromatin immunoprecipitation (ChIP) of viral genomes during latency could test whether specific histone marks are present at lytic gene promoters or the LAT promoter. I would hypothesize that repressive marks such as H3K27 me3 or H3K9 me3 are at lytic promoters, and that histone modifications that favor active transcription would be present at the LAT promoter (Kubat, et al. 2004; Cliffe, et al. 2009). HD10.6 cells proliferate rapidly and robustly. This would allow for maturation of high numbers of SNAP cells in order to scale up infections.

However, an enrichment step for viral genome collection would still be beneficial due to the low efficiency of latency established.

Reactivation Mechanisms

This human *in vitro* model was developed in order to explore the necessity of host chromatin modeling enzymes (CMEs) for the exit of HSV-1 from latency. We hypothesize that VP16 recruits CMEs to immediate early gene promoters during reactivation. As it stands, the model developed here provides a novel human context to study HSV-1 latency and reactivation *in vitro.* In order to demonstrate this VP16-dependent recruitment of CMEs to IE gene promoters, we would use ChIP and qPCR for viral genes in reactivating neurons. We would expect that HSV-1 strains with mutations in the VP16 activation domain would fail to recruit CMEs to IE gene promoters during reactivation as seen in lytic infections (Herrera and Triezenberg 2004). The low persistence of latency and low reactivation frequency may present a technical hurdle to test the aforementioned hypothesis. However as mentioned in the previous paragraph, infections can be scaled up in order to perform ChIP-qPCR analysis.

To determine which CMEs are required for viral exit from latency, we would modulate different HATs and chromatin remodelers by shRNA knock-down or pharmacological inhibition and quantify reactivation capacity. SNAP cells were successfully transduced with lentivirus vectors expressing GFP or RFP as a proof of principle (data not shown). We could therefore transduce shRNA targeting p300/CBP, PCAF/GCN5, or BRG1/BRM prior to inducing reactivation and determine whether reactivation capacity is diminished. A targeted RNA interference (RNAi) screen looking for cellular chromatin factors involved in regulation of HSV gene expression was recently performed in U2OS osteosarcoma cells (Oh, et al. 2014). The model developed here provides a platform in which RNAi screens could be done during latency

in human sensory neurons. In addition, many inhibitors are now commercially available with high specificity against histone acetyltransferases; some are even being explored as antiviral treatments (Zhao, et al. 2015).

Latency in Other Alphaherpesviruses

I hypothesize that SNAP cells, derived from dorsal root ganglia (DRG), would support HSV-2 latency. HSV-2 most commonly causes genital lesions and the DRG innervate these mucosal surfaces. SNAP cells therefore present opportunity to compare establishment efficiency and reactivation capacity between HSV-1 and HSV-2. Preferences in establishment of HSV-1 versus HSV-2 in dissociated trigeminal ganglia from adult mice appear to be specific to nociceptive subtypes and differences in LAT (Margolis, et al. 2007; Bertke, et al. 2011; Bertke, et al. 2013). HD10.6 cells matured under the protocol described in this dissertation do not produce unique subtypes of sensory neurons in terms of neurotrophin receptor expression. Therefore, a difference may not be detected in this model. On the other hand, if a significant difference is detected, an unknown factor may be important. Alternatively, as discussed earlier in this chapter, HD10.6 cells may have the potential to differentiate into unique subpopulations of sensory neurons.

SNAP cells are also likely permissive to infection by other human alphaherpesviruses, such as VZV. Currently, neurons differentiated from human embryonic stem cells, induced pluripotent stem cells, or NPCs are being used to explore *in vitro* VZV latency (Pugazhenthi, et al. 2011; Lee, et al. 2012; Markus, et al. 2015). VZV commonly establishes latency in DRG and as such these cells provide a promising candidate for a human latency model. Based on results of this dissertation presented at conferences, some labs that study VZV have already acquired HD10.6 cells from Celgene.

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APPENDIX A. Latency and Reactivation Methodology in SNAP Cells

This dissertation describes a novel context in which to study HSV-1 latency in human sensory neurons. HD10.6 cells, the cell line used for this model, were developed in the late 1990's and published on only once to describe the immortalization and validation of the cells (Raymon, et al. 1999). Due to advancements in neuronal culturing techniques and commercial availability of culture materials, we were able to modify the propagation and maturation of HD10.6 cells. Whereas we were the first to use HD10.6 cells for alphaherpesvirus infections, the techniques for latency and reactivation were modified from current *in vitro* HSV-1 latency models in rodent ganglia (Wilcox and Johnson 1987; Arthur, et al. 2001; Kobayashi, et al. 2012). This appendix captures important methodology details that were used to develop this novel cell culture model.

HD10.6 Cells Proliferation and Maturation

HD10.6 cells proliferated robustly on fibronectin-coated Nunc flasks. Proliferation medium was changed every 2-3 days. Proliferation medium comprised of advanced Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 supplemented with glutaMAX, B-27 supplement, 10 ng/mL prostaglandin E1, and fresh 0.5 ng/mL fibroblast growth factor-basic (bFGF). In order to maintain robust cultures for splitting and maturation, HD10.6 cells were split frequently and maintained at a confluency of 25–90%. When HD10.6 cells were split below 25% confluency neurospheres formed, and subsequently cells lost their adherence to the flask. Alternatively, when cells were left to proliferate to 100% confluency medium became acidic (as indicated by a red to yellow color change), and despite subsequent passaging cells stopped proliferating.

For maturation, plates were first coated with 2% Matrigel and 10 µg/mL poly-D-lysine at volumes which completely covered wells and incubated for at least 1 h at room temperature.

Matrix coating solution was reused one time an stored at 4ºC. We noted cells peeled less frequently from wells with smaller surface areas and found 12-well plates (surface area of 3.7cm^2) to be sufficient for long term experiments. For maturation, HD10.6 cells were plated at a density of 5×10^4 cells/cm^2 in proliferation media (day 0). Proliferation medium was changed to complete maturation medium 24 h after plating (day 1). Complete maturation medium comprises NeuralQ Basal Medium, supplemented with glutaMAX, GS21 neuronal supplement, 1 µg/mL doxycycline, 50 ng/mL 2.5S nerve growth factor (NGF), and 25 ng/mL each of ciliary neurotrophic factor (CNTF), glial cell derived neurotrophic factor (GDNF), and neurotrophin-3(NT-3). All cells (and infections) were incubated at 37 °C and 5% CO_2. Half-volume media changes with complete maturation media were carried out every 3 d throughout maturation.

Establishing HSV-1 Latent Infection in SNAP Cells

Virus inoculum was calculated based on a desired multiplicity of infection (MOI) or plaque forming unit (pfu) per cell using a dilution factor equation of [cell number × desired MOI (pfu / cell)] / [virus stock titer (pfu / mL) × inoculum volume (mL)]. Cell number at the time of infection (day 7) was estimated by calculating [the number of cells plated× 1.2] to account for initial proliferation and cell loss during maturation, therefore, the actual MOI for infections was only an estimate. Virus was diluted in NeuralQ Basal Medium and used at volumes sufficient to completely cover wells (150 µL in 6-well plates, 100 in 12-well plates, 50 µL in 96-well plates). Typically an estimated MOI of 0.1–1.0 pfu/cell resulted in latent infection. The optimal MOI to prevent break-through varied based on the viral strain used for infection (KOS versus 17 syn+).

On day 7 of maturation, media was removed from SNAP cells. Virus inoculum was added for 1 h with gentle rocking every 15 minutes. At 1 h post infection (hpi) the inoculum was removed and replaced with complete maturation medium with 100 µM acyclovir (for latent

infection) or without acyclovir (for lytic infection). Three to four days post infection (DPI), 100% of the media was changed (without acyclovir) for both latent and lytic infections. At this time, plates were screened for evidence of lytic infection. When a GFP-late gene reporter virus was used, screening was done by microscopy for GFP-expression in SNAP cells. Alternatively (or in the case of wild-type viral infections), media from SNAP cells was used to inoculate Vero cells which demonstrate cytopathic effect (CPE) when infected. The latter example was less desirable because some virus can be either released or residual during this early time, as well as required multiple days to see results. Cultures with break-through lytic infection should be discarded. Having control mock and lytic infections running parallel to latent infections was useful to ensure both that neurons were healthy and that virus stock was viable.

Maintenance of HSV-1 Latency in SNAP cells

While acyclovir from the single treatment at 1 hpi was in the media, the culture was considered to be "establishing latency". From 5 DPI and beyond (following the removal of acyclovir via the media change) the culture was considered to be "maintaining" latency. Cells during maintenance of latency time showed no evidence of lytic infection. If lytic infection became evident during this time, cultures were considered to have spontaneously reactivated. Maintenance of latency in SNAP cells was carried out for several weeks by performing half-volume changes every 3–4 d. In order to avoid peeling and possible spontaneous reactivation, cultures were handled with care by performing gentle media changes and careful transporting of plates.

Reactivation of HSV-1 Latently Infected SNAP cells

Latently infected SNAP cell cultures would ultimately peel from plates; this generally occurred after 4 weeks in culture. Reactivation, therefore, was induced at 7 DPI. Before inducing

reactivation, cells were screened for evidence of spontaneous reactivation, either by microscopy for GFP-expression or by collecting media for "pre-reactivation" inoculum. For large batches of reactivating cultures, pre-reactivation media was stored at -80ºC and later used to inoculated Vero cells in parallel with post-reactivation medium.

For reactivation by nerve growth factor (NGF) depletion, media was carefully removed at 7 DPI and replaced with maturation media that did not contain NGF and contained 1:1000 NGF anti-sera. After 48 h, 100% of the media was carefully removed and replaced with complete maturation medium. Fresh media was added every 3–4 d to maintain neuronal health at volumes that reconciled evaporation. Ten days post-reactivation induction, media was transferred as inoculum to 85%-confluent Vero cells for at least 3 h. Without removing SNAP cell inoculum, DMEM was added at a volume sufficient to maintain Vero cell viability. Vero cells were then incubated for 3–5 d to allow for multiple rounds of infection. Vero cells were then screened for evidence of lytic infection, either by detecting CPE or by reporter virus infection. **Figure 14** represents a schematic of latency and reactivation in SNAP cells. Appendix C describes the rationale and approaches for alternative reactivation induction stimuli that were unsuccessful in the model describe here.

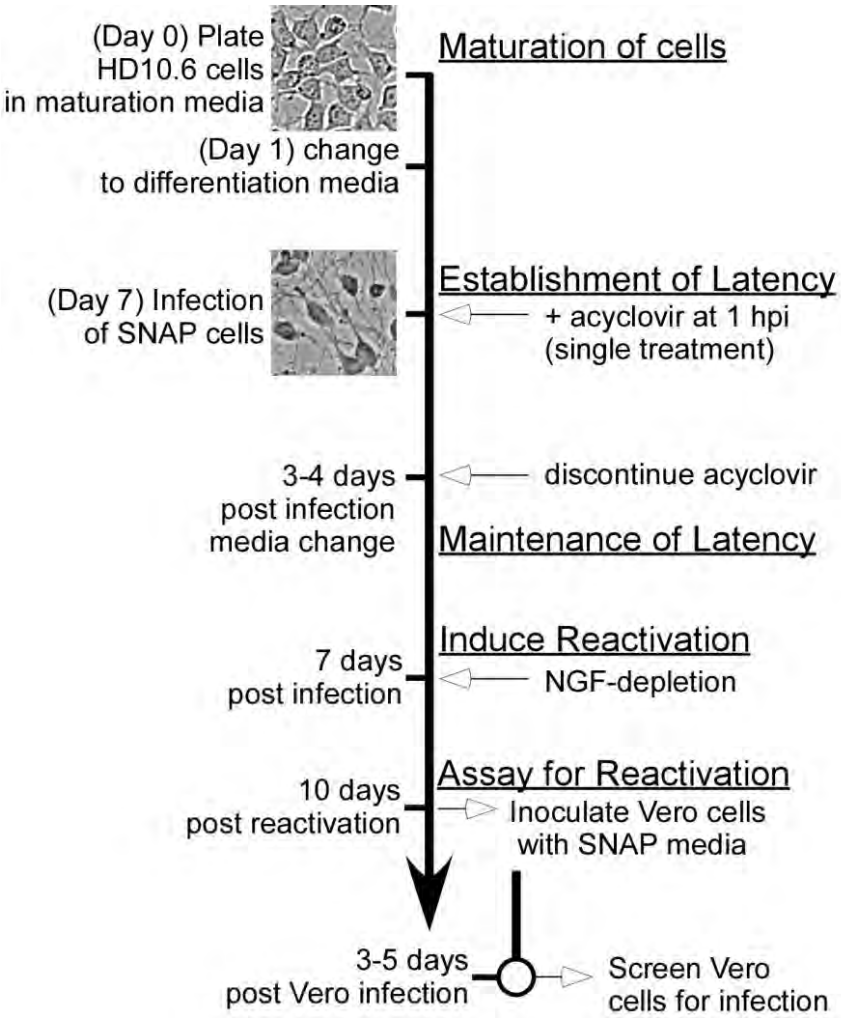

Figure 14. Schematic of latency and reactivation in SNAP cells. HD10.6 cells are plated (Day 0) in proliferation median. At 24 hours post-plating (Day 1) media is changed to complete maturation media. At neuronal maturation day 7, SNAP cells are infected with HSV-1 and at 1 hour post infection the inoculum is replaced by medium containing acyclovir 100 µM. At 3–4 d post infection, 100% of the media is changed (no acyclovir). Establishment of latency is when acyclovir is present. Maintenance of latency begins 5 d post infection and continues until reactivation. Reactivation can be induced at 7 d post infection by NGF-depletion. Ten days after inducing reactivation, medium from SNAP cells is transferred as inoculum to Vero cells for amplification of released (reactivated) virus. Vero cells are screened 3–5 d after inoculation for evidence of infection, which would indicate reactivation of HSV-1 in SNAP cells.

References for Appendix A

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APPENDIX B. Establishing Latency in Compartmented Chambers

In natural infection, HSV latency is established after virus replicates in epithelial cells and enters innervating axons for retrograde transport to neuronal cell bodies (soma) in the ganglia (McGraw and Friedman 2009). When cultured in specialized compartment chambers, axons of neurons penetrate a fluid-impermeable barrier allowing for the separation of media from soma and the distal portions of axonal outgrowth; thus, one culture system experimentally mimics two different tissue environments (Campenot 1977; Pazyra-Murphy and Segal 2008). Since the axons and cell bodies are located in different compartments, neuronal cell components can be infected and assayed separately (Curanovic, et al. 2009; Song, et al. 2016). Inoculation of the axon-only compartment more closely resembles natural infection. This is important because there is evidence that the physical entry of HSV-1 into distal axons of neurons (away from the nucleus) favors the onset of nonproductive infection (Hafezi, et al. 2012).

In conventional infections, where neurons are seeded into a dish or well, inoculum is placed directly on an entanglement of cell bodies and axons. When SNAP cells were infected in this conventional manner, productive (lytic) infections occurred. In order to establish a non-productive infection in SNAP cells, treatment with acyclovir was used to inhibit viral replication (but allow viral entry). We asked whether we could more naturally establish HSV-1 latency in SNAP cells without relying on an antiviral drug. We hypothesized that by infecting the axons only, virus would favor entry into quiescence without the use of acyclovir.

Botting Chamber Design

In order to test this hypothesis, we first had to optimize the growth and maturation of HD10.6 cells in compartmented chambers. Many versions of compartmented neuronal chambers exist; the traditional Campenot chambers consist of a 10 to 25 mm diameter Teflon ring with

septa that divide the culture into multiple compartments (Campenot 1977). In addition, there are commercially available microfluidic devices that have compartments connected by microgrooves but are fluidically isolated due to hydrostatic pressure (Park, et al. 2006). Most frequently, the three-compartmented Campenot design is used in the herpesvirus field **(Figure 15)** (Curanovic, et al. 2009; McGraw and Friedman 2009; Hafezi, et al. 2012), but microfluidic chambers have also been used (Liu, et al. 2008; Wisner, et al. 2011). Campenot chamber models are temporarily adhered into cell culture wells or dishes by a silicon grease layer which maintains a diffusion-free barrier. Groves are first made in the dish (extending beneath the compartment septa), this allows for only axonal outgrowth (not media) to traverse into the neighboring compartment (Pazyra-Murphy and Segal 2008; Curanovic, et al. 2009).

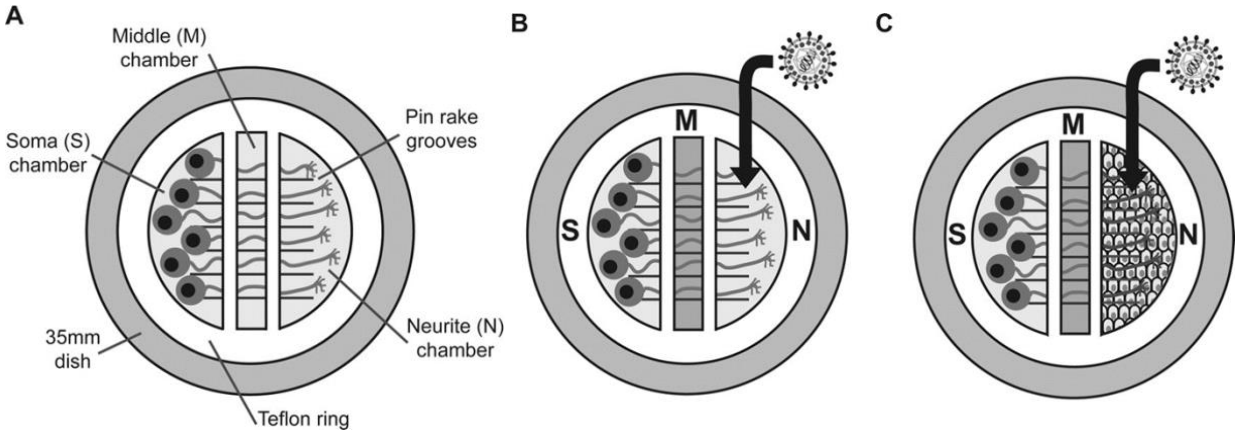

Figure 15. Campenot chamber options for HSV infection. This figure was reproduced (with permission) from McGraw, H.M. and H.M. Friedman. 2009. Herpes simplex virus type 1 glycoprotein E mediates retrograde spread from epithelial cells to neurites. *Journal of Virology* 83(10): 4791-4799 **(A)** Three-compartmented Campenot chambers have two large semicircle sections separated by a middle (M) chamber. Cells are plated in the soma (S) chamber. Axons grow through the middle chamber to the neurite (N) chamber. **(B)** Axons in the neurite chamber can be infected with virus. **(C)** Alternatively, epithelial cells can be co-cultured in the neurite chamber for infection.

For our experimental design, we wanted to maximize the number of cells with accessible axon-termini for infection, while maintaining a high enough cell body density to isolate RNA and DNA for quantitative analysis. We observed that the axonal outgrowths of SNAP cells do not develop as lengthy as the axons from primary rat neuronal cultures; a comparison based on detailed protocols describing primary rat neurons and axonal outgrowth in chambers (Curanovic, et al. 2009). We determined that the commercially available compartmented chambers did not provide a desirable plating surface area in which access to axonal termini for optimum infections, and therefore designed our own.

Laboratory member Carolyn Botting modified the design of the traditional Campenot chamber from a circle to a square device that includes five equal-sized rectangular compartments **(Figure 16a).** HD10.6 cells can be plated in alternating sections (soma compartments) so that axons traverse under septa (through grooves in the plate) into neighboring axonal compartments. Due to the shorter axonal growth of SNAP cells compared to primary rodent neurons, we seeded SNAP cells in the middle compartment of the three-compartmented Campenot design which allowed for axonal outgrowth and infection in both the right and left semicircle compartments **(Figure 16b)**. Compared to the Campenot design, the Botting design allows for three times the number of cells to be plated, while optimizing infection of SNAP cells via the two axonal compartments (**Figure 16a).** Serial images of an axonal compartment demonstrate that SNAP cell axons grew the entire distance of a single Botting rectangular compartment (**Figure 16b**).

Permissibility of Axons to HSV-1

We first asked whether SNAP cells were permissive to lytic (productive) infection by axonal-only inoculation. In order to demonstrate axonal infection and transport, we seeded the two internal alternating compartments with HD10.6 cells (# 2 and 4) and infected day 7 SNAP

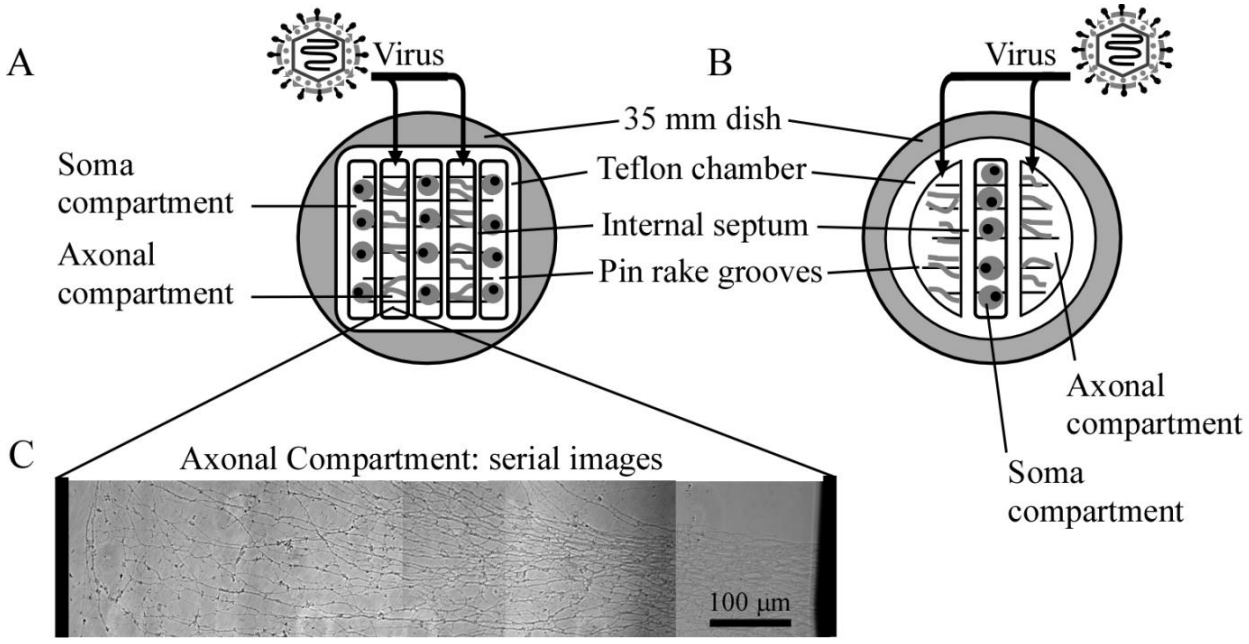

Figure 16. Botting chambers increase available axon termini. (**A**) The Botting chamber is a square Teflon chamber with five equal rectangular sections. Cells were plated in alternating soma compartments and axons grew into neighboring axonal compartments. Grooves, made with a pin rake, extend beneath the internal septa that were sealed with silicone grease. Axonal-only compartments can be inoculated with virus. (**B**) For SNAP cell infections in the Campenot chamber design, cells are seeded in the middle (soma) compartment and axons grow to the left or right axonal compartments. (**C**) Serial bright field images were aligned and demonstrate robust axonal outgrowth. Scale bar is 100 µm.

cells with HSV-1 strain 17syn+ VP26-GFP in the left-most axonal compartment (# 1) for 1 h at 37 °C (**Figure 17**). Multiplicity of infection (MOI) was difficult to calculate due to the fact there in no correlating cell number for axonal-termini, and axonal outgrowth into neighboring compartment likely varies each time cells are plated. We therefore estimated a high MOI by using 300 µL of virus inoculum that had been diluted to 10^4 plaque forming units (pfu)/mL. At 7 DPI, we found GFP-positive neurons in the left soma compartment (# 2) but GFP-negative neurons in the right soma compartment (# 4); at 14 DPI, we saw GFP-positive neurons in the right soma compartment (# 4) (**Figure 17**). We concluded that SNAP cells are permissive to HSV-1 via axonal entry and that virus is transported neurons in Botting chambers.

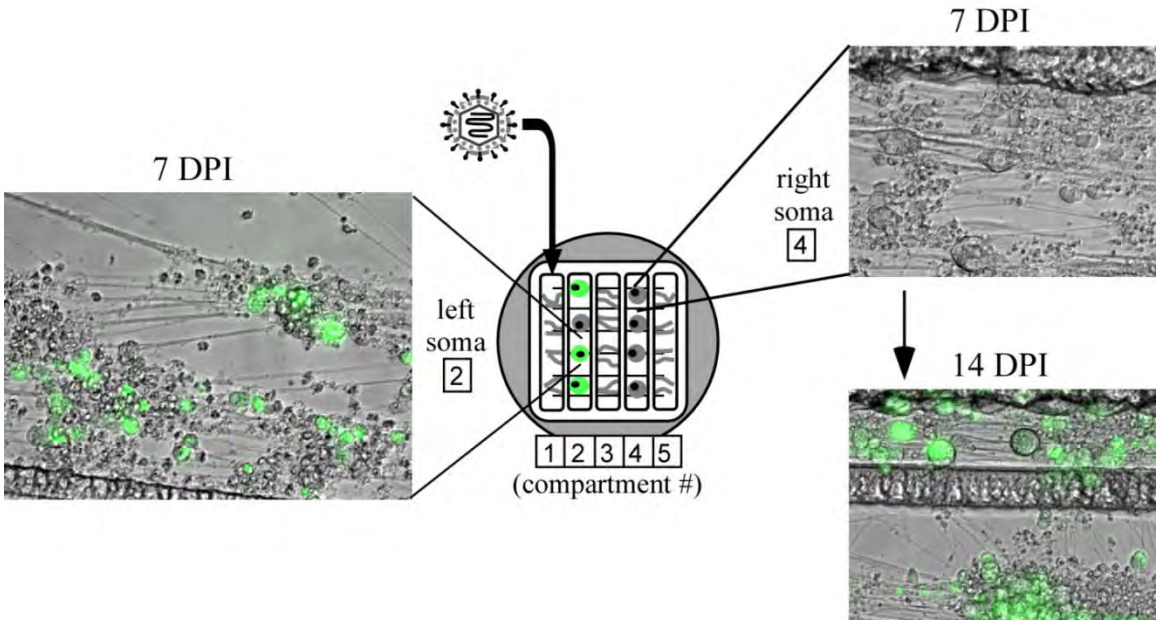

Figure 17. SNAP cells are permissive to axonal-only infection. Bright field and FITC merged microscopy images represent cells in left soma (#2) or right soma (#4) compartments at 7 days post infection (DPI) and 14 DPI. Green (GFP-positive) soma indicates a lytic (productive) infection. Only the left most axonal compartment (#1) was infected with HSV-1 strain 17 syn+ VP26-GFP at a high pfu/mL to promote productive infection.

Establishing Latency by Axonal-Only Infection

We next ask whether latency could be established without acyclovir by using axonal-only infections. HD10.6 cells were seeded in alternating compartments (#1, #3, and #5) and day 7 SNAP cells were subsequently inoculated in compartments #2 and #4 as depicted in the schematic in **Figure 16a**. Lytic infection of SNAP cells was possible through axonal-only inoculation using an estimated high MOI (300 µL of 10^4 pfu / mL). Therefore, in order to determine whether latency could be established without using acyclovir, we infected axonal compartments with a range of diluted inoculums of HSV-1 strain 17syn+ VP26, including 300 µL of 10^2, 10^3, or 10^4 pfu/mL into each axonal chambers.

In order to determine whether SNAP cells were infected productively or non-productively, we screened for GFP-expression in SNAP cells (by microscopy) 7 DPI. In addition, we checked for infectious virus release by inoculating Vero cells with media from chambers 7 DPI . SNAP-cell media containing infectious virus (productive infection) resulted in positive-Vero cell inoculations; this was evident 3–5 d after inoculation either by cytopathic effect (CPE) of GFP-expression in Vero cells. SNAP-cell media from non-productive infections did not contain infectious virus and resulted in negative-Vero cell inoculations (no CPE and no GFP-expression in Vero cells).

Using an inoculum of 10^4 pfu/mL on SNAP cell axons routinely resulted in productive infections. In comparison, using an inoculum of 10^2 pfu/mL rarely resulted in productive infection, but also had very low cellular viral DNA yield by qPCR analysis (data not shown). With an inoculum of 10^3 pfu/mL, only about 25% of the cultures progressed to productive infection. As a result, we moved forward with an inoculum of 10^3 pfu/mL and routinely screened cultures for evidence of productive infection (GFP-positive SNAP cells).

In order to validate latent infection, we asked whether we could quantify viral DNA from neurons of non-productive infections. HD10.6 cells were seeded into alternating compartments (#1, #3, and #5) and day 7 SNAP cell axons were infected with 300 μL of inoculum containing HSV-1 strain 17 syn+ VP26-GFP at 10^3 pfu/mL as depicted in **Figure 16a**. At 7 DPI, chambers were screened for productive infection as previously mentioned; SNAP cells without evidence of productive infection were putatively deemed latent. Cell bodies from SNAP cell infections were lysed and DNA was harvested for quantification by qPCR. We found viral DNA in axonal infections that were both productive (lytic) or non-productive (latent) (**Figure 18**). We also noted that viral DNA was occasionally detected in our mock infections; we used these values as a threshold of detection. We hypothesized that inactive viral DNA was retained in residual grease on the chambers from previous experiments. To resolve this contamination, we changed to a sulfuric acid – NoChromix wash which degreases the chambers and allowed for a more thorough cleaning. Latent infections using this new protocol have not been analyzed. Nonetheless, we have preliminary evidence that SNAP cells grown in compartmented chambers can establish a non-productive infection without the use of acyclovir through axonal-only infection.

Reactivation from Compartmented Chambers

A key hallmark of latency is that the viral genomes retain the capacity to reactivate and to release infectious virus. We attempted to induce reactivation from compartmented infections using a combination of NGF-depletion and superinfection with ultraviolet-inactivated virus (UVi) as described in Chapter 2. Reactivation studies were carried out using 3-compartmented Campenot chambers by seeding cells in the middle compartment as seen in **Figure 16b**, followed by inoculation (300 μL of 10^3 pfu/mL) in both the right and left axonal compartment at day 7 of SNAP cell maturation. At 7 DPI, media was changed to maturation medium without NGF and

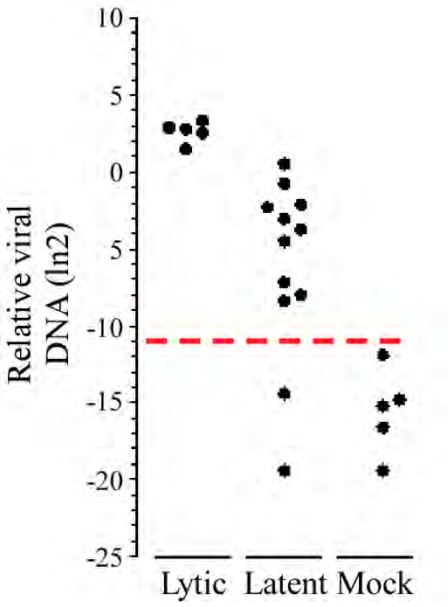

Figure 18. Non-productive viral DNA detected in neuronal soma. HSV-1 viral genomes were quantified by qPCR from axonal-only SNAP-cell infections in Botting chambers. Viral (DNA relative to cellular DNA) from cell bodies was plotted for individual Bottoming chambers. Infections were deemed productive (Lytic) or non-productive (Latent) based on evidence of infectious virus after inoculating Vero cells with SNAP-cell media. A threshold of viral genome detection (---) was set based on results from mock infected SNAP cells.

1:1000 dilution of anti-NGF antibody for 48 h, followed by superinfection with UVi 17syn+ VP26-RFP in axonal compartments. We screened for productive infection using Vero cell inoculation as previously described at 7 DPI (prior to reactivation stimuli) and again at 10 days post NGF-depletion. Chambers either had productive infection both prior to and following reactivation, which we infer to be persistent lytic infection, or had no evidence of productive infection at either time points.

We conclude that we were unsuccessful in inducing reactivation from SNAP cells in Campenot chambers. Perhaps superinfection of the axonal compartments is not the right approach as we would expect VP16 tegument protein transport to diminish as the capsid travels to the nucleus. Superinfection directly on cell bodies might be more effective, however, based on reactivation studies in Chapter 2 (**Figure 13**), it was NGF-depletion that resulted in an increased probability of reactivation.

Conclusions and Future Directions

By growing SNAP cells in Botting chambers, axonal outgrowths can be maintained in a unique fluidic environment that is separate from neuronal soma. In theory, this would allow for the establishment of latency without the use of acyclovir by inoculating axon-only compartments. SNAP cells grow and mature well in compartmented chambers. SNAP cells are permissive to HSV-1 infection at both the axonal terminals only or by inoculating cell body compartments only. We demonstrated that virus is axonally transported through Botting compartments. In addition, we have preliminary evidence that inoculation with a low titer of virus results in a non-productive, quiescent infection without acyclovir treatment. We were unable to demonstrate reactivation capacity and as such cannot state with certainty that we established HSV-1 latency using this approach. However, based on the low frequency of reactivation shown in Chapter 2, we may not have analyzed a sufficient number of replicates in order to detect reactivation.

Several steps could be taken to further advance this technique. The establishment of latency may require better optimization such that more viral genomes are present in neuronal soma; this may increase the likelihood of inducing a reactivation event. Latency establishment experiments need to be repeated using the proper degreasing technique in order to eliminate background viral DNA that might be skewing qPCR analysis. In addition, a more robust reactivation stimulus may be required. Superinfection directly onto cell bodies for example, may deliver viral tegument proteins more efficiently for reactivation. SNAP cells could even be co-cultured with epithelial cells in order to study the establishment of HSV latency and perhaps inducing cellular stress in epithelial cells would induce reactivation.

Materials and Methods

Compartmented Chamber Assembly and Cleaning

Compartmented chambers were purchased from Tyler Research. These included both 3-compartmented Campenot chambers (CAMP3 – 25mm OD) and a custom five-compartmented chamber manufactured to our specifications based on a design by Carolyn Botting (Botting chamber). Six-well cell culture plates were coated with 2% Matrigel and 10 µg/mL poly-D-lysine and incubated for 1 h at room temperature. While the matrix coating solution was still present, grooves were made in the center of the plastic well with a pin-rake (homemade pin rake was constructed from 16 insect pins (size 00) arranged in a row such that sharp ends of the pins are aligned) (Curanovic, et al. 2009). Compartmented chambers were sealed to coated plates using silicone vacuum grease which was applied to the underside of the chamber using a sterile syringe and 18 gauge needle hub (needle point removed with hemostats). 300 µL of proliferation medium (described in Appendix A) was first added without cells to axonal compartments to check for immediate leaking.

Teflon compartmented chambers were cleaned for re-use by first aspirating any media, submerging chambers in 70% ethanol for at least 1 minute, and wiping excess grease with paper towel; this was performed in a BSL-2 cell culture hood. Chambers were soaked for 1 h in sulfuric acid-NoChromix solution according to the manufacturer's protocol. Chambers were rinsed five times in ddH2O and boiled for 30 minutes in ddH2O; this cycle was repeated 3 times. Chambers were then air dried and autoclaved prior to use.

Plating of HD10.6 Cells

HD10.6 cells were seeded with proliferation medium in the desired soma compartment(s) at 1.25×10^5 cells per compartment at equal volume to media in axonal chambers (300 µL).

Proliferation media in all chambers was aspirated and replaced with maturation medium (described in Appendix A) 24 h after plating. Additional 50 – 100 µL aliquots of maturation media were added to compartments every 3 d. About 10–25% of the chambers would leak slowly, and this was detected by the presence of cell bodies in the axonal compartments 24 h after plating. Leaky chambers were re-plated or used for mock controls in which no virus inoculum was used.

Chamber Infections

For viral infections of SNAP cells in compartmented chambers, media was removed from the axonal compartments only and 300 µL of 10^2 to 10^4 pfu/mL of virus (diluted in NeuralQ Basal Medium) was added for 1 h at 37 °C and 5% CO_2. At 1 hour post infection (hpi) the inoculum was removed and complete maturation media was added. HSV-1 reporter strains 17 syn+ VP26-GFP and 17 syn+ VP26-RFP were gifts from Andrea Bertke.

Quantitative PCR

Cells lysates were combined from all three soma compartments of a single Botting chamber for DNA extraction. DNA was purified using Qiagen DNeasy Blood and Tissue Kit. Purified DNA was quantified by qPCR with primers specific for the viral ICP4 viral genes or the human 18S ribosomal RNA gene. The qPCR was performed on an ABI 7500 RT-PCR system (Applied Biosystems) using FastStart Universal SYBR Green master mix (Roche). For viral genome quantification studies, was normalized to 18S (dCt) and analyzed by $2^{-(dCT(transcript)-dCt(DNA)}$.

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APPENDIX C. Exploring Reactivation Induction

Optimization of the Model

In natural infection, herpes simplex virus (HSV) reactivates spontaneously but also as a result of physiological stressors or external triggers. Sunlight exposure, for example, is reported by some patients to precede recurrent cold sore lesions (Mills, et al. 1987; Rooney, et al. 1991) and the recurrence of herpetic keratitis increases with exposure to ultraviolet (UV) B light (Ludema, et al. 2014). Other reactivation triggers include surgical procedures (Huang, et al. 1999; Alonso-Vanegas, et al. 2016), immunosuppression (Nouri, et al. 2006; Okada, et al. 2013), fever, and even psychosocial stress (2000; Chida and Mao 2009). Experimentally, rodents do not spontaneously reactivate, and as such an induction stimulus is needed. One of the original stimuli used to reactivate latent HSV *in vitro* was the deprivation of nerve growth factor (NGF) (Wilcox and Johnson 1987).

We tried a variety of reactivation stimuli in latently infected SNAP cells based on techniques currently used in the literature. In Chapter 2 of this dissertation I describe and compare two specific reactivation techniques in detail (i) NGF-depletion and (ii) superinfection with a UV-inactivated HSV-1 virus (UViR). The purpose of this appendix is to report on the failed reactivation stimuli, as well as capture details in the rationale and technical approaches employed for the different stimuli.

It is important to point out that reactivation experiments of latently infected SNAP cells were carried out at the same time as the optimization and validation of establishment of latency in SNAP cells. The technique for establishing latency, as well as the timing of reactivation induction and detection, was therefore modified throughout the exploration of determining whether a specific stimulus trigged reactivation. For example, initially SNAP cells were infected

on day 4 of maturation, but changed to day 7 of maturation based on characterization of the maturation process. Acyclovir treatment was also modified from pretreatment (12 to 24 h prior to infection) to a single treatment (1 h post-infection.) Latently infected cultures were first screened for evidence of reactivation 3 to 4 days after induction, but later screened 7 to 10 days after induction due to evidence of a delayed lytic cycle in SNAP cells.

Furthermore, due to literature reports that 17 syn+ reactivates more frequently in mouse models compared to KOS, I switched from using an HSV-1 VP16-GFP reporter with a KOS background (DG1), to a neurotropic HSV-1 VP26-GFP reporter strain with a 17 syn+ background for reactivation studies. Many of the approaches used to induce reactivation were attempted in early experiments, before optimization of the model (described in Appendix A) and as a result warrant revisiting. **Table 3** summarizes results from failed reactivation experiments but does not completely capture all reactivation attempts. Infections were carried out in a variety of plate sizes (from 96-well assay format to 25 mm dishes) and most contained non-induced controls to determine the level of confounding spontaneous reactivation for each unique experiment.

Approaches for Reactivation

Elevated Temperature

Hyperthermia is used to induce reactivation in mice (Sawtell and Thompson 1992) and it is thought that fevers precede recurrence in people. I incubated latently infected SNAP cells at different elevated temperatures to mimic both heat shock and fever (thermal stress). Latently infected SNAP cells were either incubated at 43ºC for 1 to 3 (heat shock) or at 39.5ºC for 24 h (thermal stress). Heat shock proteins are induced by various stresses such as heat shock, ischemia, and hypoxia (Morimoto, et al. 1997; Mosser, et al. 2000). We immunoblotted for heat

shock protein 70 (Hsp70) at 30 min, 1 h, 6 h, and 24 h after recovery (back to 37ºC) from both thermal challenges (**Figure 19**). Despite evidence of induction of Hsp70 by 24 h after recovery, we did not see promising evidence of reactivation from latent SNAP cell cultures (**Table 3**).

Table 3. Summary of failed reactivation experiments

	No. of reactivated wells detected by GFP fluorescence [a]	
Description of stimulus	Induced cultures	Not induced (control wells) [b]
Heat Shock [c]	0 (96 wells)	0 (96 wells)
Thermal Stress [d]	0 (2 dishes)	2 (2 dishes)
Neurotrophin w/d [e]	0 (96 wells)	0 (96 wells)
PI3K inhibition	0 (96 wells)	0 (96 wells)
Forskolin treatment	0 (96 wells)	0 (96 wells)
Trichostatin-A treatment	0 (96 wells)	0 (96 wells)
Axotomy	1 (1 well) 1 (12 wells)	0 (1 well) 1 (12 wells)
Dexamethasone + Heat Shock	6 (6 wells) 0 (6 wells)	ND ND
Dexamethasone + neurotrophin w/d	3 (3 wells) 0 (3 wells)	ND ND
Activated media [g]	1 (24 wells) 12 (12 wells)	ND 9 (12 wells)
Ultraviolet irradiation	1 (1 well) 0 (1 well)	1 (1 well) 0 (1 well)

Not done (ND)
[a] Total number of wells tested per experimental condition is noted in parenthesis
[b] SNAP cells infected latently in parallel, but not induced with indicated stimulus
[c] Heat shock induced by incubating cells at 43˚C for 1-3 h
[d] Thermal stress induced by incubating cells at 39.5˚C for 24 h
[e] Neutrotrophin withdraw (w/d) of NGF, NT-3, GDNF, CNTF from media
[g] Media from LPS-treated microglial

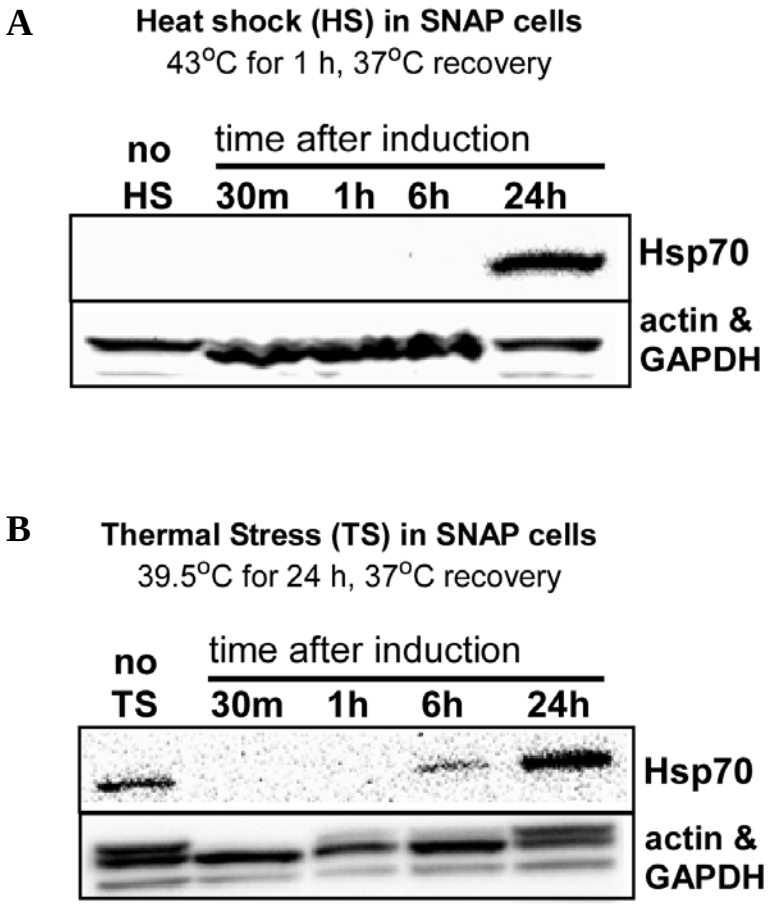

Figure 19. Heat shock and thermal stress induce Hsp70 in SNAP cells. Expression of heat shock protein 70 (Hsp70) was assessed by immunoblot analysis in SNAP cells incubated at elevated temperatures to demonstrate an activated cellular stress response. Actin and GAPDH were used as loading controls. (A) SNAP cells incubated at 43°C (heat shock) for 1 h were returned to 37°C and probed at 30 minutes (m), 1 h, 6 h, and 24 h after induction. (B) SNAP cells incubated at 39.5°C (thermal stress) for 24 h were returned to 37°C and probed at 30 m, 1 h, 6 h, and 24 h time after induction.

Neurotrophin Withdraw

Initially I attempted to induce reactivation by replacing complete maturation medium on latently infected SNAP cells with medium lacking NGF. When this did not induce reactivation, I then tried medium lacking all four supplemented neurotrophins: NGF, neurtrophin-3 (NT-3), glial derived growth factor (GDNF), and ciliary neurotrophic factor (CNTF). Three to four days after removing either just NGF or all the neurotrophins from maturation media, SNAP cell axons began to retract and cell bodies would round up and shrink, presumably becoming apoptotic. Moving forward, I only removed neurotrophins for a maximum of 48 h. Unfortunately, removal of all 4 neurotrophins did not appear to be a significant induction trigger for reactivation in latently infected SNAP cells (**Table 3**). Only by adding NGF anti-sera with NGF withdrawal (NGF-depletion), did we see evidence of reactivation of latent HSV-1 over background (experiments discussed in Chapter 2).

The resulting signaling cascade of neurotrophin supplementation has not been characterized in SNAP cells. We determined that SNAP cells express multiple neurotrophic receptors (experiments discussed in Chapter 2), but we did not determine the specific downstream signaling activity in SNAP cells as a result of neurotrophin supplementation. Exploration of where these pathways converge, in order to more directly target the pathways potentially maintaining HSV-1 in a latent state, is warranted and may provide a way to induce reactivation without compromising the overall health of cells in culture.

PI3-kinase Inhibition

In rodent sympathetic neurons, reactivation is induced by disruption of the PI3K, AKT, and mTOR pathway (Camarena, et al. 2010). I therefore explored PI3-kinase inhibition early on as a reactivation induction stimulus. Initially, I used the PX866 (Cayman Chemical) PI3-kinase

inhibitor because another lab at Van Andel Institute had experience using the compound (and had it on hand). I did not see any promising results from reactivation experiments. Later, we used LY294002, a PI3-kinase inhibitor more commonly used in the herpesvirus field, but experiments performed by myself and lab member Carolyn Botting did not yield promising evidence of reactivation. Furthermore, because NGF-depletion gave promising results, we moved forward with this method under the assumption that we were disrupting the PI3-kinase pathway upstream by blocking activity of the TrkA receptor (for which NGF binds with high affinity to).

Axotomy

Interestingly, both Botting and I noted that if neuronal cultures of latently infected SNAP cells began to peel, spontaneous reactivation could be detected by GFP late gene expression. We hypothesized that peeling caused a neuronal stress factor that triggered reactivation, similar to that induced by explanting latently infected ganglia from rodents. We attempted to recapitulate this axonal stress by excoriating the cultures of latently infected SNAP cells with a sterile needle or glass culture pipette tip (similar to a scratch assay). We did not see evidence of reactivation over background spontaneous reactivation after the axons were cut in a grid pattern (axotomy) (**Table 3**). We surmise that spontaneous reactivation of latently infected SNAP cells may in fact cause axonal retraction which precedes and leads to peeling.

Inflammatory Triggers

I attempted to recapitulate different inflammatory triggers based on reactivation stimuli used in other alphaherpesvirus models. Bovine herpesvirus 1 (BVH-1) establishes latency in sensory neurons and synthetic corticosteroid treatment reactivates virus in calf trigeminal ganglia within 90 minutes (Kook, et al. 2015). I treated latently infected SNAP cells with the synthetic corticosteroid dexamethasone (Sigma D4902) at 1 µg/mL, but did not see evidence of

reactivation over background. In addition, I combined dexamethasone treatment with heat shock or neurotrophin withdrawal (**Table 3**), but still did not see promising signs of reactivation.

As an alternative to synthetic corticosteroids, I tried using activated media harvested from inflammatory cells of the nervous system (microglial cells) as a stimulus for reactivation. BV-2 cells are immortalized mouse-microglial cells that are a suitable model for inflammation due to the ability to activate the cells *in vitro* (Bocchini, et al. 1992). Activated microglial cells change morphology in culture and release inflammatory factors such as TNF-α, IL-1β, nitric oxide, and prostaglandin E (Henry, et al. 2009; Dai, et al. 2015). I treated microglial cells with lipopolysaccharide (LPS) at low and high concentrations (250 ng/mL or 1000 ng/mL) for 24 hours and collected the media (stored at -20 ºC) (Dai, et al. 2015). Treating latently infected SNAP cells with activated media yielded inconclusive results (**Table 3**).

Briefly, Botting explored whether direct UV irradiation of latently infected SNAP cells would induce reactivation and found that doses were either cytotoxic to SNAP cells or failed to reactivate virus. It may be that UV irradiation of epithelial cells is necessary to trigger release of inflammatory signals distal to the neuronal cell body in the skin. Medium from UV irradiated epithelial cells could be collected and used as a reactivation stimulus without causing direct toxicity to SNAP cells.

Other Treatments

Protocols for HD10.6 cell maturation from Celgene, called for supplementation of forskolin in the medium. Initially, I had a hard time maintaining a quiescent infection in SNAP cells even with acyclovir treatment. I removed forskolin from maturation media after realizing it is used in culture to induce HSV reactivation (Danaher, et al. 2005; Danaher, et al. 2013).

Unfortunately, when I treated latently infected SNAP cells with forskolin (at 50 µM or 85 µM) it did not induce reactivation over background (**Table 3**).

The histone deacetylase (HDAC) inhibitor trichostatin-A, has also been used to induce reactivation in cell culture models (Danaher, et al. 2005). I treated latently infected SNAP cells with the trichostatin-A (at 33 nM) without positive reactivation results (**Table 3**). Other HDAC inhibitors, such as sodium butyrate, may be effective at inducing reactivation (Danaher, et al. 2005); I did not test sodium butyrate on latently infected SNAP cells. With advances in the field of epigenetics, novel HDAC inhibitors are being discovered and synthesized. Latently infected SNAP cells would provide a useful screening tool to explore the effects of HDAC inhibitors on reactivation frequency.

Future Reactivation Stimuli

As noted, optimization of HSV latency in SNAP cells was ongoing while reactivation experiments were being carried out. It may be valuable to revisit the stimuli described here in a more robust (multiple 96-well plate) manner under the optimized infection conditions described in Appendix A. Furthermore, I would add compounds such as hexamethylene bisacetamide (HMBA), which compliments viral transcriptional (Burris, et al. 2008), to list of compounds to screen for reactivation.

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