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Inducible heat shock protein 70 enhances human papillomavirus type 31 genome replication, viral capsid protein nuclear localization and progeny virion morphogenesis in human keratinocytes

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BY

Hebin Song

Bachelor of Medicine, Peking University Health Science Center, 1998

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biomedical Sciences

The University of New Mexico
Albuquerque, New Mexico

August, 2009

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ABSTRACT

Human papillomaviruses (HPVs) are small, non-enveloped double stranded DNA viruses that demonstrate a strict species and cell type tropism for human epithelial cells. The association between high-risk HPV types and cervical cancer is well established. Additionally, HPVs have been implicated as causes in development of several other epithelial cancer types.

Increasing data indicate heat shock proteins (HSPs) including inducible HSP70 (HSP70i) are involved in the replicative cycles of different viruses including adenoviruses, polyomaviruses (PyV), and some RNA viruses. Cell-free

system studies implicate HSP70i in HPV11 genome replication with E1 and E2 proteins, and there is evidence that HSP70 is involved in capsid assembly and disassembly for PyV and PV. HSP70 expression is increased in HPV16 E6/E7 gene transduced human primary keratinocytes, and frequently detected in early stage uterine cervical cancer at levels in conjunction with lesion severity. In this study we carry out analyses with the natural host cell to assess HSP70i's role in the viral infectious life cycle. For these studies we used the organotypic (raft) culture system to recapitulate the full viral life cycle of the high-risk human papillomavirus type 31 (HPV31). Upon heat shock of HPV31 infected organotypic tissues, we find high and sustained expression of HSP70i coincident with enhanced HPV genome replication and virion production. Whereas there is no detectable effect on total L1 expression levels, we find that HSP70i interacts with L1, colocalizes with and enhances L1 nuclear localization in differentiated cells. Adenovirus-mediated gene transfer was used to study the effects of HSP70i in naturally HPV infected differentiating tissues and showed results similar to those in heat shocked rafts. In HPV31 infected monolayer cells ectopically expressing viral capsid proteins, without obvious impact on L1 expression levels, our results suggest wild type HSP70i interacts with and promotes L1 translocation into nucleus concomitant with increased HPV genome replication and virion production. Alternatively, HSP70i ATPase domain mutant (HSP70i(K71A)) impedes virion production while viral genome levels, L1 expression and localization demonstrate the same pattern as control. These results indicate that HSP70i is involved in diverse aspects of the viral life cycle

including genome replication, capsid protein transportation and virion morphogenesis. We conclude that HSP70i contributes directly to these HPV replicative viral activities and the production of infectious progeny virions.

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Introduction

A. Introduction to Papillomaviruses

i). Classification

Papillomaviruses (PV) are a large and diverse group of viruses, and likely occur in most mammals. The PVs had been originally classified with the polyomaviruses (PyV) in one family, the *Papovaviridae*. This was based on similar, nonenveloped capsids and the common circular double-stranded DNA genomes. As it was later recognized that the two virus groups have different genome sizes, completely different genome organizations, and no major nucleotide or amino acid sequence similarities, they are now officially recognized by the International Committee on the Taxonomy of Viruses (ICTV) as two separate families, *Papillomaviridae* and *Polyomaviridae* (229).

The PV genome is divided into an early region (E), encoding various genes that are expressed immediately after initial infection of a host cell, and a late region (L) encoding the capsid genes L1 and L2. The L1 ORF is the most conserved gene within the genome and has therefore been used for the identification of new "PV types". A new PV isolate is recognized as such if the complete genome has been cloned and the DNA sequence of the L1 ORF differs by more than 10% from the closest known PV type. Differences between 2% and 10% homology define a subtype and less than 2% a variant. This definition was agreed upon between all PV scientists working on PV taxonomy and diagnosis (58, 59). Over one hundred PV types have been detected in humans based on the isolation of complete genomes. There is also strong evidence that

PV genomes are very static, and sequence changes by recombination or mutation are very rare events.   Mutational changes apparently occur at frequencies similar to those of the DNA genomes of the infected host organism (229).

ii). Papillomavirus Structure

PVs are non-enveloped, icosahedral symmetry, DNA viruses with a ~ 55 nm diameter. The PV genome is a double-stranded circular DNA molecule ~8,000 base pairs in length. It is packaged within the capsid along with cellular histone proteins, which serve to condense DNA (78).

The virus particle is comprised of 72 pentamers of the major capsid protein (L1) on the outer surface, arranged on a T=7 icosahedral lattice (reviewed in (88, 227)). A minor capsid, L2, is located mostly internal to the L1 shell.   The viral genomic DNA is packaged within the L1/L2 capsid as a minichromosome (reviewed in (227)). The recombinant L1 capsid alone can assemble into the virus-like particle (VLP) structure *in vivo* and *in vitro* (39, 40, 109, 123, 187, 204, 210). Thus, the L1 protein contains all the information needed for the particle assembly. Self-assembled VLPs composed of L1 are the basis of a successful group of prophylactic HPV vaccines designed to elicit virus-neutralizing antibodies that protect against initial HPV infection.

Each capsid contains 12-72 molecules of L2 and the arrangement within the virion remains unclear (31). Although the minor capsid protein, L2, is not

required for capsid formation, it is known to perform several important functions, including facilitating the encapsidation of the viral genome into nascent virions as well as a number of essential roles in the infectious entry of the virus into new host cells (22, 95, 96, 106, 111, 181). Therefore, L2 is of interest as a possible target for more broadly protective HPV vaccines (98, 108).

PVs have circular double-stranded DNA genomes with sizes close to 8 kb. All genes are encoded on one DNA strand, which include an early region (E), encoding various genes that are expressed immediately after initial infection of a host cell, and a late region (L) encoding the capsid genes L1 and L2. Brief functional descriptions of the genes are stated below.

E1 encodes a protein that binds to the viral origin of replication in the long control region (LCR) of the viral genome. E1 uses ATP to exert a helicase activity that forces apart the DNA strands, thus preparing the viral genome for replication by cellular DNA replication factors (61).

The E2 protein serves as a master transcriptional regulator for viral promoters located primarily in the long control region. The protein has a transactivation domain linked by a relatively unstructured hinge region to a well-characterized DNA binding domain. E2 facilitates the binding of E1 to the viral origin of replication (76, 144). E2 also utilizes a cellular protein known as Bromodomain-4 (Brd4) to tether the viral genome to cellular chromosomes (139). This tethering to the cell's nuclear matrix ensures faithful distribution of viral genomes to each daughter cell after cell division. It is thought that E2 serves as

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a negative regulator of expression for the oncogenes E6 and E7 in latently HPV-infected basal layer keratinocytes (179, 186). Genetic changes, such as integration of the viral DNA into a host cell chromosome, that inactivate E2 expression tend to increase the expression of the E6 and E7 oncogenes, resulting in cellular transformation and possibly further genetic destabilization (86).

The E3 gene is not known to be expressed as a protein and does not appear to serve any function.

E4 proteins are expressed at low levels during the early phase of viral infection, but increased dramatically during the late phase of infection (65). The E4 protein of many papillomavirus types is thought to facilitate virion release into the environment by disrupting intermediate filaments of the keratinocyte cytoskeleton (66).

The E5 are small, very hydrophobic proteins that de-stabilize the function of many membrane proteins in the infected cell (28). The association of HPV E5 proteins to cancer is likely through the activation of signal cascade initiated by epidermal growth factor upon ligand binding (35).

The E6 protein can bind and inactivate the tumor suppressor protein p53 (51, 143). Since the expression of E6 is strictly required for maintenance of a malignant phenotype in HPV-induced cancers, it is an appealing target of

therapeutic HPV vaccines designed to eradicate established cervical cancer tumors (99, 107, 116).

The E7 protein can inactivate the pRb family of tumor suppressor proteins (9, 50). Together with E6, E7 serves to prevent cell apoptosis and promote cell cycle progression, thus priming the cell for replication of the viral DNA (93, 135, 137). E7 also participates in immortalization of infected cells by activating cellular telomerase (130, 200).

E8 protein is only expressed in a few PV types. The E8^E2 gene product causes repression of early HPV transcription and replication (117).

L1 is the major capsid protein and spontaneously self-assembles into pentameric capsomers. Purified capsomers can continue to form capsids, which are stabilized by disulfide bonds between neighboring L1 molecules (39, 40, 88, 109, 123, 187, 204, 210). Compared to other PV genes, the amino acid sequences of most portions of L1 are well-conserved between types (102). However, the surface loops of L1 can differ substantially, even for different members of a particular PV species. This probably reflects a mechanism for evasion of neutralizing antibody responses elicited by previous PV infections (34).

In addition to cooperating with L1 to package the viral DNA into the virion, L2 has been shown to interact with a number of cellular proteins during the infectious entry process. After the initial binding of the virion to the cell, L2 must be cleaved by the cellular protease furin (181). The virion is internalized, through

different pathways including caveola-mediated and clathrin-mediated process (27, 197), into an endosome, where acidic conditions and reduction of an intramolecular disulfide are thought to lead to exposure of membrane-destabilizing portions of L2 (33, 106). The cellular proteins beta-actin (223) and syntaxin-18 (26) may also participate in L2-mediated entry events. After endosome escape, L2 and the viral genome are imported into the cell nucleus where they traffic to a sub-nuclear domain known as an ND-10 body that is rich in transcription factors (54). Small portions of L2 are well-conserved among different PV types, and experimental vaccines targeting these conserved domains may offer protection against a broad range of HPV types (173).

In short, three oncogenes, E5, E6, and E7, modulate the transformation process, three regulatory proteins, E1, E2 and E2^E8, modulate transcription and replication, and two structural proteins, L1 and L2, compose the viral capsid (reviewed in (149)). The E1, E2, L1, and L2 ORFs are particularly well conserved among all members of the family. Most cis-responsive elements are in the LCR between L1 and E6, a segment with little sequence conservation.

iii). Papillomavirus Life Cycle

The unique aspect of HPV biology is the essential dependence on epithelial differentiation for the completion of the life cycle (Figure 1.1). In the current knowledge of HPV infection, the viruses gain access to the mitotically active cells in the basal layer of epithelium presumably through small wounds,

known as breaches, in the skin (70, 194). Following successful infection of these cells, early gene expression products, E1 and E2 proteins, facilitate viral genome episomal maintenance and segregation during mitosis (175, 225). At the same time, viral genome replication occurs at low level and is maintained episomally (69, 132). The viral oncogenes E6 and E7 promote uncontrolled growth and cell cycle disregulation by inactivating the tumor suppressor p53 and pRb (150, 174, 189, 217). PV genomes can be maintained in stem cells of the epithelial basal layer for decades (64, 153).

The expression of the viral late genes, L1 and L2, is exclusively restricted to terminally differentiation of infected keratinocytes, ensuring that the progeny virions are released only in the outermost layers of the epithelium (41, 205). As the viral capsid proteins are most likely to be targeted by the host immune responses, and the outer layers of stratified epithelia are subject to relatively limited surveillance by the immune system, this gene expression strategy represents a form of immune evasion by HPVs (208). The ability of HPV to avoid immune clearance and persist over the course of years plays a necessary role in the high-risk HPV and cancer association.

Progeny virus morphogenesis occurs in the cell nucleus. PVs have evolved a mechanism for releasing virions into the environment through desquamation with the epithelial cells which is a confidential, non-inflammatory release (29, 64, 201).

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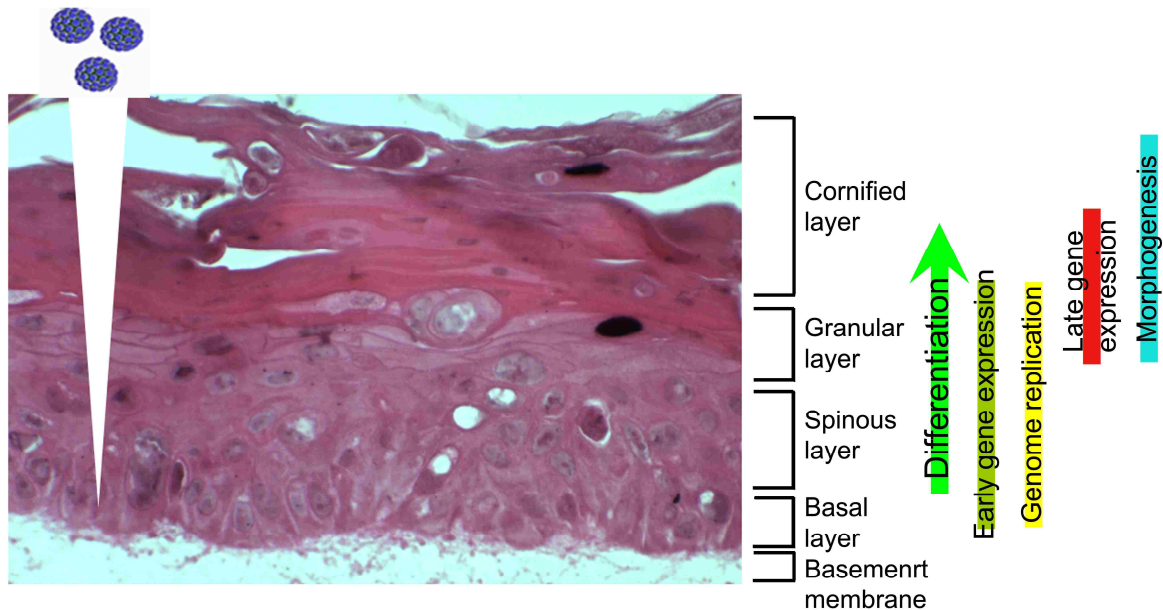
Figure 1.1. The differentiation dependent life cycle of papillomaviruses. Papillomaviruses infect basal epithelial cells *via* a breach in the differentiated epithelium. Infected basal cells exhibit early gene expression and low level genome replication. As infected cells divide, differentiate in the epithelia, increased early gene expression and genome replication occur. Late gene expression (capsid proteins) is activated only as infected cells approach terminal differentiation, and morphogenesis of progeny virions occur. As these cells approach the surface of the epithelia they are sloughed off, and the progeny virions are released.

B. Human Papillomavirus Infections and Diseases

HPVs infect the skin and mucous membranes of humans. Infection with HPVs is ubiquitous, the health of both men and women are impacted (4). Approximately 130 HPV types have been identified. Some HPV types can cause benign warts, certain types of PVs convey a high risk for malignant progression, while others have no symptoms (reviewed in (48, 136, 151, 228)). About 30-40 HPV types are typically transmitted through sexual contact and infect the anogenital region.

HPVs are the most common sexually transmitted infection in the United States (68, 216). Most sexually active men and women will acquire genital HPV infection at some point in their lives (12). By the age of 50 more than 80% of American women will have contracted at least one type of genital HPV (68). It was estimated that in the year 2000, there were approximately 6.2 million new HPV infections among Americans aged 15-44; of these, an estimated 74% occurred to people between ages 15-24 (216).

All HPVs are believed to be capable of establishing long-term "latent" infections in small numbers of stem cells present in the skin, because a wide variety of different types can be detected at random sites of healthy skin (3, 5). Although these latent infections may never be fully eradicated, immunological control is thought to block the appearance of symptoms such as warts. Immunological control is likely HPV type-specific, meaning that an individual may become immunologically resistant to one HPV type while remaining susceptible to other types. Recent studies suggest that HPV may eventually be cleared in most people with well-functioning immune systems. Conditions of immune suppression in humans lead to activation of latent infections or increased susceptibility to reinoculation from active infections resulting in overt lesions (reviewed in (57, 74, 103)). It appears that in some cases the virus does remain in the body indefinitely, producing symptoms if the immune system weakens (202).

Once an HPV viron invades a cell, an active infection occurs, and the virus can be transmitted. Several months to years may elapse before benign warts or squamous intraepithelial lesions (SIL) develop and can be clinically detected. Persistent infection with "high-risk" HPV types - different from the ones that cause benign warts - may progress to precancerous lesions and invasive cancer (202).

Genital or anal warts (condylomata acuminata or venereal warts) are the most easily recognized sign of genital HPV infection. Although a wide variety of HPV types can cause genital warts, types 6 and 11 account for about 90% of all cases (85, 209). Most people who acquire genital wart-associated HPV types clear the infection rapidly without ever developing warts or any other symptoms. People may transmit the virus to others even if they are not displaying overt symptoms of infection. HPV types that tend to cause genital warts are not the same ones that cause cervical cancer. However, since an individual can be infected with multiple types of HPV, the presence of warts does not rule out the possibility of high-risk types of the virus also being present (209). The types of HPV that cause genital warts are usually different from the types that cause warts on other parts of the body, such as the hands or inner thighs. People do not get genital warts by touching warts on their hands or feet (209).

Recurrent respiratory papillomatosis is a rare condition caused by HPV types 6 and 11, in which warts form on the larynx or other areas of the respiratory tract (18, 196). These warts can recur frequently, may require

repetitive surgery, may interfere with breathing, and in extremely rare cases can progress to cancer (146).

About a dozen HPV types (including types 16, 18, 31 and 45) are called "high-risk" types because they can lead to cervical cancer, as well as anal cancer, vulvar cancer, and penile cancer (170-172). Infection with one or more high-risk HPV types is believed to be a prerequisite for the development of cervical cancer (214). However, most HPV infections are cleared rapidly by the immune system and do not progress to cervical cancer. Because the process of transforming normal cervical cells into cancerous ones is slow, cancer occurs in people who have been infected with HPV for a long time, usually over a decade or more (84, 196).

Several types of HPV, particularly type 16, have been found to be associated with oropharyngeal squamous-cell carcinoma, a form of head-and-neck cancer (53). Engaging in oral sex with an HPV-infected partner may increase the risk of developing these types of cancers (53). HPV-induced cancers often have viral sequences integrated into the cellular DNA (110).

The American Cancer Society estimates that in 2008, about 11,070 women in the United States will be diagnosed with invasive cervical cancer, and about 3,870 US women will die from this disease. Avoiding sexual contact with an infected person is the only 100% effective prevention method; however, many people are unaware that they are infected with HPV. Condoms offer some protection, but exposed skin can transmit the virus. Two vaccines are currently

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available (see "HPV vaccines" below) to women between the ages of 9 and 26 (79, 87, 127).

C. HPV Prevention

i). Pap test

Persistent infection with one or more of about a dozen of high-risk HPV types is an important factor in nearly all cases of cervical cancer. The development of HPV-induced cervical cancer is a slow process that generally takes many years. During this development phase, pre-cancerous cells can be detected by regular cervical cytology Papanicolaou screening, colloquially known as "Pap" smear testing (188). The Pap test is an effective strategy for reducing the risk of invasive cervical cancer. The Pap test involves taking cells from the cervix and putting them on a small glass slide and examining them under a microscope to look for abnormal cells. This method is 70% to 80% effective in detecting HPV-caused cellular abnormalities (156). A cervical Pap test is used to detect cellular abnormalities. Detailed inspection of the cervix by colposcopy may be indicated if abnormal cells are detected by routine Pap test. This allows targeted surgical removal of condylomatous and/or potentially precancerous lesions prior to the development of invasive cervical cancer. All women are encouraged to get a yearly pap test solely to detect cellular abnormalities caused by HPV. Since the Pap test was developed there has been a 70% decrease in

cervical cancer deaths over the last 50 years. Pap test testing has proven to be one of the most successful screening tests in the history of medicine (211).

ii). Vaccines

Although the widespread use of Pap testing has reduced the incidence and lethality of cervical cancer in developed countries, the disease still kills several hundred thousand women per year worldwide. HPV vaccines, Gardasil and Cervarix, which prevent infection with some of the sexually transmitted HPV types that cause the majority of malignant disease, may lead to further decreases in the incidence of HPV-induced cancers (131).

The HPV vaccine is made up of the HPV L1 capsid protein. The US Food and Drug Administration approved Gardasil, a prophylactic HPV vaccine which is marketed by Merck. The vaccine trial, conducted in adult women with a mean age of 23, showed protection against initial infection with HPV types 16 and 18, which together cause 70% of cervical cancers, and can cause other cancers, such as anal cancer and head and neck cancer. The vaccine also protects against HPV types 6 and 11, which cause 90 percent of genital warts (89, 90). Gardasil vaccine is delivered in a series of three shots over six months at a cost of approximately $360 (US dollars).

The vaccine has recently reported to show high efficacy against HPV type 16/18 associated CIN 2 (cervical intraepithelial neoplasia, grade 2) and cross protection against other high-risk HPV types such as HPV31, 45, and etc. (167).

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In addition, females already infected with one or more vaccine HPV types before vaccination would be protected against disease caused by the other vaccine HPV types.

Since the current vaccine will not protect women against all the HPV types that cause cervical cancer (166), women should continue to seek Pap smear testing, even after receiving the vaccine. Cervical cancer screening recommendations have not changed for females who receive HPV vaccine. Both men and women are carriers of HPV. Possible benefits and efficacy of vaccinating men are being studied (20, 160).

In addition to preventive vaccines, laboratory research and several human clinical trials are focused on the development of therapeutic HPV vaccines. In general, these vaccines focus on the main HPV oncogenes E6 and E7. Since expression of E6 and E7 is required for promoting the growth of cervical cancer cells and cells within warts, it is hoped that immune responses against the two oncogenes might eradicate established tumors (15, 184).

D. Heat shock proteins

Heat shock proteins (HSP), also known as "cellular stress proteins" and "molecular chaperones", are a class of functionally related proteins whose expression is increased when cells are exposed to elevated temperatures or other stress including viral infections, inflammation, toxic chemicals, etc. (55,

104). Increased synthesis of HSPs was first reported in 1974 in *Drosophila* cells (192). The dramatic upregulation of the heat shock proteins is a key part of the heat shock response and is transcriptionally induced primarily by heat shock factors (HSFs) (220). Scientists have not discovered exactly how heat-shock (or other environmental stressors) activates the HSF. However, some studies suggest that an increase in damaged or abnormal proteins brings HSFs into action.

HSPs are classified according to their molecular size including HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs like HSP27 (104, 124). These proteins are ubiquitous and essential for both normal cellular function and for cellular defenses to maintain cell homeostasis against various forms of stress. Although altered HSP expression is found in nearly every tumor type, it is not clear whether the association is causal or correlative. Little is known about how HSP protect cells from apoptosis, and their ability to do so, especially in the context of malignant progression, requires additional studies (45, 46). HSPs function as intra-cellular chaperones for other proteins. They interact with diverse proteins substrates to assist in their folding and assisting in the establishment of proper protein conformation and prevention of unwanted protein aggregation, especially under cell stress conditions. Consequently, these proteins assist in recovery from stress either by repairing damaged proteins (refolding) or by promoting their degradation, thereby supporting cell survival. HSP also exhibit other functions, by helping to stabilize partially unfolded

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proteins, HSPs aid in transporting proteins between cellular organelles within the cell (23, 195, 215, 222).

The 70-kDa family of HSPs (HSP70) in particular, plays a vital role in cellular protection and has been detected in various tissues subjected to stress (2, 17). HSP70 stress proteins exist as two main forms. The cognate form referred to as HSC70 is constitutively expressed in the non-stressed cell as a molecular chaperone. The predominant form is highly inducible and up-regulated in response to stressful stimuli to function as a molecular chaperone, and is referred to as inducible HSP70 (hereafter called HSP70i). HSP70i protein in unstressed cells is found in both the cytoplasm and nucleus (80).

The HSP70 proteins have three major functional domains (71, 119):

- The N-terminal 44-kDa adenosine triphosphatase (ATPase) domain that contains the ATP binding site and retains ATPase activity. It binds ATP and hydrolyzes it to ADP. The hydrolysis of ATP drives conformational changes in the other two domains and involved in the folding and refolding of substrates.

- The 18-kDa substrate binding domain represents an internal fragment of HSP70 that contains a groove that can bind neutral, hydrophobic amino acid residues. The groove can interact with peptides up to seven residues in length.

- The 10-kDa C-terminal domain is rich in alpha helical structure that is proposed to act as a 'lid' for covering the substrate binding domain. When an HSP70 protein is ATP bound, the lid is open and peptides bind and release relatively rapidly. When HSP70 proteins are ADP bound, the lid is closed, and peptides are tightly bound to the substrate binding domain. Also, this domain serves as a site for the binding of co-chaperones, such as the HSP40 protein family.

ATP-ADP cycle is necessary in the HSP70-substrate complex formation, proper peptide folding and releasing. HSP70 proteins exhibit fast on-and-off rates for substrate binding when ATP is bound. Interactions with peptide can stimulate ATP hydrolysis and leads to a conformational change in HSP70 that stabilizes HSP70-substrate complexes (8, 193). An interdomain communication between the ATPase domain and substrate binding domain of HSP70 appears to exist (30, 145). The affinity of HSP70 proteins for peptides is increased by closing the lid domain on the peptide binding groove and trapping substrates. Ultimately, with the exchange of ADP for ATP, peptide substrates are released (161, 218).

The HSP70i ATPase domain K71A mutant (hereafter called HSP70i(K71A)) demonstrates a prominent loss of its binding to ATP while retaining its native ADP-binding properties (11, 159, 221). Thus, this mutant is

proposed to bind its substrates at a higher affinity and not to release the substrates as does wild type HSP70i.

Intracellular heat shock proteins are highly expressed in cancerous cells and are essential to the survival of these cell types. Hence small molecule inhibitors of HSPs, especially HSP90 show promise as anticancer agents (62). The potent HSP90 inhibitor 17-AAG is currently in clinical trials for the treatment of several types of cancer (199).

HSPs are useful as immunologic adjuvants in boosting the response to a vaccine (16, 158). Furthermore, some researchers speculate that HSPs may be involved in binding protein fragments from dead malignant cells and presenting them to the immune system. Therefore HSPs may be useful for increasing the effectiveness of cancer vaccines (19, 158).

E. Heat Shock Proteins and Virus Infections

HSPs are involved in many steps in the life cycles of viruses. Cellular or virally-encoded stress proteins cause altered transcription, cellular transformation, viral genome replication, and increased virion assembly (reviewed in (206)). Increasing data indicate HSPs including HSP70, HSP90, HSP40 are involved in the life cycle of different viruses including adenovirus (Ad) (82, 133), PyV (43), dengue virus (180). Little is known about the role of heat shock proteins in HPV life cycle activities. Immunohistochemical (IHC) analysis

detects HSP70 frequently in uterine cervical cancer, especially in the early stages (169), and enhanced IHC staining intensity with increasing lesion severity (38). HSP70 expression is increased in HPV16 E6/E7 gene transduced human primary keratinocytes (125). In a cell free system, HSP70 and HSP40 mediate HPV11 DNA replication (126, 129) and are involved in disassembly of PVs (42). Evidence from closely related PyV and SV40 viruses suggests that stress proteins are involved in virion assembly and disassembly (42, 43, 206).

F. Gaps in the Current Knowledge, Rationale for Work, Central Hypothesis and Specific Aims

Virtually all cervical cancers are caused by HPV infections (214). HPVs are the necessary but insufficient cause of all cervical cancers as the incidence of HPV infection is much higher than HPV related cervical cancer reports (214). Progression from HPV infection to cancer can take up to 30 years (92). Epidemiological studies indicate tobacco use, other genital infections, impaired immune system particularly related to HIV infection, hormonal factors including early age at first birth, use of hormonal contraceptives, multi-parity, and nutritional factors contribute to the HPV-cervical cancer process (36, 47, 56, 67, 75, 77, 94, 152, 198, 207). There are only rudimentary explanations of how these co-factors might contribute to malignant progression in the HPV-infected cervix. A common feature of each of these putative co-factors is that they can increase the levels of HSPs and/or viral persistence in the cervical environment

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(6, 36, 47, 56, 67, 75, 77, 94, 152, 190, 198, 207). Understanding the role of HSPs in the PV life cycle may allow us to establish the means to inhibit PV infections and thereby prevent HPV-associated cancers and diseases.

The life cycle of HPVs has been difficult to study owing to its dependence on epithelial differentiation. The productive phase of the HPV life cycle, which includes viral DNA amplification, capsid protein synthesis, and assembly of progeny virions, naturally takes place in a small fraction of superficial differentiated cells (205). In this study, we use the three-dimensional tissue culture system (also termed the organotypic or "raft" tissue culture system), which is the only *in vitro* system proven to consistently mimic epithelial differentiation to the extent that the complete HPV life cycle can be studied and that infectious virions can be purified. Meyers and coworkers were the first to reproduce the complete HPV life cycle *in vitro* using a continuous cell line, CIN-612 9E (hereafter called "9E") established from a CIN-1 (cervical intraepithelial neoplasia, grade 1) biopsy and persistently infected with HPV31 (14, 140). This system achieves an environment permissive for the complete HPV viral life cycle, and provides us a unique opportunity to address questions relating to specific modulators in a controlled environment where we can mimic specific phenotypes and directly assay their effects on the HPV replicate life cycle.

The organotypic tissue culture system provides an excellent system to study the differentiation dependent HPV life cycle activities. In addition, a simple monolayer culture system has been developed recently that can produce

infectious virions independently of viral DNA replication and epithelial cell differentiation by cotransfection HPV capsid protein expression plasmid as well as the full-length HPV genome into HEK293T cells (178). In this study, we modified this "293T-HPV virion assembly system" (178) for HPV31 using the 9E cell line, which endogenously maintains HPV31 episomal genomes. In brief, 9E monolayer cells are transfected with HPV31 L1/L2 plasmid and virion particles are produced. We termed this revised system as "9E-L1/L2 monolayer cell culture system". This monolayer system is more time-efficient and easily adapted to experimentation and manipulation compared to the raft system.

Present data suggest that HSPs have important roles in HPV life cycle activities. However, the functions of HSPs in the HPV life cycle under authentic replicative conditions, i.e. in natural host epithelial cell and/or tissues, are not known. This represents a significant gap in the current knowledge of HPV molecular biology. Therefore, my *central hypothesis is that HSPs play essential roles in the PV replicative life cycle*.

Three specific aims were designed to test this hypothesis:

1. Identify the effects of HSP induction on HPV31 viral activities in keratinocyte raft tissue culture - the natural replicative environment.

2. Investigate the roles of specific HSP on HPV31 viral functions.

3. Determine the mechanisms involved in the impact of specific HSPs on HPV31 life cycle activities.

Due to the abundance of research on HPV viral activities that has been performed using inappropriate cell types and cell free system, many aspects of HPV biology under authentic replicative conditions still remain unclear. Therefore, in this research, I have attempted to define the molecular roles that HSPs play in HPV life cycles activities in the natural host cell types, human keratinocytes, and in a viral life cycle authentic tissue culture system. This work will provide a foundation for defining the molecular roles that HSPs play in HPV life cycles in authentic replication environments.

Chapter 2

Materials and Methods

A. Culture of Cell Lines and Tissues

i). Cell lines

The human embryonic kidney 293 (HEK293) cells were grown in monolayer with Dulbecco's modified Eagle's high glucose medium (DMEM; Gibco) plus 10% fetal bovine serum (FBS), 100 U/ml gentamycin, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich) (83).

The CIN-612 9E (hereafter called 9E) human keratinocyte cell line was established from a CIN-1 biopsy and maintains HPV31 genomes extrachromosomally (100). 9E cells were grown in monolayer culture in the presence of mitomycin C-treated J2 3T3 fibroblast feeder cells with E medium containing 5% FBS and 100 U/ml Nystatin (Sigma-Aldrich) as previously described (162, 163).

Cell lines were passaged or harvested by dispersal with 0.1% trypsin–0.5 mM EDTA at 37℃.

ii). Organotypic (raft) tissue culture system

9E cells were cultivated as organotypic epithelial (raft) tissue cultures to induce differentiation as described previously (162, 163). Unless otherwise stated, the raft epithelial tissues were allowed to initiate stratification and differentiation for 6 days at the air-medium interface under normal growth conditions in E medium containing 5% FBS, 100 U/ml Nystatin (Sigma-Aldrich)

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and 10 μM 1,2-dioctanoyl-sn-glycerol (C8:0; Sigma Chemical Co.) a protein kinase C activator that enhances differentiation of HPV-infected cells (141). 9E raft tissues were heat shocked and harvested on different days at the air-liquid interface. HPV-infected raft tissues reach maximum differentiation by day 14 after lifting to the air-medium interface and this is our end point for tissue growth (164, 203). For heat shock treatments, raft tissues were incubated at 43ºC for 90 minutes. For Ad-mediated HSP70i transduction, monolayer 9E cells atop collagen matrices were exposed to various multiplicities of infection (MOI) of Ad-mediated transduction 24 hours before lifting to the air-medium interface. 9E raft tissues were harvested on different days at the air-medium interface.

Entire raft tissues, including the dermal equivalent, were harvested for tissue section histological analyses by fixation in 4% formalin overnight. The tissues were embedded in paraffin and cut into 4-μm-thick cross-sections. Alternatively, to harvest raft tissues for protein, DNA, and virions, stratified keratinocyte tissues were peeled away from the dermal equivalent, and the tissue was frozen at -80℃ until extraction (see below).

iii). 9E-L1/L2 monolayer culture system

9E cells were propagated in E media with J2 feeder cells as described above. After one passage without J2 feeder cells, 9E cells were transfected with a codon optimized HPV31-L1/L2 expressing plasmid using FuGene 6 transfection kit (Roche). Briefly, for each 100 mm plate of 9E cells, 1.5 μg of plasmid and 9 μl

FuGene 6 Reagent were mixed in serum free E media to make 600 µl total volume of transfection complex. After 30 minutes incubation at room temperature, the complex was added to the 9E cells and cultured at 37ºC in a cell culture incubator. To investigate the effect of HSP70i expression on viral activities, Ad-mediated HSP70i transduction was performed with various MOI. At 24 hours post transduction, the HPV31-L1/L2 plasmid transfection was carried out as described above. At 48 hours post-transfection, cells were harvested for protein, DNA, and virion assays described below.

B. Purification and Quantification of Viruses

i). Purification and quantification of recombinant adenoviruses

Adtrack (Ad-v), the vector for recombinant Ads, expresses green fluorescent protein (GFP) allowing direct detection of transduction efficiency. Ad-HSP70i (AP70) expresses the complete human HSP70i cDNA and was generated as previously described (63, 82, 91). Ad-HSP70i(K71A) expresses the HSP70i containing a K71A mutation in the ATPase domain (134, 221). The primary Ad stocks were propagated in HEK293 cells for several passages to obtain high titer preparations, which contain higher Ad particles per unit volume. Viral particles were purified by CsCl ultracentrifugation, and the titer of viral stocks was determined by plaque assay in HEK293 cells as described (13). The titers were at 10^{13} pfu/ml.

ii). Purification and quantification of HPV virons

a). Purification and quantification of HPV virons from raft tissues

HPV31 virions were isolated from 10 raft tissues per treatment group by a protocol and observing strict BSL2 conditions as detailed previously (163). Following the centrifugation of viral DNA-containing particles at 130,000 x g, the supernatant was carefully decanted and the pellet was air dried. Pellets were resuspended in 500 µl of buffer (50mM NaCl, 50 mM sodium phosphate, pH 7.4) and transferred to a 2-ml Dounce homogenizer to disperse clumps. The resulting homogenate was centrifuged at 8,000 x g in a microcentrifuge at 4°C for 10 min. The supernatant was reserved, and the pellet was re-extracted as described above; virion-containing supernatants were pooled. The crude virus preparations from these pooled supernatants were subjected to dot-blot hybridization for quantification of vDNA-containing particles (i.e., <u>v</u>iral <u>g</u>enome <u>e</u>quivalents, or VGE) using a ^{32}P-labeled HPV31 DNA probe as reported (162, 163).

b). Purification and quantification of HPV virons from 9E-L1/L2 monolayer cells

A transfection-based method for infectious PV production from 9E-L1/L2 monolayer cells was modified from that previous described for HEK 293TT cells (32, 178). At 72 hours post Ad-mediated transduction, which was also 48 hours post HPV31-L1/L2 plasmid transfection, cells were trypsinized, pelleted, and

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resuspended at 5 x 10^7 cells/ml in PBS/9.5 mM MgCl$_2$. Cells were lysed and subjected to 3 x freeze-thaw cycles. DNase treatment and virion maturation were performed as described (32). Cell lysate supernatants were collected and layered atop of 1.25 g/ml - 1.4 g/ml CsCl step gradient. The viral band was extracted by side puncture after centrifugation at 20,000 x g for 18 hours. Virions were purified and concentrated using Amicon Ultra-4 filter devices (Millipore) as needed. Virion purity was assessed by SDS-PAGE and Coomassie staining. Virion stocks were quantified by dot blot hybridization based on VGE as described above (162, 163).

C. Protein Isolation and Analysis

i). Total protein isolation or cell protein fractionation

a). Total protein isolation

Raft tissues were resuspended per raft in 0.5 ml of RIPA buffer (150 mM NaCl; 50 mM Tris, pH 8.0; 5 mM EDTA; 1% Triton X-100; 0.1% SDS; 1% DOC; 1 mM DTT; 1 mM PMSF; 1 µg/ml Leupeptin; 1 µg/ml Aprotinin). Tissues were homogenized thoroughly with volume-appropriate sterile Dounce homogenizer, then centrifuged at 13,000 x g for 15 min at 4°C. Supernatants were collected and protein concentrations were determined by Bradford Assay.

9E-L1/L2 monolayer cells were trypsinized, pelleted, and resuspended per 100 mm plate in 0.5 ml of RIPA buffer (150 mM NaCl; 50 mM Tris, pH 8.0; 5 mM EDTA; 1% Triton X-100; 0.1% SDS; 1% DOC; 1 mM DTT; 1 mM PMSF; 1 µg/ml Leupeptin; 1 µg/ml Aprotinin). The solutions were passed 10 times through a 23-

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gauge needle, then centrifuged at 13,000 x g for 15 min at 4℃. Supernatants were collected and protein concentrations were determined by Bradford Assay.

b). Cell protein fractionation

9E-L1/L2 monolayer cells were trypsinized, pelleted, and nuclear/cytoplasm protein fractionations were extracted using NE-PER nuclear and cytoplasmic extraction reagents kit (Thermo Scientific). Briefly, cell pellets were resuspended per 100mm plate in 200 μl CER I and 11 μl CER II, then centrifuged at 16,000 x g for 5 min at 4℃. The sup ernatants were collected as cytoplasmic protein fractions. The insoluble pellets were resuspended per 100 mm plates in 100 μl NER, subjected to 4 x freeze-thaw cycles with vigorous vortex, then centrifuged at 16, 000 x g for 15 minutes at 4℃. The supernatant were collected as nuclear fractions. Protein concentrations were determined by Bradford Assay.

ii). Immunoprecipitation (IP)

a). Raft tissue protein sample immunoprecipitation

For IP, 800 μg of total protein per treatment was pre-cleared with EZview Red Protein G Affinity Gel (Sigma). Lysates were then incubated with either 10 μg of rabbit anti-HSP70i (Stressgen, SPA-812) or with 10 μl of rabbit anti-HPV31 VLP antisera (Ozbun Lab, 60 mg/ml protein) for 20 h at 4℃. The antigen-antibody complexes were precipitated with EZview Red Protein G Affinity Gel,

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boiled in SDS sample buffer, and the released proteins were resolved by SDS-PAGE and analyzed by immunoblot. Mouse anti-HSP70i (SPA-810) or mouse anti-HPV L1 (Abcam) antibody were utilized to reciprocally detect precipitated proteins.

b). Cell fractionation protein sample immunoprecipitation

Fractionated cellular protein samples were analyzed by the same process as in raft tissue proteins described above, except that mouse anti-HPV L1 antibody-cocktail (H16.J4, H16.A6, and H31.V5 gifted from N. Christensen, Pennsylvania State University College of Medicine) were utilized to IP HPV L1 protein.

iii). SDS-PAGE and immunoblot

Proteins were analyzed by SDS-PAGE along with molecular weight markers followed by transfer to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The membrane was blocked 1 h at room temperature in 5% milk–TBST (TBS with 0.1% Tween 20) and incubated with primary antibody at room temperature for 3 h. Peroxidase-conjugated secondary antibody was applied for 50 min at room temperature and detected using the ECL Plus reagent (Amersham Biosciences) according to the manufacturer's instructions. Primary antibodies for HSPs were obtained from Stressgen and included: HSP40 (SPA-450, 1:1000), HSP60 (SPA-806, 1:1000), HSP70i (SPA-

810, 1:1000), HSP70i (SPA-812, 1:1000), Hsc70 (SPA-815, 1:1000), HSP90 (SPA-830, 1:1000), and HSP110 (SPA-1103, 1:1000). Anti-β-tubulin antibody (T0198, Sigma-Aldrich, 1:1000) and anti-HDAC-1 antibody (H3284, Sigma-Aldrich, 1:1000) were used to verify equal protein loading on all blots.

D. Immunohistochemistry (IHC), Immunofluorescence (IF), and Confocal Microscopy

Raft tissue sections were deparaffinized in xylene, rehydrated through a series alcohol rinses, and endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 in methanol for 15 min. Tissues were washed in phosphate-buffered saline (PBS), unmasked by boiling in 10 mM sodium citrate (pH 6) for 30 min, cooled to room temperature, washed in PBS. For IHC detection of HSP70i and HPV31 L1, blocking was in 1.5% horse serum (Vector Labs) in PBS for 30 min at room temperature. Rabbit anti-HSP70i (SPA-812; Stressgen) was used at a 1:200 dilution; mouse monoclonal antibody K1H8 (DAKO) was used at a 1:100 dilution to detect HPV L1. Primary antibodies were applied in 1.5% horse serum–PBS and incubated from 0.5 h at room temperature to overnight at 4ºC according to empirically determined detection sensitivity for each. The sections were washed in PBS, and the primary antibody-antigen interaction was detected with the Vectastain ABC and DAB kit (Vector Labs) as described by the manufacturer. Sections were counterstained with Eosin Y. For IF detection of HSP70i and HPV31 L1, blocking was in 5%

goat serum (Vector Labs) in PBS for 30 min at room temperature. Tissue sections were incubated 1 h at 37℃ with anti-HPV L1 mouse monoclonal antibody K1H8 (DAKO) at 1:100 dilution and anti-HSP70i rabbit polyclonal antibody (SPA812, Stressgen) at 1:200 dilution. After extensive washing with PBS, tissues were again blocked for 30 min with 5% goat serum and subsequently incubated for 1 h at 37℃ with Texas Red-conjugated goat anti-mouse IgG (Invitrogen) and FITC-conjugated goat anti-rabbit IgG (Invitrogen). Tissues were washed with PBS and then mounted with cover slips by using VectaShield containing DAPI (4', 6'-diamidino-2-phenylindole; Vector Labs). For IF detection of HSP70i and HPV31 L1, 9E monolayer cells were grown on Lab-TekII Chamber Cassette Slide System, fixed with 3.7% paraformaldehyde, and permeablized with 0.1% Triton X-100 in PBS. After the permeablization, the same steps as in the raft tissue sections were performed except Alexa Fluor 555-conjugated goat anti-mouse IgG (Invitrogen) and Alexa Fluor 647-conjugated goat anti-rabbit IgG (Invitrogen) were used as secondary antibodies. Photomicroscopy was performed by using a Zeiss LSM 510 META confocal microscope with proper objectives and the appropriate filters.

E. *In Situ* Hybridization

Raft tissue sections were deparaffinized in xylene, washed in 100% ethanol and PBS. After pepsin solution incubation and deionized water wash, 20 µl of ZytoFast CISH HPV31 DNA probe (T-1036, ZytoVision) was added onto the

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sections, then sealed with cover slip using rubber cement. After denaturation at 75℃ for 10 min, hybridizations were incubated from 1 h to overnight at 37℃ to empirically determine detection sensitivity. Tissue sections were washed in PBS, then deionized water, and the hybridization was detected with the AP-Biotin T-1052 kit (ZytoVision) as described by the manufacturer.

F. Preparation and Analysis of DNA

9E raft tissues were resuspended in 3 ml per raft tissue of lysis buffer (10 mM Tris•Cl, pH 7.4; 25 mM EDTA; 1% SDS), and homogenized thoroughly in a volume appropriate sterile Dounce homogenizer. For the 9E-L1/L2 monolayer cell cultures, cells were trypsinized, pelleted, and resuspended in 3 ml per 100 mm plate of lysis buffer. The homogenates or cell lysates were supplemented with 100 µg/ml proteinase K and 50 µg/ml RNase A and incubated at 56℃ overnight with agitation. DNAs were sheared by passage 10 times through an 18-gauge needle. Lysates were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) followed by extraction with an equal volume of chloroform-isoamyl alcohol (24:1). The DNA was ethanol precipitated using 0.3 M sodium acetate and 2 volumes of 100% ethanol. The pellets were resuspended in 200 µl of double-distilled H2O per initial raft tissue or 100mm plate of cell. DNAs were quantified by spectrophotometry.

To assay for the presence of extrachromosomal versus integrated HPV31 DNA, 7.1µg of total genomic DNA was digested with *BamHI,* which does not cut

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HPV31, or with *HindIII* to yield a single cut in the extrachromosomal HPV31 genome. The DNA samples were analyzed by 0.8% agarose gel electrophoresis, transferred to nylon membrane (RPN3050B, Amersham), then Southern blot hybridization using a ^{32}P-labeled HPV31 DNA probe as previously described (165). Following high-stringency washes, the Southern blot was analyzed using a phosphorimager (Molecular Dynamics). To quantify the total number per of viral genomes cell, 7.1μg of total genomic DNA was analyzed by dot blot hybridization as described above. HPV31 genome copies were normalized to detection of the GAPDH gene as a loading control.

Chapter 3

Inducible heat shock protein 70 enhances HPV31 viral genome replication

and virion production during the differentiation-dependent

life cycle in human keratinocytes

HSPs are present in all cells in all life forms. They interact with diverse proteins substrates to assist in their folding especially during cell stress to prevent misfolded or otherwise damaged molecules (177, 206). Increasing data indicate HSPs including HSP70, HSP90, HSP40 are involved in the life cycle of different viruses including Ad (82, 133), PyV (43), dengue virus (180). Little is known about the role of heat shock proteins in HPV viral activities. IHC analysis detects HSP70 frequently in uterine cervical cancer, especially in the early stages (169), and enhanced IHC staining intensity with increasing lesion severity (38). HSP70 expression was increased in HPV16 E6/E7 gene transduced human primary keratinocytes (125). In cell free system, HSP70 and HSP40 mediate HPV11 DNA replication (126, 129) and are involved in disassembly of PVs (42). Present data are indicative that HSPs have important roles in HPV life cycle activities. However, the functions of HSPs in HPV life cycle in authentic replicative conditions, i.e. in cell and/or tissue, for the most part are not known. This represents a significant gap in the current knowledge of HPV molecular biology. We hypothesize that induced expression of certain HSPs, like HSP70i, enhance HPV31 activities. Using the organotypic (raft) epithelial tissue system, we defined the specific effects of HSPs induction have on HPV viral activities including virus production, viral genome replication, and viral capsid protein expression and localization. Also, using Ad-mediated gene transfer, we determined the impact of specific HSPs expression on HPV viral replication and virus production.

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A. Heat shock induces expression of HSPs in HPV-infected cells. HSPs are expressed in response to heat stress and other biological insults (reviewed in (104)). To investigate the roles for HSPs in the HPV life cycle in host keratinocytes, we first strove to define the expression patterns and longevity of HSP induction following heat stress of HPV-infected epithelial tissues. 9E rafts were grown and subjected to heat shock as shown in Table 1. Each harvest and assay point consisted of halves from four individual raft tissues to account for biological and experimental variability. Groups of raft tissues were heat shocked after lifting to the air-medium interface in single- (day 6), double- (days 6, 8), and triple- (days 6, 8, 10) dose fashions, and harvested at day 6, day 10, or day 14. Each assay point included non-heat shock rafts as controls. Total proteins were extracted and analyzed by SDS-PAGE and immunoblot with a panel of HSP antibodies. The data show heat shock induced HSP70i and to a lesser degree HSP40 protein levels at all stages of epithelial differentiation, with HSP70i most highly induced and sustained in a heat shock dose-dependent manner (Fig. 1). The levels of HSP60 and HSP90 increased only slightly, if at all, upon heat shock. This experiment was performed with three separate raft groups and demonstrated similar results.

Table 3.1 Schedule of heat shock[1] and harvest for assay of virus life cycle.

Day post lifting[2]:	6	8	10	14[3]
Group 1				
Group 2	HS[1]			
Group 3	HS	HS		
Group 4	HS	HS	HS	

[1] Heat shock is at 43°C for 90 min, denoted by HS 4 h p rior to harvest.
[2] Days after lifting to the air-liquid interface, which is considered day 0.
[3] Virus production is an endpoint assay and was only performed on day 14.
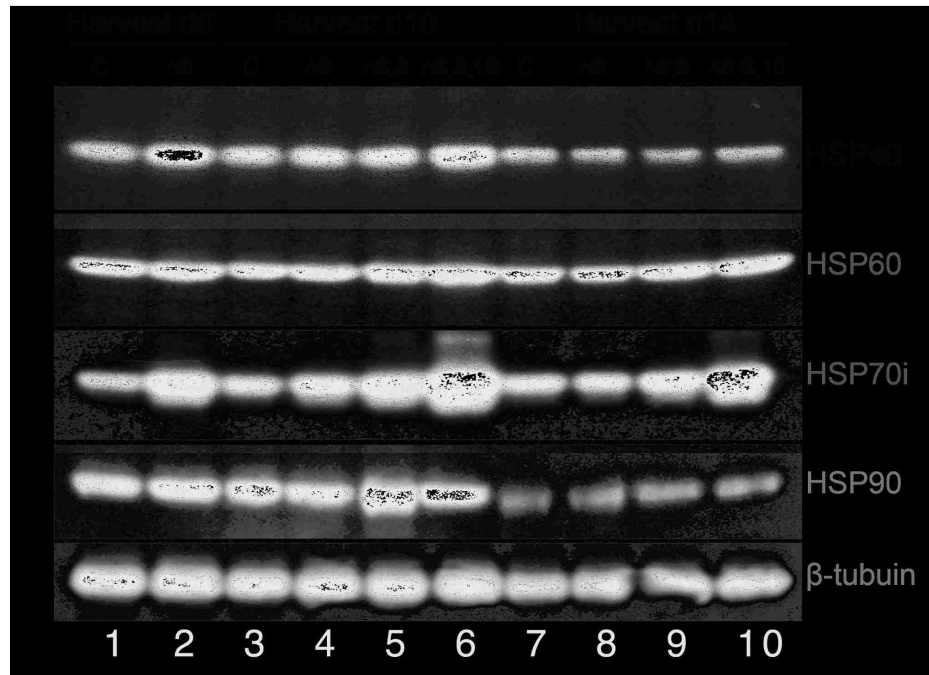
Figure 3.1. HSP levels are induced by heat shock in HPV31-infected raft tissues.
9E raft epithelial tissues were subjected to heat shock (Δ) at 43°C for 90 min on day 6,
days 6 and 8, days 6, 8 and 10, or not at all (C). One-half of four individual rafts from
each group were harvested on day 6, day 10, or day 14 after lifting to air-medium
interface with indicated heat shock treatments. Total proteins (80 µg) from each set
were analyzed by SDS-PAGE and immunoblot with primary antibodies to the proteins
indicated. A representative immunoblot is shown for one of three individual replicate
experiments performed.

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B. HPV virion production is augmented coincident with heat shock and increased HSP levels. To delineate the effects of heat shock and induction of HSPs on HPV31 life cycle activities, four sets of raft tissues were differentially exposed to heat shock and analyzed for virion production (Table 3.1). Each group consisted of 10 raft tissues; a control group was not exposed to heat shock, and the other groups were exposed to one, two, or three heat treatments, on day 6, days 6 and 8, or days 6, 8 and 10, respectively. All four groups were harvested at day 14 after lifting to the air-liquid interface. Virions were isolated and the virion preparations were quantified for viral DNA containing particles to reflect the number of VGE (162, 163). To compare virion production among the four differentially heat shocked tissue groups, the numbers of VGE per raft were normalized to the untreated control, which was set to 1. This experiment was performed three separate times reflected in the data shown in Fig. 3.2 A, and revealed that HPV virion production increased in a heat-shock and HSP induction level-dependent manner. Compared to the untreated control, heat shock and HSP induction enhanced HPV virion production as much as ≈10 fold under the conditions described. Virion preparations conferred equivalent infectivity levels based on VGE dosages in human HaCaT keratinocytes as we have reported (162, 163) (data not shown).
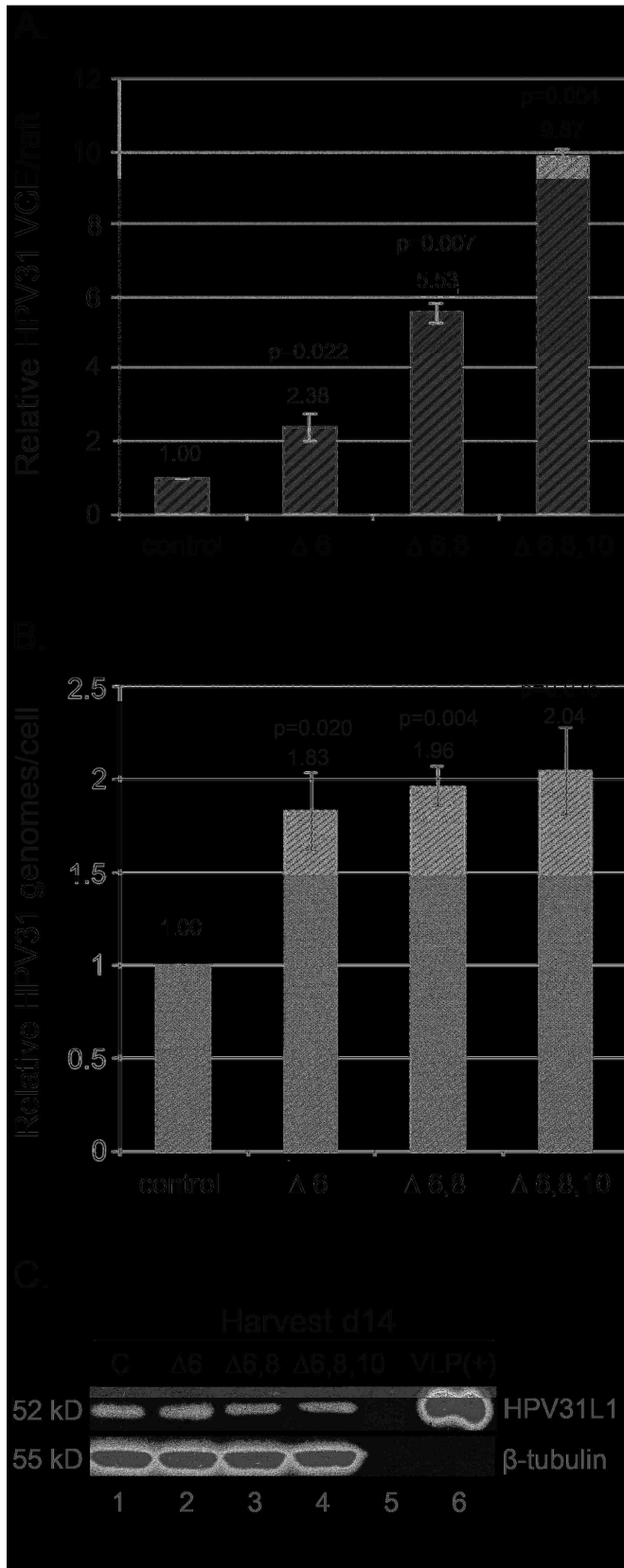
Figure 3.2. Heat shock and induction of HSPs leads to dose-dependent increases in HPV31 replication activities in 9E epithelial tissues. Heat shock (Δ) treatments were as indicated and as shown in Table 1. In each case the control (C) rafts were not exposed to heat shock and all raft tissues were harvested on day 14 after lifting to the air-liquid interface. **(A)** Virion production was quantified in each group to reflect the number of VGE per raft normalized to the control group. The control rafts typically yielded ~10^7 VGE per raft. **(B)** Total HPV31 genome levels in raft tissues were determined by DNA dot blot hybridization using GAPDH gene detection as a loading control. Results were normalized to the control group. **(C)** Raft total protein was extracted and analyzed by SDS-PAGE and immunoblot with primary antibodies shown respectively. HPV31 VLPs were used as a positive control for L1 protein. For A & B the results represents the means ± standard errors of the means (SEM) from three individual replicates of each experiment.

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C. HPV genome amplification increases in response to heat shock and induction of HSPs. To determine the viral basis for the increased HPV virion production, we next investigated whether there was an increase in HPV genome replication. Total DNA was harvested from raft tissues with or without heat shock in the same process as described above. Southern blot and dot blot on the total DNA of the rafts was used to quantify the total HPV genome copies per raft, which were normalized to non-heat shocked controls for each group. We found that heat shock and HSP70i level induction coincided with increased HPV genome copies per cell in raft tissue in a dose dependent manner (Fig. 3.2 B). Yet, compared to total virion levels that increased up to $\approx$10 fold (Fig. 3.2 A), heat shock only resulted in HPV genome level increases up to ~2-fold (Fig. 3.2 B). Although the increase was statistically significant, the data suggest heat shock-enhanced virion production involves more than an increase in viral genome replication.

D. The heat shock response does not influence total HPV major capsid protein L1 expression levels. As the HPV virion is composed of L1 and L2 capsid proteins and the viral genome, we next assayed for levels of the major HPV capsid protein in total raft lysates by SDS-PAGE and immunoblot assay. The results demonstrated that total L1 protein expression was not detectably affected by the heat shock response in differentiated tissues (Fig. 3.2 C),

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indicating that the ~10 fold increase in virion production was not simply due to enhanced capsid protein expression.
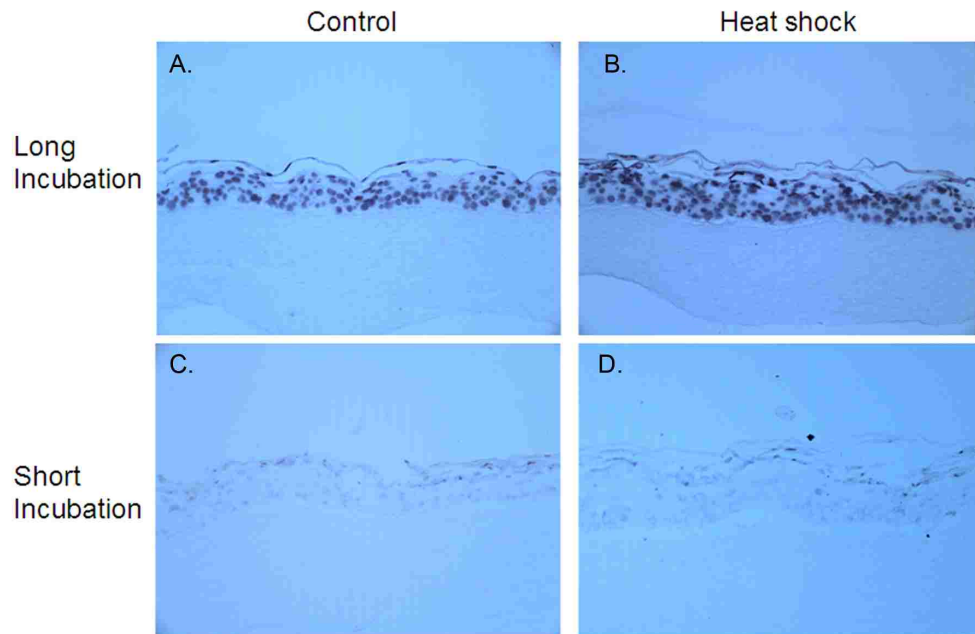
Figure 3.3. Heat shock results in enhanced HPV31 viral genome in all layers of 9E raft tissues. Raft tissues were grown under normal conditions **(**control: **A, C)** or with heat shock exposure on days 6, 8, and 10 **(B, D)** Tissues were harvested after 14 days at the air-medium interface, fixed, and cross sections were subjected to ISH staining for HPV31 genome. Long incubation **(A, B)** and short incubation **(C, D)** indicate overnight or 1 hour incubation time applied during the ISH process.

E. The heat shock response does not influence viral genome levels *in situ*.

To delineate the expression patterns of the HPV genome among various layers of epithelium throughout the raft tissues, *in situ* hybridization assays were

performed. The results demonstrated that HPV31 viral genome expression appeared to be slightly increased by the heat shock and HSPs induction response in differentiated tissues (Fig. 3.3), in agreement with the dot blot results shown previously (Fig. 3.2 B). However, there were no obvious differences among cell layers in both control and heat shocked tissues, suggesting that the ~10-fold increase in virion production was not due to higher enhanced viral genome levels in the top layer where viral morphogenesis occurred.
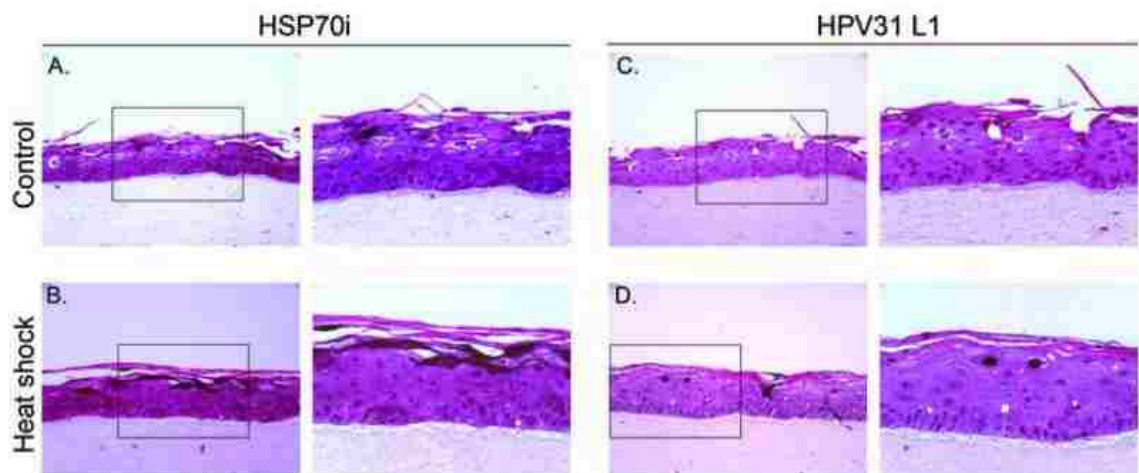
Figure 3.4. Heat shock results in increased detection of HSP70i and HPV31 L1 in upper differentiated layers of 9E raft tissues. Raft tissues were grown under normal conditions **(**control: **A, C)** or with heat shock exposure on days 6, 8, and 10 **(B, D)** Tissues were harvested after 14 days at the air-liquid interface, fixed, and serial cross sections were subjected to IHC staining for HSP70i **(A, B)** and HPV L1 protein **(C, D)**. Right panels are higher magnifications of boxed areas in left panels.

F. HSP70i and L1 proteins colocalize in the nucleus in upper epithelial layers. HSP70 is important for many viral activities (38, 42, 43, 125, 126, 129, 169, 206), and we find HSP70i to be the most highly induced and sustained in a heat shock dose-dependent manner in differentiating raft epithelial tissues. Therefore, we focused on HSP70i as a possible key mediator of enhanced HPV virion production. HSP70i expression patterns were investigated in the epithelial tissues following heat shock as described above. IHC staining performed in histological tissue sections showed strong HSP70i expression in all tissue layers. Although all heat-treated tissues showed higher HSP70i levels compared to the control, there were no obvious differences among the heat-shocked groups (data not shown). Yet, reduced primary antibody incubation time revealed HSP70i expression to be the greatest in the cytoplasm and nucleus of cells of the upper tissue layers (Fig. 3.4 A, B). This upper highly differentiated tissue layer is where HPV late proteins accumulate and virion morphogenesis occurs (140, 142). Using tissue sections in serial to those detecting HSP70i, IHC staining for the HPV L1 major capsid protein demonstrated higher detectable nuclear L1 levels in the same layer of cells where high levels of HSP70i were detected (Fig. 3.4 C, D). The serial nature of the sections suggested that the HSP70i and L1 proteins were colocalized in those differentiated cells. To more directly investigate the relationship between HSP70i and L1 localization, we performed co-IF staining within the same tissue sets. Results showed that L1 detection was low and diffuse in control rafts not exposed to heat shock (Fig. 3.5 A, B; B arrowhead and

inset) but was more easily detected in the upper layers of tissues exposed to heat shock (Fig. 3.5 C-E). Whereas HSP70i was detected throughout the tissue layers regardless of heat treatment, HSP70i levels were increased in the nuclei and upper tissue layers upon heat shock (compare panels A and C in Fig. 3.5). Furthermore, L1 detection became concentrated in the nuclei of differentiated cells where HSP70i levels were highest after heat shock, and cells with the highest levels of L1 protein had substantial co-localization of L1 with HSP70i (Fig. 3.5 D, E). These data taken with those in Fig. 3.4 and Fig. 3.2 C, suggest that increased L1 nuclear detection is due to the localized concentration, not because of higher total L1 levels. However, it is difficult to count out the possibility that a few cells in the upper layers with higher local HSP70i levels express more L1 protein, which is undectable by immunoblot of whole raft tissue lysates with an averaging effect among tissue layers.
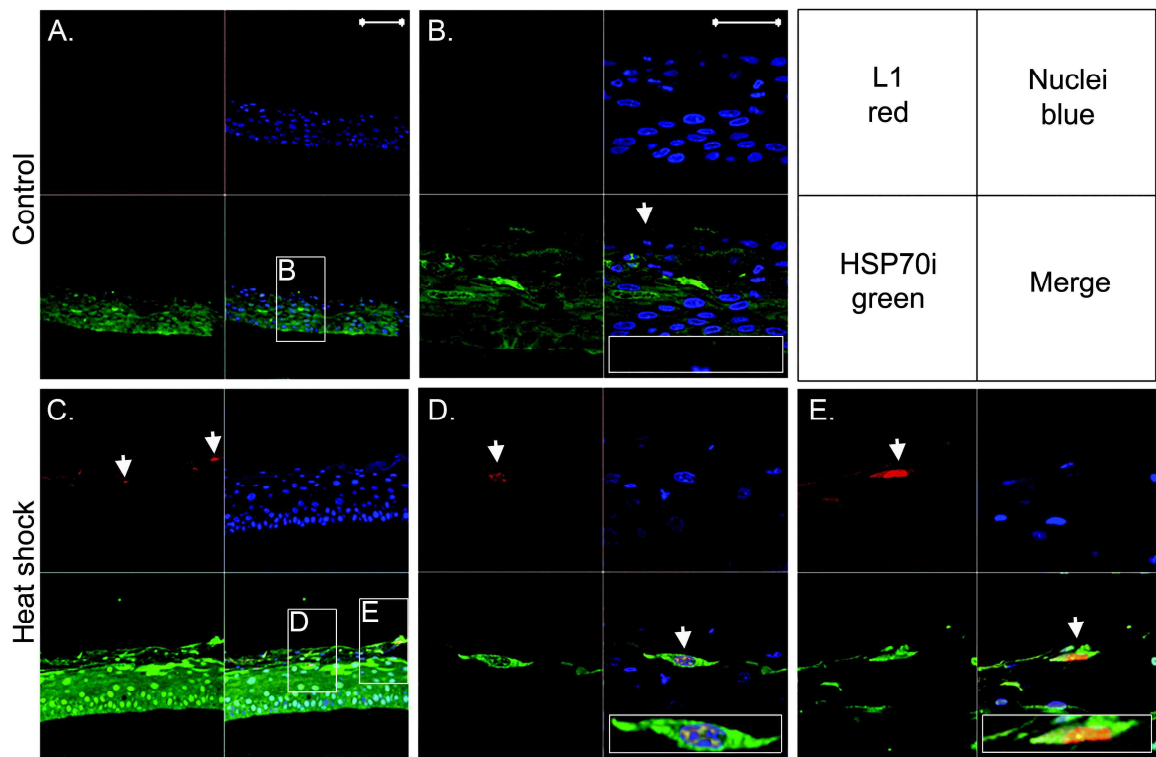
Figure 3.5. Colocalization of HSP70i and HPV31 L1 in 9E raft tissues upon heat shock. Raft tissues from control **(A,B)** and heat shocked sets **(C, D, E)** as in Fig. 3.4 were deparaffinized and stained by IF. The panel key is shown in the upper-right corner: L1 detection (red), DAPI stained nuclei (blue), HSP70i (green), and three-image merge. The areas boxed in panels A and C are shown at higher magnification as indicted in panels B, D, and E. Scale bar in A is 100 µm, scale bar in B is 50 µm. Arrowheads mark cells with positive L1 staining.

G. Ad-mediated gene transfer results in increased HSP70i expression in raft tissues and enhanced HPV31 activities. Heat shock is a complicated and multi-dimensional response. Therefore, to more precisely address our hypothesis that HSP70i plays a main role in enhancing HPV31 replicative activities, we employed Ad-mediated gene transfer to specifically transduce HSP70i into 9E cells. After optimization to obtain ~100% transduction efficiency in 9E monolayer cells (data not shown), monolayer cells atop collagen matrices

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were Ad-vector exposed 24 hours prior to lifting to the air-medium interface. Cells were infected at various MOI of Ad-HSP70i (AP70) or Ad-vector only (Ad-v), or were uninfected as controls. Raft tissues were harvested at 2, 8, and 14 post lifting for total protein, total DNA, histology, and on day 14 for virion production. Immunoblot showed that HSP70i was successfully transduced and maintained specifically without obvious effects on other HSP expression levels (HSP40 is shown as a representative), and HSP70i expression levels were dependent on the MOI of Ad-mediated gene transfer (Fig. 3.6 A). IHC showed increased HSP70i detection in the cytoplasm and the nucleus of the upper epithelial layers.   Concurrently, enhanced L1 staining was observed in the nucleus of differentiated cells (Fig. 3.6 B), in a pattern similar to that observed in heat shocked raft tissues (Fig. 3.4). IF staining in AP70 transduced rafts also showed colocalization of HSP70i and L1, similar to that in heat shocked rafts, albeit at lower levels (Fig. 3.6 E-F). Additionally, increased HSP70i expression correlated with enhanced HPV virion and genome levels in rafts, without detectable affecting L1 protein levels, similar to what we observed with heat shocked rafts (Fig. 3.7). These data further corroborate our hypothesis that HSP70i positively regulates HPV genome replication and L1 localization activities in the epithelial rafts tissues.

Figure 3.6. Effects of adenovirus-mediated HSP70i expression on HPV31 L1 expression and localization in 9E rafts. (A) Total protein immunoblot for HSP70i and tubulin as a loading control. **(B)** IHC for HSP70i and L1. AP70 represents Ad-HSP70i, Ad-v represents Ad vector only. Inserts demonstrate the amplified arrow pointing area. **(C-G)** Confocal microscopy on deparaffinized raft tissues mock transduced **(C, D)**, transduced with AP70 **(E, F)**, or control transduced with Ad-v **(G)** and stained by IF. The panel key is shown in the upper-right corner: L1 detection (red), DAPI stained nuclei (blue), HSP70i (green), and three-image merge. The areas boxed in panels C and E are shown at higher magnification as indicted in panels D and F respectively. Scale bar in A is 100 µm, scale bar in B is 50µm. Arrowheads mark cells with positive L1 staining.

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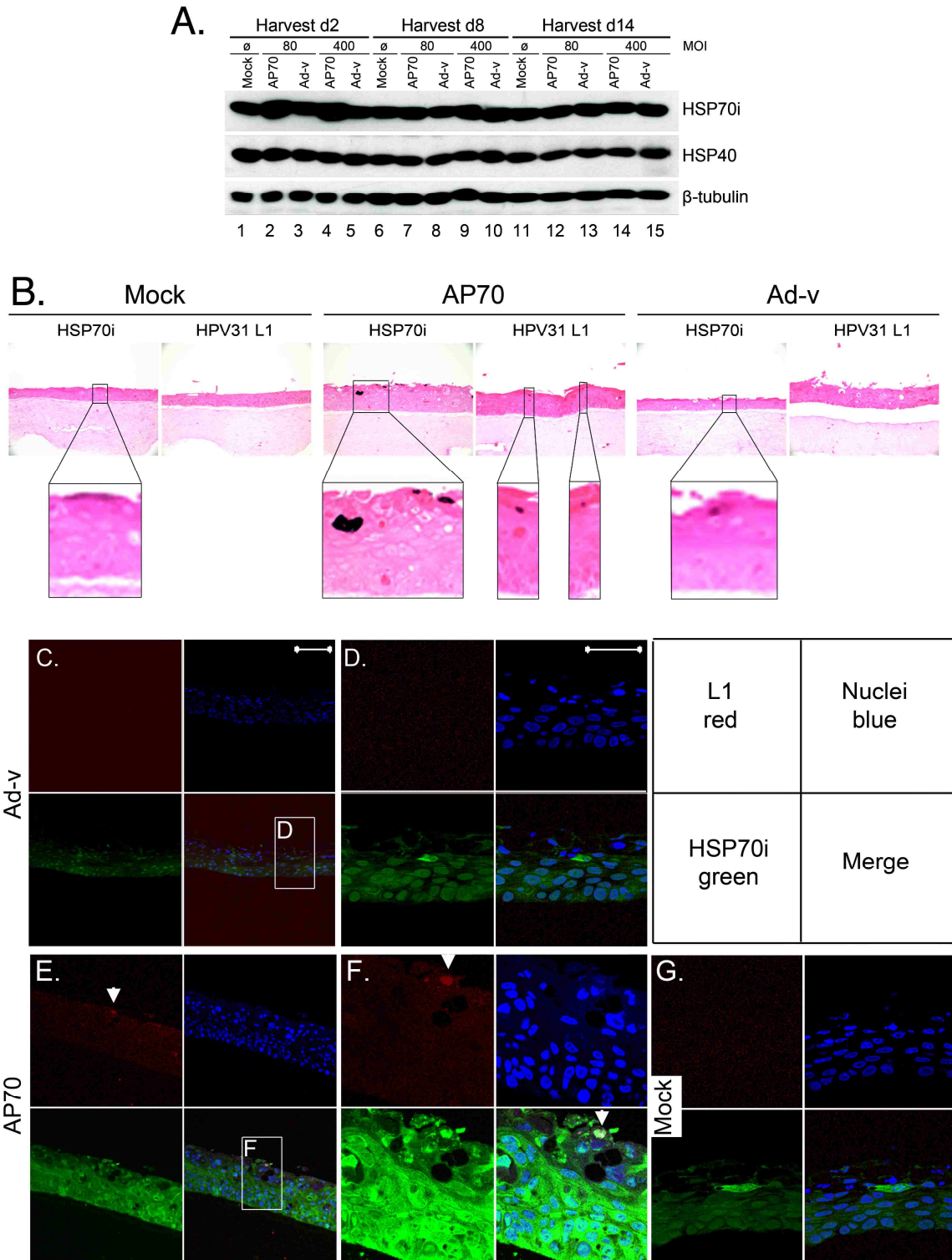
Figure 3.6. Effects of Ad-mediated HSP70i expression on HPV31 L1 expression and localization in 9E rafts. See figure legend previous page.
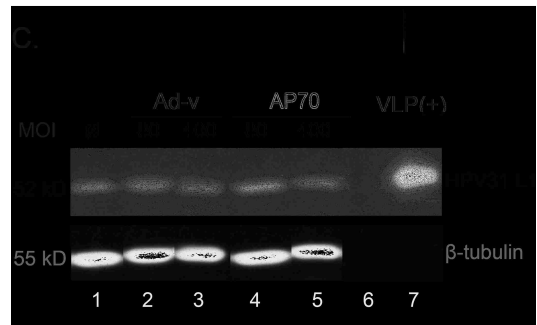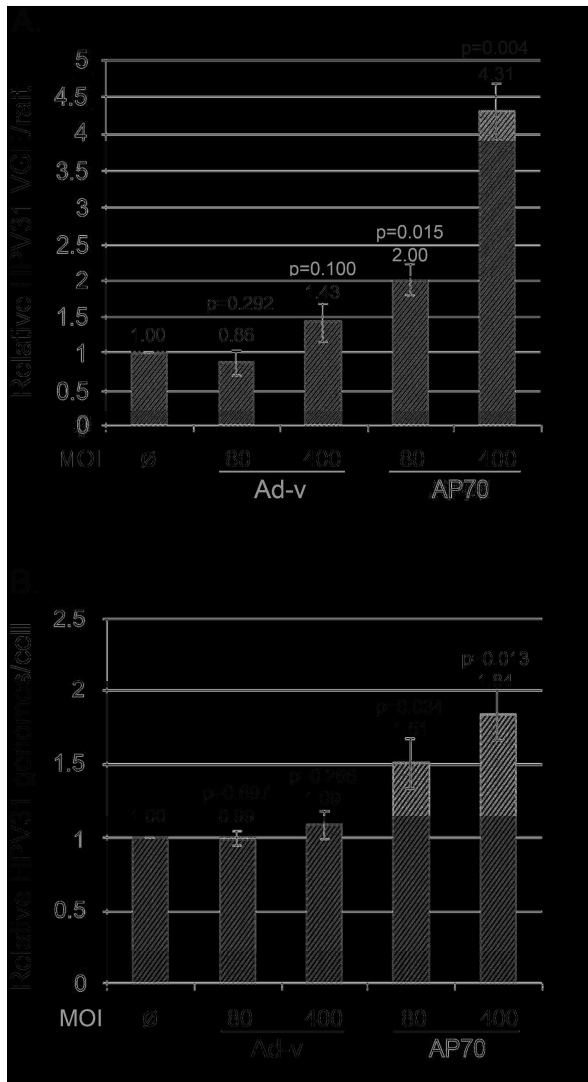
Figure 3.7. Effects of Ad-mediated HSP70i transfection on HPV31 viral activities in 9E raft tissues. (**A)** For virion production propose, rafts tissue were harvested on day 14 only, virion relative quantification (numbers shown on each bar) was determined by dot blot hybridization reflecting VGE per raft normalized to mock. (**B**) HPV31 DNA was determined by total DNA dot blot hybridization using GAPDH gene as internal loading control and normalized to mock. (**C**) Raft total protein was extracted and analyzed by SDS-PAGE and immunoblot with primary antibodies shown respectively. HPV31 VLP as a positive control for L1 protein detection. For A&B, the results represent the means ± SEM from three individual replicate experiments.

H. HSP70i directly interacts with L1. To determine whether HSP70i directly binds with HPV31 capsid protein during viral life cycle activities in differentiated epithelium, co-IP experiments were performed. Because a low fraction of the differentiated cells in raft tissues are permissive for HPV capsid protein expression, HSP70i-L1 co-IP assay requires the analysis of unusually high

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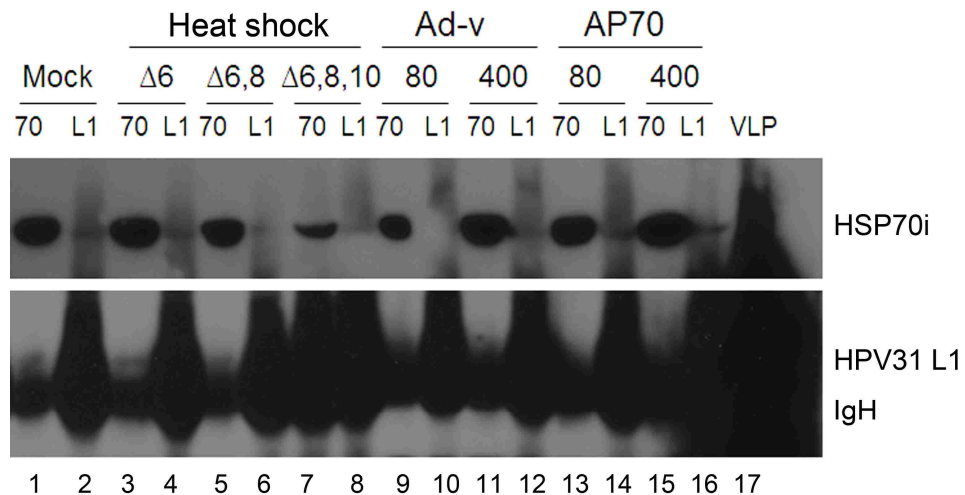
Figure 3.8. Association of HSP70i with HPV31 L1 in 9E raft tissues. Raft total protein was extracted and 800 µg analyzed by IP, SDS-PAGE and immunoblot with primary antibodies shown respectively. Mock: non heat shock non Ad-transduction control, Ad-v: Ad-transduction control, AP70: HSP70i transduction. 70: pull down with rabbit anti-HSP70i, L1: pull down with rabbit anti-HPV31 VLP, IgH: Ig heavy chain. Mouse anti-HSP70i MAb (SPA-810) and mouse anti-L1 MAb (Abcam) were used for detection. HPV31 VLPs were used as a positive control for L1 protein.

amounts of total protein from raft tissue protein extracts. We can detect HSP70i-L1 interactions starting with 800 µg of total raft tissues (Fig. 3.8). In untreated, heat shocked and Ad-transduced 9E rafts, a fraction of HSP70i proteins consistently co-IP with HPV31 L1 (Fig. 3.8, even-numbered lanes). Due to the need to use a large amount of antisera to co-IP detectable L1 and HSP70i proteins, and the similar size of L1 monomers and Ig heavy chains (50-55 kDa), L1 detection is obscured in some samples by the Ig heavy chain running slightly below the L1 protein. Nevertheless, L1 can clearly be seen co-IP with HSP70i in untreated and heat shocked raft lysates (Fig. 3.8, lanes 1, 3, 5).

In summary, our results revealed that increased HSP70i protein levels enhanced HPV31 virion production, genome amplification and L1 nuclear localization with no detectable effect on L1 expression levels. These findings support our hypothesis of HSP70i involvement in the HPV viral life cycle activities in an authentic replicative environment. Yet, questions arose from these results. Concurrent with increased HSP70i expression, what additional factors contribute to the 10-fold increase in progeny virions besides the ~2-fold viral genome increase? What mechanisms control L1 localization during virion morphogenesis? Certain findings are intriguing, including that L1 directly interacts with HSP70i, L1 proteins are significantly concentrated into the nucleus and colocalized with the increased HSP70i. Therefore, we hypothesize that in addition to the 2-fold viral genome increase, HSP70i interacts with L1 and promotes its re-localization from the cytoplasm into the nucleus, thereby contributing to the 10-fold increase in progeny virus morphogenesis.

Chapter 4

Nuclear accumulation of HPV31 major capsid protein L1 is enhanced

significantly by HSP70i and is dependent upon the ATPase domain

In Chapter 3 we concluded that HSP70i is involved in HPV viral life cycle activities including viral genome replication and progeny viron morphogenesis in an authentic replicative environment. Our goal in this section was to reveal the mechanism of HSP70i's contribution to these viral activities. HPV L1 capsid protein contains information needed for capsid morphogenesis and thus can self-assemble into VLPs *in vivo* and *in vitro* (39, 40, 109, 123, 187, 204, 210). Under natural replicative conditions, only capsid assembly that occurs in the nucleus and packages L2 and viral genomes can produce infectious progeny HPV virions. Therefore, L1 has to be expressed and localized into the nucleus to function in virion synthesis. During this process, L1 expression and localization can be the limiting steps in viral life cycle. The results from Chapter 3 indicate that increased HSP70i expression concurs with higher L1 in nuclei while total L1 levels appear not to be affected. To more directly investigate the impact of HSP70i expression on L1 expression and localization, we adapted the 293TT-HPV virion assembly system (178) to HPV31 morphogenesis in human keratinocytes using the 9E cell line. In brief, 9E monolayer cells are transfected with an expression plasmid for HPV31 L1/L2 and virion particles are produced. We termed this system as 9E-L1/L2 monolayer cell culture system and utilized it to investigate HPV31 viron packaging activities without the complexity of differentiation.

A. HPV31 virion production is enhanced with increased HSP70i expression.

HPV virions were isolated from 9E-L1/L2 monolayer cell culture system by CsCl centrifugation in which purified DNA containing viral particles can be obtained.

The virion preparations were quantified for viral DNA containing particles to reflect the number of VGE per plate by a protocol developed previously (162, 163). To compare virion production among the different Ad-transducted groups, the numbers of VGE per plate were normalized to the untreated control, which was set to 1. This experiment was performed three separate times reflected in the data shown as Fig. 4.1 A. The results revealed that HPV virion production increased in an HSP70i level-dependent manner. Compared to the untreated control and the Ad-v control, HSP70i transduction enhanced HPV virion production as much as ~11 fold under the conditions described, similar to that in the heat shocked raft tissues. Virion production in the Ad-v control groups demonstrated no differences from mock-transduced controls suggesting that the virion production enhancement was not derived from Ad-transduction.

B. HPV31 viral genome replication is augmented with increased HSP70i expression. Total DNA was harvested from 9E-L1/L2 cells with various Ad transductions as described above. Southern blot and dot blot hybridization were used to quantify the total HPV genome copies per plate, which were normalized to non-transduced controls. We found that increased HSP70i expression levels coincided with increased HPV genome copies per cell in a dose dependent manner (Fig. 4.1 B). Yet, compared to total virion levels that were enhanced up to ~11-fold (Fig. 4.1 A), increased HSP70i only resulted in HPV genome level augmentation up to ~4-fold (Fig. 4.1 B). Although the increase was statistically

significant, the data suggest HSP70i enhanced virion production involves more than simply an increase in viral genome replication.

A.

B.

C.
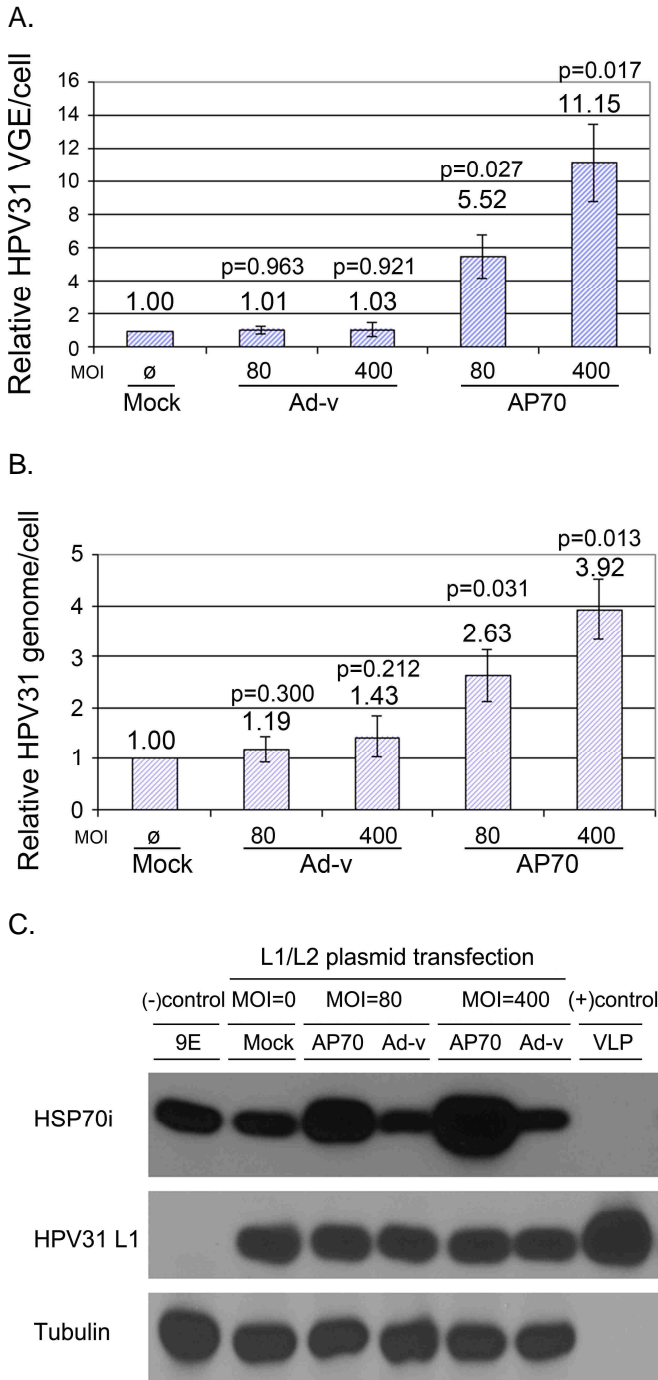
Figure 4.1. Effects of Ad-mediated HSP70i transduction on HPV31 activities in 9E-L1/L2 monolayer system. (A) Relative virion quantification (average numbers shown on each bar) was determined by dot blot hybridization reflecting the numbers of VGE per 100 mm cell culture dish normalized to mock tranduced controls. **(B)** HPV31 viral DNA relative quantification was determined by total DNA dot blot hybridization using GAPDH gene as internal loading control and normalized to mock transduced controls. **(C)** Total protein was extracted and 40 µg analyzed by SDS-PAGE and immunoblot with primary antibodies to the proteins as indicated. HPV31 VLP was used as a positive control for L1 protein. The values in A & B represent the mean ± SEM from each of three individual replicate experiments.

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C. HSP70i expression does not readily influence total HPV major capsid protein L1 expression levels. Total protein samples extracted from 9E-L1/L2 monolayer cells were analyzed by SDS-PAGE and immunoblot with anti-HPV31 L1 antibody (Abcam), anti-HSP70i antibody (SPA-810). Anti-β-tubulin was used as a loading control. Compared to the mock non-tranduction control, HPV31 L1 expression levels were not influenced by various Ad-transductions or with increasing HSP70i protein levels (Figure 4.1 C), indicating that the ~11-fold increase in virion production was not due to enhanced L1 capsid protein expression.
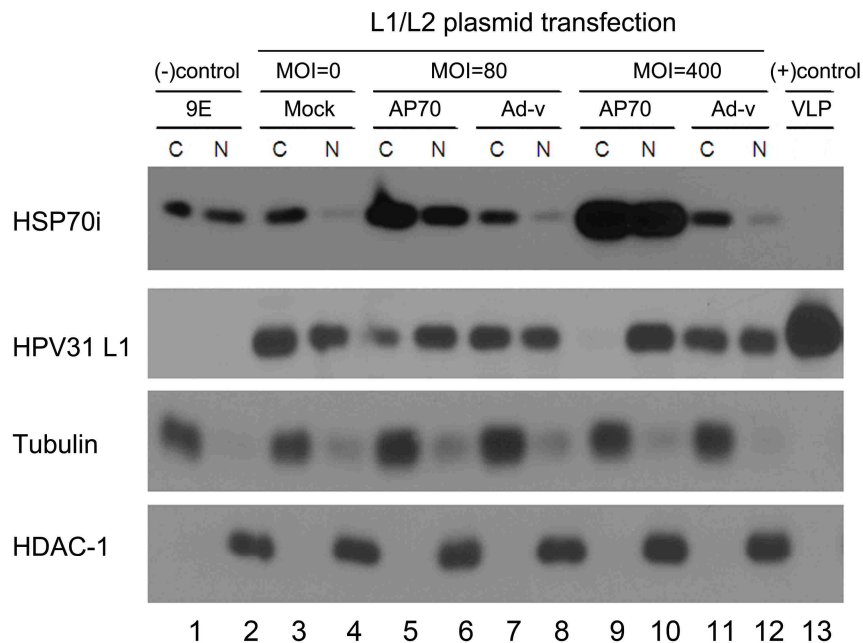
Figure 4.2 Effects of Ad-mediated HSP70i expression on HPV31 L1 protein cellular localization. 9E-L1/L2 monolayer cell cytoplasmic (C) and nuclear (N) fractionated proteins were extracted and 20 µg analyzed by SDS-PAGE and immunoblot with primary antibodies to the proteins indicated. Tubulin was used as cytoplasmic protein indicator, HDAC-1 was used as nuclear protein indicator, and HPV31 VLPs were used as a positive control for L1 protein.

D. HPV31 major capsid protein L1 relocalization from cytoplasm into nucleus is enhanced by HSP70i expression. IHC and IF data from raft tissue analyses in Chapter 3 showed L1 protein was concentrated into the nucleus under increased HSP70i conditions. Our attempts to separate cytoplasmic and nuclear protein fractions from raft tissue were unsuccessful (data not shown). However, clean cytoplasmic and nuclear fractionation was achieved in protein lysates from the 9E-L1/L2 monolayer cell system. Samples were analyzed by SDS-PAGE and immunoblot with anti-HPV31 L1 antibody (Abcam), anti-HSP70i antibody (SPA-810). Tubulin detection was utilized as cytoplasmic protein indicator and HDAC-1 was utilized as nuclear protein indicator (Fig. 4.2). 9E cells without L1/L2 plasmid transfection were utilized as a negative control, wherein L1 was undetected and HSP70i was present in both the cytoplasm and the nucleus (Fig. 4.2, lanes 1-2). In 9E cells with L1/L2 transfection, the mock without Ad-transduced as well as the Ad-v controls had L1 proteins in both the cytoplasm and the nucleus and HSP70i was mainly detected in the cytoplasm (Fig. 4.2, lanes 3-4, 7-8, 11-12). With increased HSP70i expression, more L1 was detected in the nucleus and a higher proportion of HSP70i was also found in the nucleus (Figure 4.2, compare lanes 3-4 to lanes 5-6, 9-10). The Ad-v transduction control demonstrated the same patterns as the mock transduced control (Figure 4.2, compare lanes 3-4 to lanes 7-8, 11-12), suggesting that the Ad-transduction alone had no effect on L1 or HSP70i localization or levels. These results illustrated that the L1 protein was re-localized from the cytoplasm

into the nucleus in an HS70i expression dose-dependent manner, although total protein immunoblots showed no detetable change of total L1 expression levels (Fig. 4.1 C).   The data in Fig. 4.1 and Fig. 4.2 suggest that, in addition to augmenting vDNA levels, HSP70i promotes L1 transport into the nucleus and thus appears to contribute to the enhanced virion production.

A.

B.

C.
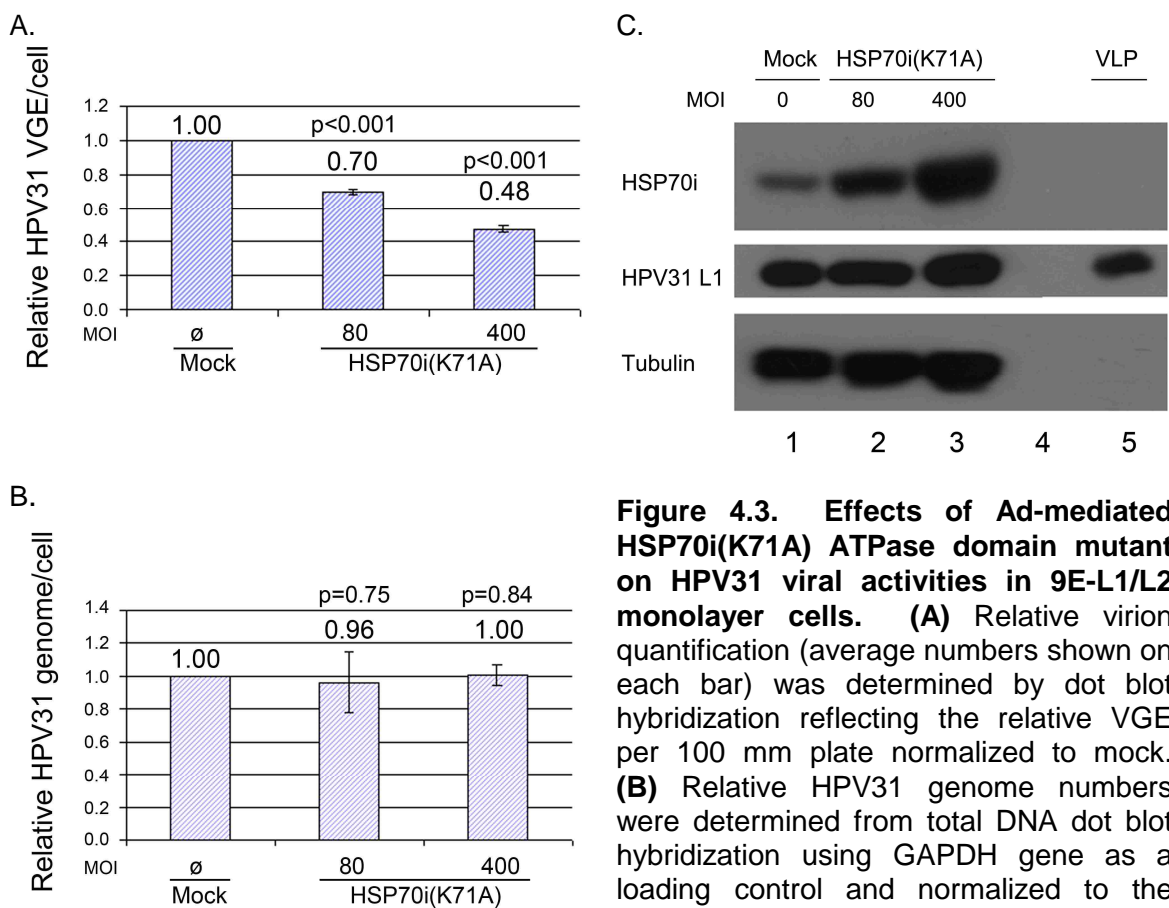
Figure 4.3.   Effects of Ad-mediated HSP70i(K71A) ATPase domain mutant on HPV31 viral activities in 9E-L1/L2 monolayer cells.   (A) Relative virion quantification (average numbers shown on each bar) was determined by dot blot hybridization reflecting the relative VGE per 100 mm plate normalized to mock. **(B)** Relative HPV31 genome numbers were determined from total DNA dot blot hybridization using GAPDH gene as a loading control and normalized to the mock transduced.   **(C)** Total protein was extracted and analyzed by SDS-PAGE and immunoblot with primary antibodies shown.   HPV31 VLPs were used as a positive control for L1 protein.

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E. HSP70i(K71A) ATPase domain mutant inhibits HPV31 virion production without obvious impact on vDNA and L1 levels. To investigate the effect of HSP70i expression and functional inhibition on HPV31 activities, we first tried an HSP70i inhibitor (KNK437), which was reported to specifically inhibit HSP70i expression in other cell lines, such as HeLa cells, under stress conditions (112, 113, 224). However, our results indicated that KNK437 did not inhibit the expression and induction of HSP70i in 9E cells (data not shown). Therefore, we tested the HSP70i(K71A) ATPase domain mutant to assay the impact on viral activities in the 9E-L1/L2 monolayer cell system using the same Ad-transduction method. The K71A mutant loses its ATP binding properties while retaining native ADP-binding, therefore it binds substrates at a higher affinity and not to release the substrates as does wild type HSP70i (11, 221). Virions were isolated by CsCl centrifugation and the virion preparations were quantified for viral DNA containing particles to reflect the number of VGE per plate as described above (162, 163). To compare virion production with increasing HSP70i(K71A) expression levels, the numbers of VGE per plate were normalized to the untreated control, which was set to 1. This experiment was performed three separate times, average results are shown with SEM (Fig. 4.3 A). The data revealed that HPV virion production was significantly inhibited in an HSP70i(K71A) level-dependent manner. Compared to the mock-transduced control, HSP70i(K71A) transduction inhibited HPV virion production as much as ~55% under the conditions described. Southern blot and dot blot hybridization

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on the total DNA was used to quantify the total HPV genome copies per plate, which were normalized to GAPDH and non-transduction controls. Cells expressing the HSP70i(K71A) mutant demonstrated no differences in viral genome levels (Fig. 4.3 B). Total protein samples were analyzed by SDS-PAGE and immunoblot to detect HSP70i(K71A) mutant and L1 expression. Our data revealed that increasing HSP70i(K71A) expression had no discernable impact on total L1 protein levels (Fig. 4.3 C). The results illustrated in Fig 4.3 imply that the decreased virion production was not due to lower viral genome or L1 protein levels.

F. HSP70i(K71A) ATPase domain mutant fails to promote nuclear accumulation of HPV31 L1. To investigate the mechanism by which virion production was decreased with increasing HSP70i(K71A) mutant expression while total vDNA and L1 levels were not affected, cytoplasmic and nuclear protein fractions were extracted from 9E-L1/L2 monolayer cell as described above. Fractionated samples were analyzed by SDS-PAGE and immunoblot for HPV31 L1 and HSP70i (Fig. 4.4). Although the HSP70i antibody does not discriminate between wild type or the K71A ATPase domain mutant, the basal levels of HSP70i can be seen in both cytoplasmic and nuclear fractions (Fig. 4.4, mock lane 1-2). Ad-transduction caused an increase in HSP70i(K71A) levels, but th proteins remained in both cytoplasmic and nuclear fractions (Fig. 4.4, lanes 3-6). In the mock transduced control, L1 was detected in both cytoplasmic

and nuclear fractions (Fig. 4.4, lanes 1-2). In contrast to the complete nuclear localization of L1 in response to high wild type HSP70i levels (Fig. 4.2, lanes 9-10), increased levels of HSP70i(K71A) had no net effect on L1 cellular distribution. These data taken with those in Fig. 4.1 C, Fig. 4.2 and Fig. 4.3 C, suggest that a functional HSP70i ATPase domain is critical for the nuclear localization of L1.

G. HSP70i and L1 proteins colocalize in 9E-L1/L2 monolayer cells. HSP70i and L1 expression patterns were investigated in the 9E-L1/L2 monolayer epithelial cells that were exposed to Ad-v, Ad-HSP70i wild type, or the Ad-HSP70i(K71A) mutant. IF for HPV31 L1 and HSP70i were performed on cells grown on chamber slides.   Results showed that L1 detection was diffuse predominaty cytoplasmic in mock transduced controls (Fig. 4.5 A) and the Ad-v transduction controls (Fig. 4.5 B, C). L1 was readily detected in the nuclei of
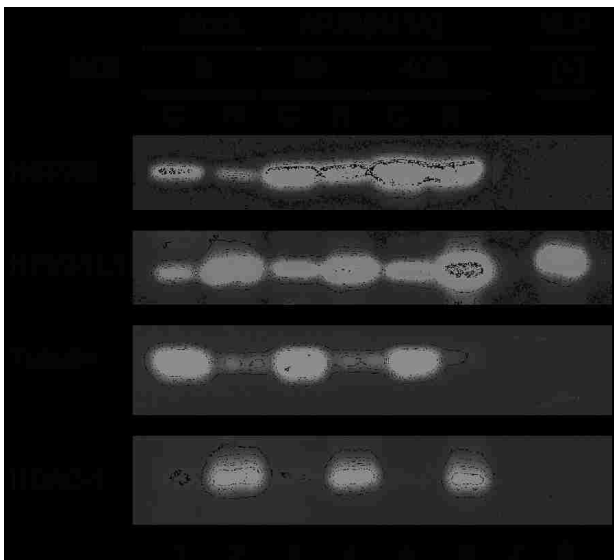
Figure 4.4. Effects of Ad-mediated HSP70i(K71A) ATPase domain mutant on HPV31 L1 protein cellular localization. 9E-L1/L2 monolayer cell cytoplasmic and nuclear protein fractions were extracted and 20 µg analyzed by SDS-PAGE and immunoblot with primary antibodies shown. Tubulin was used as a cytoplasmic protein indicator and HDAC-1 was used as a nuclear protein indicator. HPV31 VLPs were used as a positive control for L1 protein detection.

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cells expressing high levels of wild type HSP70i (Fig. 4.5 D, E). However, in cells transduced with HSP70i(K71A) mutant, L1 was dispensed in the nucleus and cytoplasm (Fig. 4.5 F, G), demonstrating same patterns as in mock and Ad-v transduction controls. These findings agree with L1 protein cellular distribution shown in Fig. 4.2 and Fig. 4.4. Furthermore, substantial co-localization of L1 with wild type HSP70i was detected in the nucleus (Fig. 4.5 D, E), whereas co-localization of L1 with HSP70i(K71A) mutant was disseminated through the nucleus and cytoplasm (Fig. 4.5 F,G). These data taken with those in Fig. 4.3 and Fig. 4.4 suggest that the HSP70i ATPase domain function is critical for the transportation and accumulation of L1 into the nucleus, and the increased L1 nuclear detection is primarily due to the localized concentration, not because of increased total L1 expression levels.

Figure 4.5 (next page). Colocalization of HSP70i and HPV31 L1 in 9E-L1/L2 monolayer cells. 9E-L1/L2 monolayer cells were stained by IF. **(A)** Non transduction mock control; **(B, C)** Ad-v transduction control; **(D, E)** Ad-mediated wild type HSP70i transduction; **(F, G)** Ad-mediated HSP70i(K71A) mutant transduction. The panel key is shown in the upper-right corner: L1 detection (red), DAPI stained nuclei (blue), HSP70i (green), and three-image merge. Scale bar in A is 50 µm. Arrowheads point to cells expressing HSP70i.

	L1 red	Nuclei blue
	HSP70i green	Merge

Mock

A.

Ad-vector

B. **C.**

Ad-HSP70i

D. **E.**

Ad-HSP70i(K71A)

F. **G.**

MOI=80   MOI=400
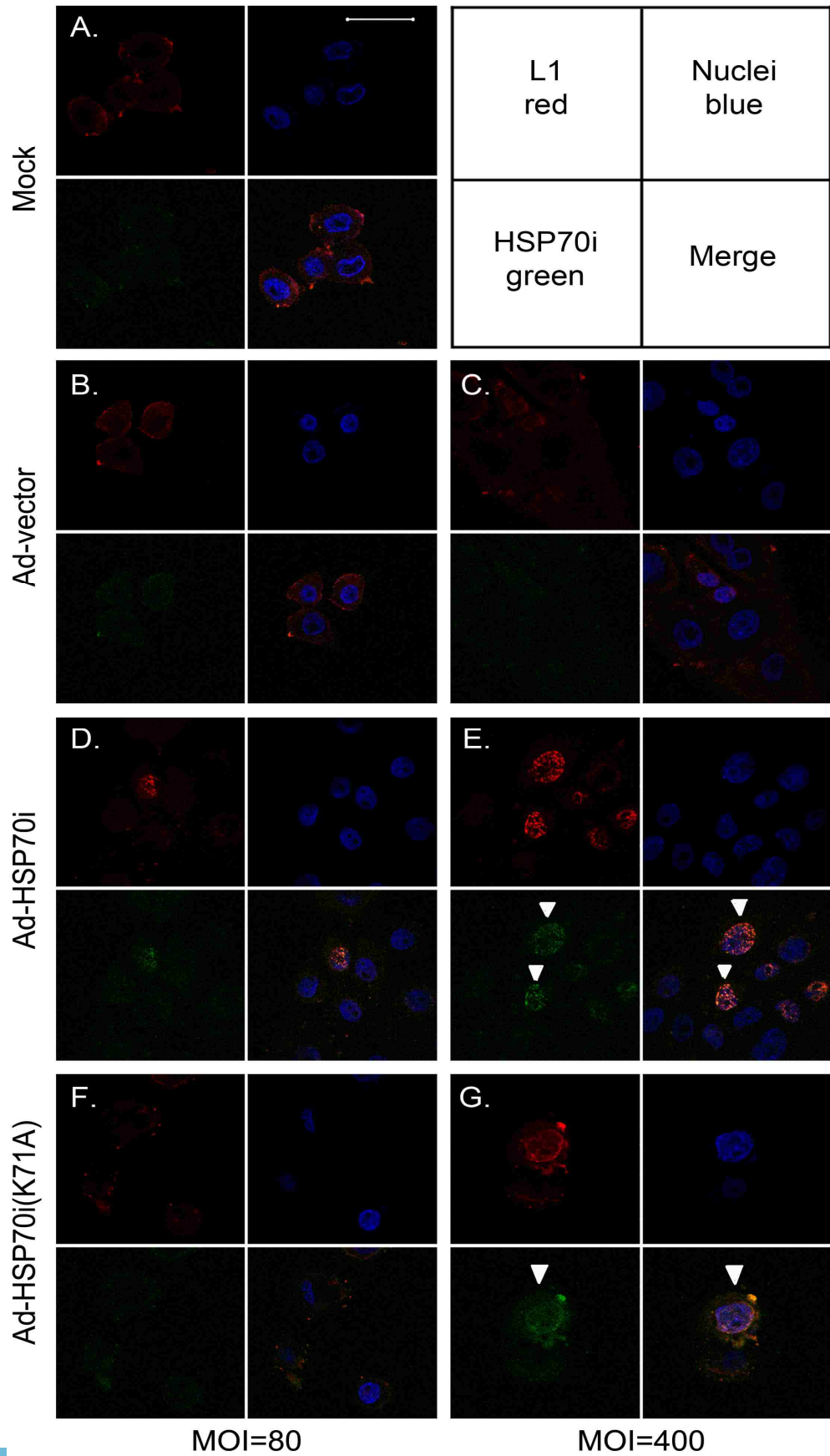
H. HSP70i directly interacts with L1. To assay for the association of HSP70i expression and distribution with HPV31 capsid protein L1, co-IP experiments were performed. Cytoplasmic and nuclear protein fractions obtained from mock transduced 9E-L1/L2 cells or Ad-transduced 9E-L1/L2 cells were analyzed by IP with either rabbit anti-HSP70i polyclonal antibody or a mouse anti-L1 monoclonal antibody cocktail (H16.J4, H16.A6, H31.V5). Mouse anti-HSP70i antibody and mouse anti-L1 antibody were used to detect proteins by immunoblot. As seen in Fig. 4.6, an association between HSP70i and L1 was detected in reciprocal Co-IPs of 9E-L1/L2 cells expressing high levels of both wild type and mutant (K71A) HSP70i (Fig. 4.6 lanes 9-17). While wild type HSP70i demonstrated reciprocal interactions with L1 dominantly in nuclear fractions (Fig. 4.6 lanes 9-12), HSP70i(K71A) mutant yielded reciprocal interactions with L1 in both cytoplasm and nucleus (Fig. 4.6 lanes 13-16). These results indicate that L1 interacts with HSP70i protein, their association is detected with increased HSP70i levels. Also, the HSP70i ATPase domain function, depicted as ATP-ADP cycle, is necessary in the L1 binding-folding-releasing cycle which leads to the L1 nuclear localization. These data together with those in Fig 4.3, Fig. 4.4 and Fig. 4.5 suggest that the HSP70i(K71A) mutant, without inhibiting viral DNA replication, impeded HPV31 virion production by limiting L1 accumulation into the nucleus.
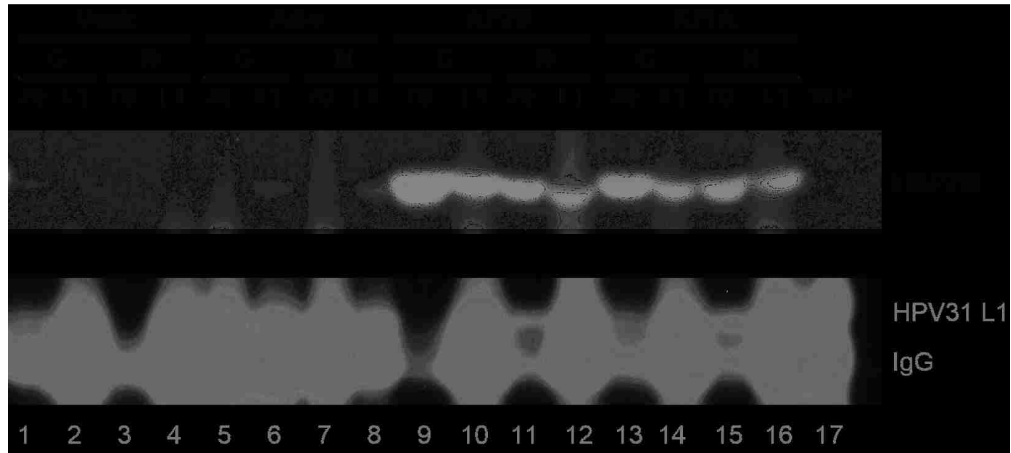
Figure 4.6. Association of HSP70i with HPV31 L1 in 9E-L1/L2 cells. Reciprocal co-IP of HSP70i and L1 using 100 µg of cytoplasmic (C) and nuclear (N) protein fractions from 9E-L1/L2 monolayer cells with Ad-transduction as indicated. Lysates were pre-cleared with protein G-agarose and subjected to IP with either rabbit anti-HSP70i antibody or mouse anti-L1 antibody cocktail (H16.J4, H16.A6, and H31.V5) as indicated atop each lane. Proteins were detected by immunoblot using mouse anti-HPS70i (Stressgen; upper panel) and mouse anti-L1 (Abcam; lower panel) and HRP-goat anti-mouse secondary antibody. A preparation of HPV31 VLPs was run as a control for L1 detection. Mock: non-transduction control, Ad-v: transduction control, AP70: HSP70i transduction, K71A: HSP70i (K71A) ATPase domain mutant transduction, IgH: Ig heavy chain.

As a summary, our data demonstrate that in the 9E-L1/L2 monolayer system, wild type HSP70i enhances HPV31 virion production, genome amplification and L1 transportation from the cytoplasm into the nucleus with no detectable effect on total L1 protein expression. These results fully support those found in raft tissues. Fractionation of proteins into nuclear and cytoplasmic components revealed that the transport of L1 protein into the nucleus is dependent on HSP70i levels and specifically the ATPase function. This is likely due to the fact that the ATPase function is critical for the ATP-ADP cycle of HSP70i and correlated substrate binding-folding-releasing activities.

Therefore, without release of L1, the HSP70i(K71A)-L1 complex can enter and exit the nucleus leading to its existence in both the cytoplasm and nucleus. As the nuclear localization of L1 is critical for the progeny virion morphogenesis, these data support our hypothesis that in addition to the enhancement of viral genome levels via a mechanism that does not involve the ATPase function, HSP70i interacts with L1, promotes its transportation from the cytoplasm into the nucleus and contributes to the augmented progeny virus production.

Chapter 5

Discussion

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A productive viral infection resulting in the morphogenesis of infectious progeny virions typically involves the coordinated synthesis of a large number of viral proteins in a relatively short time span. As an obligate intracellular parasite, the hijacking virus must then commandeer a number of cellular proteins. To help with the often rate limiting step of protein folding, many viruses utilize cellular chaperones at various stages of their life cycles (reviewed in (206)). In addition, viruses often need to restrict cellular processes such as signal transduction, cell cycle regulation and induction of apoptosis in order to create a favorable environment for their proliferation and to avoid premature cell death. Chaperones also are involved in the control of these cellular processes. HSP70 proteins are homologues of *E. coli* DnaK chaperones, and as central components of the cellular chaperone network, are frequently recruited by viruses at various life cycle stages (138). The HSP70 family members play key roles in cellular protein folding by acting as polypeptide binding and release factors that interact with non-native regions of proteins at different stages of their metabolism. HSP40 co-chaperones (DnaJ in *E. coli*) regulate complex formation between HSP70 and client proteins (71).

A handful of studies using cell-free systems or undifferentiated cells indicate that HSPs function at various steps in the life cycles of HPVs. Expression of high-risk HPV E6 and/or E7 proteins in human keratinocytes results in the up-regulation of HSP60, HSP70i, and HSP110 (120, 121, 125, 148). This suggests that the modulation of chaperone expression by HPV16

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E6/E7 proteins may be involved in HPV16 E6/E7 induced immortalization. The HPV E7 oncoprotein may have chaperone functions. The hTid-1, a human homolog of the Drosophila tumor suppressor protein Tid56 and member of the DnaJ family, has the ability to form complexes with the HPV18 E7 oncoprotein (191). The ability of HPV E7 to interact with a cellular DnaJ protein suggests that the viral oncoproteins may target common regulatory pathways through J-domains. Furthermore, E7 oligomers bind native *in vitro* translated pRb without a requirement of unfolding pRb, this high affinity of E7 to pRb action is named chaperone holdase activity (21). The ability to bind up to ~72 molecules of pRb by the E7 homogeneous spherical oligomeric particles form could be important either for sequestering pRb from Rb-E2F complexes or for targeting it for proteasome degradation (1). Thus, both the dimeric and oligomeric chaperone holdase activity forms of E7 can bind pRb and various potential targets (1). It is presently unknown whether the potential chaperone holdase activity of E7 plays an essential role in the viral life cycle; however, such an activity could explain the large number of cellular targets reported for this oncoprotein.

HSP70 chaperones have functions in the disassembly of PV and the closely related PyV in cell-free systems. After PyV entry, HSP70 and PyV VP1 proteins can be co-immunoprecipitated (42). Viral genome replication requires the viral origin recognition protein E2 and the viral replicative helicase E1. *In vitro* mixing experiments showed that HSP40 stimulates HPV11 E1 binding to the origin and promotes dihexameric E1 formation (129). The HPV E2 protein

can partially inhibit DNA unwinding, but HSP70i or HSP40 appear to displace E2 from the E1-ori complex to promote unwinding and viral genome replication (126, 129). Chaperones also appear to play an essential role in virion morphogenesis. HSP70 chaperone mediated assembly of PyV has been reported *in vitro* (43). In addition, the HSC70 binds and colocalizes with VP1 to the nucleus, suggesting a role for HSP70 family chaperones in regulating the quality and location of capsid assembly (43). In monolayer cultures of osteosarcoma cell line HuTK-143B cells, HSC70 was found to be important for nuclear translocation of ectopically over-expressed L2 into PML bodies, HSC70-L2 complex formation was confirmed by coimmunoprecipitation and immunofluorescence colocalization. Completion of virus assembly results in displacement of HSC70 from virions indicate that HSC70 transiently associates with viral capsids during the integration of L2 (73).

In natural HPV infections, the life cycle occurs during and is linked to epithelial differentiation (140, 142, 164, 165). HPV morphogenesis occurs in the upper layers of the epithelium, where the viral late genes L1 and L2 are expressed and serve as structural proteins to encapsidate the amplified viral genomes in the nucleus. Therefore, our goal was to determine a role for cellular chaperones during the viral life cycle functions. In HPV31-infected epithelial tissues, we used two methods for upregulating HSP expression. Heat shock was employed to induce various members of the HSP family, and we found that virus activities were augmented up to ~10-fold with increasing doses of this non-

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specific treatment. Of the chaperones tested, HSP70i expression levels were induced the greatest correlating with higher viral genome levels and increased virion production. As the heat shock response is multi-factorial, we chose to focus on HSP70i as its levels were the most pronounced and long-lived. In the second approach, we used Ad-mediated gene delivery to specifically increase HSP70i levels in HPV31 infected tissues. Our results show that increased HSP70i levels correlated with an approximately two-fold increase in viral genome copies regardless of the treatment. However, this alone could not explain the 4- to ~10-fold increase in virion production observed co-incident with higher HSP70i protein levels. Although the total detectable levels of L1 proteins were not significantly altered, immunostaining of HPV31-infected tissues were found to consistently have increased detectable L1 protein in the nuclei of the upper epithelial layers where virion morphogenesis is believed to occur. In nuclei with detectable L1 protein, HSP70i was found co-localized with L1 causing us to speculate that the subcellular localization of L1 is influenced by HSP70i expression.

The viral activities following heat shock were very similar to those resulting from Ad-mediated HSP70i transduction. The advantages of using heat shock include the high and sustained HSP70i expression levels even out to day 14 where our tissues were harvested for virion production. However, the main limitation of heat shock includes the complicated biological response to heat stress, which is not specific to HSP induction. The requirement for raft tissues to

be cultivated at the air-liquid interface maintaining a dry apical cell layer necessitated Ad-mediated gene transduction to be performed directly prior to lifting to the air-liquid border. Although Ad-mediated HSP70i transduction is a more specific approach, the required experimental design results in waning expression of HSP70i from days 8 to 14, the time when HPV genome amplification and late gene expression peak in raft tissues (164, 165). We found that Ad-mediated gene transduction was not robustly sustained in these upper tissue layers out to the time needed for virion morphogenesis. Although this is not a surprising result with regard to maintenance of Ad-transduced genes, we feel it explains the lower peak virion production levels of ~4-fold in this system compared to the ~10-fold increase in the heat shocked tissues. Alternatively, or in addition, heat stress may provide supplementary important factors for HPV virion maturation.

Self-assembly of L1 proteins occurs both *in vivo* and *in vitro*, leading to formation of capsid-like structures, known as VLPs (109, 185). Because of its propensity for self-assembly, L1 exists stably in two oligomeric configurations: homopentameric capsomers and complete viral capsids composed of 72 capsomers that may also incorporate a variable number of L2 molecules (31, 72, 122, 154, 155). Presumably, the complete capsids cannot enter the nucleus because the final particle diameter of 50-55 nm is larger than the maximal functional diameter of the nuclear pore (39 nm) through which active transport occurs (168). Furthermore, complete cytoplasmic assembly would be counter-

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productive as it would exclude the encapsidation of viral genomes that reside in the nucleus. Thus, it is reasonable to predict that L1 must remain in a sub-assembled status prior to nuclear transport, accumulation, and virion morphogenesis. The interaction between the newly synthesized HPV L1 monomers or capsomers and cellular chaperones, including HSP70i, might impede inter-capsomeric interactions and prevent premature capsid assembly in the cytoplasm. Once the L1 proteins enter the nucleus and are released from HSP70i, they would be free to fully assemble into capsids or virion. This is consistent with a process believed to occur for PyV major capsid protein VP1 (49). That increased HSP70i did not detectably alter L1 expression levels, but does interact with L1 and is associated with L1 nuclear detection suggests HSP70i directly affects L1 cellular localization, and may play a role in capsid assembly. Our IF and co-IP data indicate that only a fraction of the L1 and HPS70i interact at a given time in 14d raft tissues. Little is known about the stoichiometry of L1 capsomers to HSPs during the HPV life cycle; thus, further studies are required to determine if the ratio of L1 major capsid protein to HSP70i affects nuclear import of L1 and viral morphogenesis during the late stage of the viral life cycle.

Although the epithelial differentiation achieved in the raft tissue culture system is excellent for the study of the complete differentiation-dependent life cycle of HPV, the work described in Chapter 3 approaches the limitations of using the raft system to define the mechanistic role of HSP70i in the HPV life

cycle. In addition to the complexity of differentiation with numerous impacting factors engaged, only a few superficial cells are permissive for virion production and cell fractionation is inefficient due to differentiation-induced nuclear breakdown (reviewed in (105)). Also, the effects of mutant proteins or chemicals cannot be investigated due to the need for 14 days of cell division and differentiation. Therefore, we adapted a differentiation-independent monolayer system for expression of L1 and L2 capsid proteins in order to package HPV genomes (178). We employed monolayer 9E cells that maintain endogenous episomal HPV31 genomes and transfected them to overexpress L1 and L2. Using this 9E-L1/L2 monolayer cell culture system, we can more definitively investigate modes of HSP70i function and consequences of its inhibition. This enabled us to better investigate the mechanisms whereby HSP70i helps facilitate nuclear transport and virion morphogenesis.

From this 9E-L1/L2 monolayer system, increased HSP70i augmented HPV virion production and viral genome levels but did not detectably affect total L1 protein expression in a manner remarkably similar to effects observed in raft tissues. Analysis of cytoplasmic and nuclear fractions substantiated the fact that the subcellular localization of L1 is influenced by HSP70i expression and function. Without detectable impact on HPV total vDNA and L1 levels, the finding that the HSP70i ATPase mutant impeded L1 nuclear accumulation and virion production causes us to postulate that the efficient L1 localization and

release in the nucleus is an important function of HSP70i and a limiting step for virion morphogenesis.

Prior studies have shown that heat shock proteins are involved in HPV activities (42, 125, 126, 129, 147). Importantly, those prior studies were carried out in in irrelevant cell types or in cell free systems. These non-physiological conditions provide indicators for viral activities but fail to reveal what is occuring in the natural viral life cycle. Our studies provide the clear evidence that, in the context of the complete viral life cycle and in the natural host human keratinocytes, HSP70i plays a role in viral DNA replication, capsid protein localization and viral morphogenesis. This finding is significant, as the role of HSP70i in authentic HPV replicative conditions had not been reported before.

Our results are noteworthy because they support epidemiological studies which first correlated the relationship between increased HSP70i as a determinative factor for the development of cervical cancer. Increased HSP70i may culminate in an increased viral burden that results from increased virion morphogenesis as well as from the amplification of viral genome copies. Our current study provides molecular evidence that links the dosage-dependent effects of HSP70i to measurable effects in the HPV life cycle events such as changes in genome amplification and virion morphogenesis. Epidemiologists define HPV viral load in qualitative terms with respect to genome copies, both episomal and integrated, in tissue biopsies and exfoliated cervical cells (219). HSP70i-mediated regulation of the HPV life cycle activities may facilitate the

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carcinogenic potential of the host tissue. Upon increased HSP70i expression, enhanced virus production may raise the chances of infecting secondary sites, thus escalating viral persistence, which is thought to be necessary for malignant progression (219). Additionally, increased HSP70i correlates with genome amplification, which could potentially result in multiplied templates from which the E6 and E7 oncogene transcripts are produced. Boosted oncogene expression is also directly correlated with increased carcinogenic potentiality of the tissue (118). Our findings suggest the possibility that HSP70i-mediated manipulation of multiple HPV life cycle functions, such as the induction of genome copies, the translocation of late gene transcripts/capsid proteins, and the coincident virion assembly, may accumulatively determine the risk for cervical cancer progression.

In conclusion, we present evidence that HSP70i impacts multiple steps in HPV life cycle including viral genome amplification, the nuclear accumulation of viral capsid protein L1, and viral morphogenesis. Our findings agree with previous studies in less physiologically relevant systems wherein viral proteins were over-expressed in irrelevant cell types or tested in cell free systems. HSP70i promotes viral genome replication by displacing E2 from the E1-ori complex to assist unwinding (126, 129). HSP70i's binding to newly synthesized L1 protein can prevent its self-assembly into fully capsid in cytoplasm and support its nuclear transportation. During these processes, the ATP-ADP cycle function of HSP70i ATPase domain is required for the release of L1 in the nucleus and subsequent virion assembly. With regard to *in vivo* pathogenesis,

the relationship between increased HSP70i has been correlated epidemiologically as a determinative factor for the development of cervical cancer (38, 44). Therefore, our findings lend molecular support to the possibility that HSP70i-mediated enhancement of multiple HPV life cycle functions may add to the risk for cervical cancer progression. These investigations are foundational for the understanding of HSP70i's diverse functions in the HPV life cycle, as well as for the potential design of novel therapeutic approaches.

Chapter 6

Summary, Significance and Future directions

Human papillomaviruses are significant infectious agents, due to their strong association with cervical cancer, as well as association with various other epithelial cancers (25, 81, 101). Cervical cancer is the second most common cancer killer of women world wide, and causes approximately 300,000 death per year worldwide (172). This pressing fact emphasizes the importance of continuing to explore HPV biology and cervical cancer research. Although numerous co-factors contribute to the progression to cervical cancer, high-risk HPV infection of the cervical epithelial tissue is considered to be the necessary cause of cervical malignancy (36, 37, 128, 157, 176). Therefore, a comprehensive understanding of HPV replicative life cycle activities in authentic human keratinocytes, a comprehensive understanding of the characteristics of HPV transmission and persistant infection, and a comprehensive understanding of mechanisms controling the involvements of co-factors in HPV-related malignancy progression are of crucial importance for combating this important human pathogen.

The development of two VLP-based vaccines targeting types of high-risk HPVs have shown great promise as effective strategies against HPV infection and associated diseases. Characteristics of these vaccines, such as safety, high immunogenicity, and long-term protection against development of cervical neoplasia have been reported. Clinical trials have demonstrated high effectiveness against formation of HPV-related precancerous lesions (10, 89, 90, 114, 167, 212, 213). At present, the most inhibitory aspects of the HPV vaccines

is the cost. With the three vaccination series totaling for $360, the possibility of combining this treatment together with other childhood vaccines or requiring vaccination for school enrollment turns out to be complicated. In addition to the difficulty of cost effective HPV vaccination faced by the developed countries, distribution of the HPV vaccines poses even more difficulties. Women in developing countries account for more than 80% of annual cervical cancer cases and deaths worldwide. Unfortunately, due to the high cost, the HPV vaccines may turn out to be of little consequence to populations of these countries (7, 24, 60, 183). Therefore, research on HPV biology must be continued to expand our basic knowledge of the viruses and support the development of more affordable and accessible prophylactics and antivirals.

The differentiation-dependent life cycle of HPVs is an interesting, yet challenging, aspect of HPV biology. For decades, production of complete HPV virions in standard cell culture as well as detection of infection were hindered by this characteristic of HPVs, and the understanding of events involved in HPV infections has suffered. Because of the difficulties associated with studying HPVs, many researchers have relied on experimentation with VLPs and PsVs in unnatural conditions, which often require the use of cell types of questionable relevance to HPV biology. Thus, confusion surrounding viral life cycle events exists. Recently, a simplified culture technique has been established, and infectious HPV virions can be obtained (32, 178). Moreover, research to characterize the qualities of these virions have been conducted, and the virus

produced from this system appears to be indistinguishable from authentic virus produced from differentiation-based methods (52, 97, 182). In this body of work, in addition to the three-dimensional tissue culture system (also termed the organotypic or "raft" tissue culture system) to study the involvement of HSP70i in HPV biology, we modified this monolayer system to study specific mechanisms of the HSP70i roles during HPV31 late viral activities. Using these two systems, all experiments have been conducted in cell types relevant to natural HPV infection in order to determine events during authentic HPV31 viral activities, and to enhance our knowledge of HPV infection biology under natural conditions.
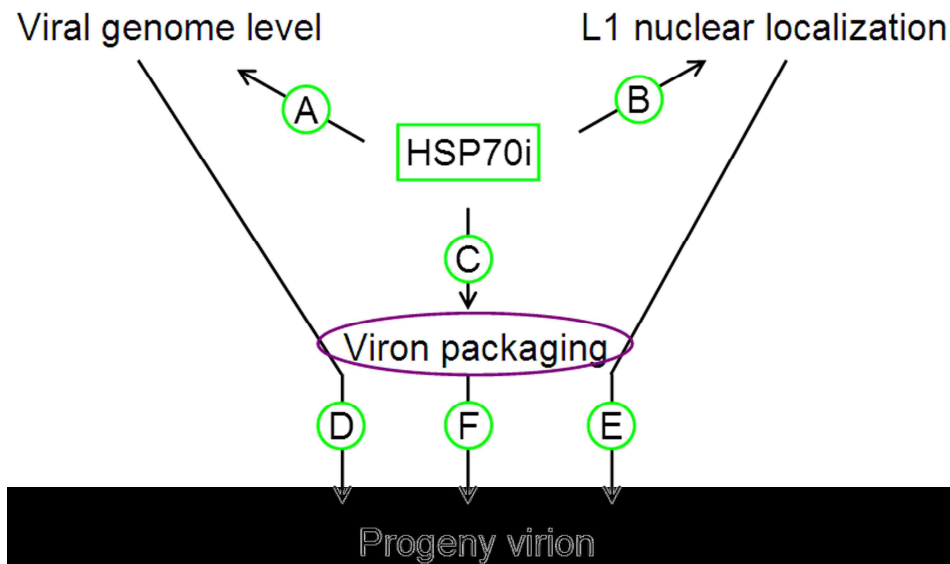
Figure 6.1. Model for HSP70i's involvement in HPV31 life cycle activities. (A) Upon induction of HSP70i, HPV31 viral genome replication increases and results in higher genome levels. **(B)** HSP70i interacts with and promotes HPV31 L1 capsid protein relocalization from the cytoplasm into the nucleus. The relocalization of L1 provides required components for virion morphogenesis in the correct cell compartment. **(C)** Colocalization and interaction of HSP70i with L1 in the nucleus accommodates virion morphogenesis. **(D, E, F)** Enhanced viral genome levels, L1 transportation into nucleus, and virion packaging cooperate to the progeny virion production.

Interest in how heat shock proteins affect virus activities has grown substantially in recent years, increasing our appreciation of how viruses manipulate host cells. The complexity of virus and HSP interactions only becomes more apparent as we understand more about how viruses utilize chaperones. In this report, HSP70i's involvement was demonstrated for multiple HPV31 replicative life cycle events. Figure 6.1 illustrates HSP70i's involvement in HPV31 life cycle activities and the established downstream effects as well as putative events that have yet to be investigated.

Monitoring of HPV31 and HSP70i involvement during viral activities has revealed novel findings. Research presented herein demonstrated that HSP70i expression and function play critical roles in HPV31 viral DNA replication, L1 capsid protein nuclear transportation, and virion morphogenesis.

Although we have not defined the downstream effect of higher viral genome levels and viron production concurring with increased HSP70i expression that were observed, this enhancement is generally associated with increased degree and possibility of clinical lesions. Figure 6.2 shows a proposed model for the interaction among HPV infection, viral gene expression, and the contribution of cofactors in progression to malignancy. The bridge-like role of HSPs, including HSP70i, provides interpretation of the connection between the previously reported factors involved in malignancy development and HPV viral infection activities (36, 47, 56, 67, 75, 77, 94, 152, 198, 207). Briefly, those factors increase HPV viral activities concurrent with the induction of HSPs. The
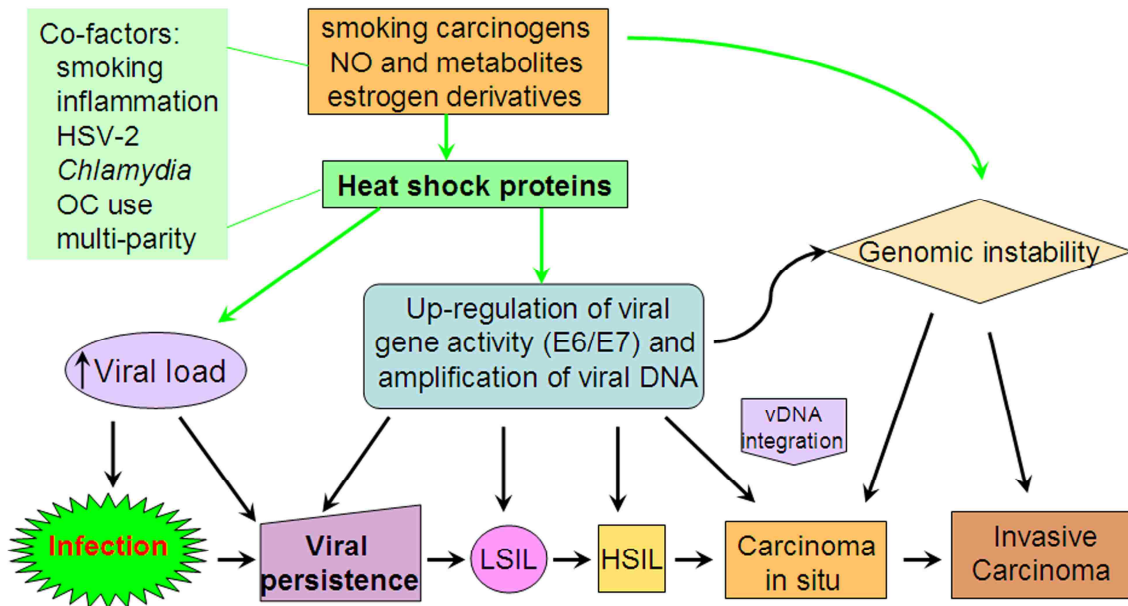
Figure 6.2. Model for the involvement of HSPs in the interaction among HPV infection, viral gene expression, and the contribution of cofactors in progression to malignancy.

higher levels of viral genome can provide template for oncogenes E6/E7 expression and increase the chance of integration which is an indication of severe lesion. Higher viral production may increase the chances of re-infection or transmission of the high-risk HPV, in this way, viral persistence is escalated, which is thought to be necessary for malignancy progression (219).

HSP70 family chaperones have now been implicated in the life cycles of a number of eukaryotic DNA and RNA viruses. The use of chaperones by viruses is perhaps not surprising, given the requirements for viral protein synthesis at high levels and for the regulation of structural proteins inherently poised for assembly. However, a mechanistic role for chaperones has not yet been fully

described for controlling either the quality or the subcellular location of viral capsid assembly.

The characterization of the co-localization of HSP70i with L1 and re-localization from post-translation into the nuclei of infected cells described here allow us to hypothesize an *in vivo* regulatory role for HSP70 chaperones in the HPV replicative life cycle. HSP70i binds the L1 immediately after translation to inhibit premature assembly in the cytoplasm, although other inhibitory factors may also be involved. HSPs may inhibit assembly until they release the cargo while they are in the nucleus. Thus, HSP70i family chaperones may also control the location of assembly *in vivo*. The direction of the equilibrium could depend on co-chaperones found in particular cellular compartments or other regulatory factors (reviewed in reference (226)). Further characterization of this reaction will be required before concluding that a unique interaction is occurring.

Due to the previously mentioned difficulties associated with studying HPVs, the field of HPV-HSPs interactions within host cells remains largely open for exciting new research. With the advent of new technology to study HPVs, combined with many interesting new techniques being applied to virological study, much is yet to be learned about HSPs and HPVs. By native PAGE analysis, fractionated cellular lysates may reveal the assembly-status of L1 in cytoplasm and nucleus with various HSP70i expressions. Immuno-gold electron microscopy to detect the expression and distribution pattern of HSP70i and L1 in 9E monolayer cells or tissue may also provide more details of the association of

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them (115). Other HSPs such as HSP40, a co-chaperone of HSP70, may also engage in this process. The work presented here focused exclusively on HPV31 but has introduced some fascinating questions concerning the common mechanisms used by distinct viral types during infection. Expansion of the research presented here to other HPV types will inform our understanding of how HPVs have evolved and how these difference and similarities relate to the divergent roles of HSPs played in HPV-related disease.

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