The Effect of Productive HPV16 Infection on Global Gene Expression of Cervical Epithelium

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Abstract

Human papillomavirus (HPV) infection is the world's most common sexually transmitted infection, and is responsible for most cases of cervical cancer. Previous studies of global gene expression changes induced by HPV infection have focused on the cancerous stages of infection, and therefore, not much is known about global gene expression changes at early pre-neoplastic stages of infection. We show for the first time, global gene expression changes of early stage HPV16 infection in cervical tissue using 3-dimensional organotypic raft cultures that produce high levels of progeny virions.

cDNA microarray analysis showed that a total of 594 genes were upregulated and 651 genes were downregulated at least 1.5-fold with HPV16 infection. Gene ontology analysis showed that biological processes including cell cycle progression and DNA metabolism were upregulated, while skin development, immune response, and cell death were downregulated with HPV16 infection in cervical keratinocytes. Individual genes were selected for validation at the transcriptional and translational levels including UBC, which was central to the protein association network of immune response genes, and top downregulated genes RPTN, SERPINB4, KRT23, and KLK8. In particular, KLK8 and SERPINB4 have shown to be upregulated in cancer, which contrasts our results.

Organotypic raft cultures that allow full progression of the HPV life-cycle have allowed us to identify novel gene modulations and potential therapeutic targets of early stage HPV infection in cervical tissue. Additionally, our results suggest that early stage productive infection and cancerous stages of infection are distinct disease states expressing different transcriptomes.

Importance

Persistent HPV infection is responsible for most cases of cervical cancer. Transition from precancerous to cancerous stages of HPV infection is marked by a significant reduction in virus production. Most global gene expression studies of HPV infection have focused on the cancerous stages. Therefore, little is known about global gene expression changes at precancerous stages. For the first time, we measured global gene expression changes at precancerous stages of HPV16 infection in human cervical tissue producing high levels of virus. We identified a group of genes that are typically overexpressed in cancerous stages to be significantly downregulated at the precancerous stage. Moreover, we identified significantly modulated genes that have not yet been studied in the context of HPV infection. Studying the role of these genes in HPV infection will help us understand what drives the transition from precancerous to cancerous stages, and may lead to development of new therapeutic targets.

Introduction

Human papilloma virus (HPV) infection is the world's most common sexually transmitted infection with approximately 291 million women worldwide infected with the virus at any given point in time (1). While low-risk HPV types cause benign warts in the anogenital area, persistent infection with high-risk HPV types can give rise to various cancers of epithelial origin. In particular, HPV is responsible for most cases of cervical cancer, which is the third most common cancer in women worldwide and the most

common cancer in women in developing countries (2). For this reason, the mechanism of HPV infection and HPV-mediated oncogenesis has been extensively studied. Early viral proteins E6 and E7 have been identified as oncoproteins that play a critical role in tumorigenesis and tumor maintenance by inhibiting tumor suppressor genes *TP53* and *RB1*, respectively (3–10).

HPV initially infects dividing cells of the basal layer of the epidermis via microabrasions. Most infections are naturally resolved, but in some cases, the virus establishes a persistent infection, which may subsequently progress to precancerous lesions that are histologically graded as cervical intraepithelial neoplasia (CIN) I to III. When left untreated, these neoplastic lesions may ultimately develop into carcinoma in situ and invasive cancer. In persistently infected cervical tissue with normal or low-grade dysplasia the HPV genome is maintained episomally and infectious viral particles are produced. In contrast, progression to severe dysplasia and invasive cancer is marked by integration of viral genome into the host genome that typically results in disruption of the E2 gene, subsequent upregulation of oncogenes E6 and E7, and abrogation of virion production (11, 12).

In the past, many studies have looked into changes in whole genome expression profiles of precancerous and cancerous lesions in order to better understand the progression of persistent HPV infection to cervical cancer. However, most of these studies focus on neoplastic lesions and cancerous lesions (13–21) and, therefore, there is a gap in knowledge of global gene expression in earlier pre-neoplastic stages of the disease when HPV establishes productive infection in the host. In two recent studies, human keratinocytes persistently infected with HPV16 were used to measure global

gene expression changes (22, 23), but they used keratinocytes derived from foreskin, which may not be appropriate for modeling infection in cervical tissue since HPV may have tissue-specific effects (24). Furthermore, these studies used monolayer cell cultures that do not produce mature virions by disallowing the virus to progress through its differentiation-dependent replication life-cycle (25, 26). In other studies, overexpression tools were used to examine the effect of specific HPV oncoproteins on global gene expression (27, 28). Since these studies only examine the effect of individual viral proteins, they do not account for the full picture of HPV infection in the natural environment.

In this study, we used oligonucleotide microarrays to measure global gene expression changes in early passage HPV16-infected human cervical keratinocytes (16HCK). Organotypic raft cultures were used in order to allow the virus to go through its full life-cycle. A total of 594 and 651 genes were at least 1.5-fold upregulated and downregulated, respectively. Gene ontology analysis of upregulated genes identified biological processes that were significantly represented including the cell cycle process and DNA metabolism. In contrast, biological processes that were significantly represented with downregulated genes included epidermis development, extracellular matrix disassembly, and regulation of NF-κB signaling.

Results

Microarray analysis of HPV16 infection in cervical tissue. Global gene expression changes in cervical tissue infected with HPV16 at a pre-neoplastic state were measured by conducting cDNA microarray analysis (GEO accession no. GSE109039) on 10-day organotypic raft tissue from early passage human cervical keratinocytes (HCK) persistently infected with HPV16 (16HCK). In order to create 16HCK cell lines, human cervical tissue from biopsies were acquired, processed, and cultured with keratinocyte-selective medium to grow primary HCKs. The HCKs were then electroporated with the HPV16 genome to establish persistent infection and immortalization. Since HPV replication is differentiation-dependent, monolayer cell culture systems cannot produce HPV virions and provide limited insight into the effect of productive infection that happens *in vivo*. Also, it is difficult to study early stages of HPV infection in clinical samples because early stages of infection are largely asymptomatic and patients typically present at a neoplastic or cancerous stage. Moreover, clinical samples may be infected with any of the numerous types and variants of HPV making it difficult to study the effect of a specific virus in a controlled environment. Therefore, we used organotypic raft cultures as previously described, which allows us to observe the full HPV life-cycle in 3-dimensional (3D) tissue at a precancerous stage of infection when HPV particles are maximally produced. The organotypic raft tissue was harvested at 10 days of culture for microarray analysis when particle production is most active, and 20 days of culture when particle accumulation is at its maximum to measure the viral titer showing that virus particles are produced at high levels (Table 1). By infecting each cell line with the same virus and subjecting the raft cultures to the same condition,

we are able to minimize variables and observe the specific effect of HPV infection on cervical tissue.

The experiment was conducted in three individually derived 16HCK cell lines in duplicates and raft tissue from uninfected HCKs served as control. Out of the 34,575 genes that were analyzed with cDNA microarray, a total of 1,245 genes were modified at least 1.5-fold ($p<0.05$) and 533 genes were modified at least 2-fold ($p<0.05$) with HPV16 infection as compared to uninfected control (Table S1). Of those genes that were significantly modulated at least 1.5-fold, 594 genes were upregulated and 651 genes were downregulated. Table 2 and 3 show the 50 most upregulated and downregulated genes in the microarray analysis. Amongst the top 50 upregulated genes are those associated with cell cycle (*CDKN2A*, *CDC7*, *NASP*, *MDC1*, *NFIX*, *FOXQ1*). In contrast, the 50 most downregulated genes span from those involved in differentiation (*RPTN*, *LCE1D*, *LCE3C*, *LCE1E*, *S100A7*), ECM-modulation (*KLK6, 8, 10, 13* and *MMP 9, 10*), immune regulation (*LCN2*, *SPNS2*, *FAM3D*, *IL1RN*, *PSG4*, *IL1F7*) to antimicrobial response (*RNASE7*, *PRSS3*, *PRSS2*). This suggests that HPV infection is driving the cell cycle, and disrupting epidermal differentiation and ECM homeostasis while evading the immune and antimicrobial responses by downregulating them.

While most previous global gene expression studies have focused on neoplastic and cancerous stages of HPV infection (13–21), two previous studies have modeled pre-neoplastic, early stage HPV infection similar to our study (22, 23). However, most of the modulated genes in the two studies did not overlap with our results. One of these studies reported that 135 genes were modulated with HPV infection, but only 38 (28.1%)

of these were modulated in the same direction and 4 of them were modulated in the opposite direction in our microarray analysis (22). In the other study, a total of 966 genes were reported to be modulated with HPV infection, but only 15 (1.6%) of these were modulated in the same direction in our microarray analysis (23). The two major differences between our study and the two previous studies is that we use cervical keratinocytes instead of foreskin keratinocytes, and that we use organotypic raft cultures instead of monolayer cultures that do not allow HPV to complete its life cycle. Using keratinocytes derived from cervical tissue, and organotypic raft cultures that allow the virus to complete its life cycle in 3D tissue enables us to capture the whole picture of an early stage HPV infection in the cervix.

While fold-change of the 50 most upregulated genes ranged between 12.1 to 2.5, that of the 50 most downregulated genes ranged between 57.5 and 6 indicating that a greater degree of gene modulation occurred in the downregulated genes than the upregulated genes. Similarly, when the cutoff for inclusion was increased to at least 5-fold modulation, only 6 genes were upregulated in contrast to 72 genes that were downregulated as shown in Fig. 1. This suggests that HPV16 is disrupting the host's physiology mostly by dampening many of the normal processes that may interfere with the virus's survival and replication.

Gene ontology analysis. In order to identify biological pathways that are significantly affected with HPV16 infection, we conducted gene ontology (GO) analysis using the online tool GOrilla (29). The GO analysis result was then summarized using REVIGO to combine similar GO terms for simplified visualization (30). 144 GO terms that were significantly represented in upregulated genes were summarized to 82 GO

terms (Table S2), and 77 GO terms that were significantly represented in downregulated genes were summarized to 52 GO terms using REVIGO (Table S3).

Amongst the genes that were upregulated with HPV16 infection, many of the represented GO terms are associated with cell cycle progression (cell cycle process, cell division, cell cycle phase transition, regulation of mitotic cell cycle, and cell cycle) and DNA metabolism (DNA metabolic process, DNA repair, cellular response to DNA damage stimulus) as shown in Fig. 2A. This suggests that persistent infection with HPV16 drives cellular proliferation in cervical tissue as expected due to the presence of viral oncogenes E6/E7 and consistent with previous gene expression studies (16, 19, 27, 31). It is known that HPV oncoproteins E6 and E7 inhibit tumor suppressor genes *TP53* and *RB1* respectively, and that this is the main mechanism through which the virus promotes proliferation and tumorigenesis. "Translesion synthesis," "neuron projection regeneration," and "retina morphogenesis in camera-type eye" were amongst the upregulated DNA metabolism GO terms which have not yet been reported by previous global gene expression studies of HPV16 infection. Translesion synthesis is a cellular DNA damage tolerance process of recovering from stalled replication forks by allowing DNA replication to bypass certain lesions (32, 33). Eight genes of the translesion synthesis GO category were upregulated in our analysis including *POLE2*, *UBA7*, *MAD2L2*, and *RPA2* which have not yet been reported in previous global gene expression studies of HPV infection. So far, not much is known about the exact role of translesion synthesis in HPV infection. One previous study speculated that HPV oncoprotein E6 may have inhibitory effects on translesion synthesis (34) while another study reported that p80, a cellular cofactor of HPV31 replication, may downregulate

translesion synthesis (32). Our study shows for the first time that translesion synthesis is upregulated in a productive raft culture model of HPV16, suggesting that this process may facilitate viral replication and production.

Within the genes that were downregulated with HPV16 infection, the GO terms that were significantly represented are associated with skin development (epidermis development, keratinocyte differentiation), immune response (positive regulation of IKK/NF-κB signaling, antimicrobial humoral response), and cell death (cell death, positive regulation of JNK cascade) as shown in Fig. 2B. Downregulation of genes associated with skin development have also been observed in previous studies of HPV-positive tumors (13, 17, 35). Similarly, downregulation of genes under GO terms associated with immune response and cell death have been shown in previous studies of HPV-positive tumors and E6 transgenic mice (18, 22, 23, 28, 36, 37). Overall, these results suggest that HPV16 perturbs normal epidermal development while having a hyperproliferative and immunosuppressive effect on cervical tissue. Downregulated GO terms that have not yet been reported in previous global gene profiling studies of HPV infection included “positive regulation of cell migration” and “regulation of platelet-derived growth factor production.” In contrast to our results, previous studies of global gene expression at cancerous stages of HPV infection have shown upregulation of genes involved in cell migration, which is an indicator of invasive, or metastatic cancer (20, 36, 38). Specifically, one study showed that *MMP9*, a well-established metastatic gene, is upregulated in cervical carcinoma cell lines and tissue samples, whereas our microarray analysis shows that this gene is downregulated 17.1-fold (38). Moreover, *LCN2*, which is overexpressed in various cancers and prevents degradation of *MMP9*,

was downregulated 9.1-fold in our microarray analysis (39–46). These opposing trends in modulation of cell migration genes highlights the fact that our study investigates early productive stages of HPV infection, whereas the other studies focus on the cancerous stages of infection when viral production is significantly decreased. We speculate that cell migration genes interfere with efficient viral replication and assembly and, therefore, are suppressed during the productive stages of infection, whereas these genes are upregulated to facilitate tumor development and metastasis once the infection enters the cancerous stages.

Upregulation of cell cycle and DNA metabolism. While GO analysis revealed biological processes that are significantly affected by persistent infection of HPV16, it does not provide information on interactions amongst the genes. Therefore, we further examined the interaction amongst individual proteins based on published data using the online tool STRING (47, 48). STRING protein-protein interaction network analysis allows us to identify signaling pathways, interactions amongst signaling pathways, and central proteins that have maximum interactions with other proteins within a category. A protein association network was created for each of the five aforementioned categories of biological processes that were broadly represented in the GO analysis: cell cycle, DNA metabolism, skin development, immune response, and cell death.

From the upregulated genes, a protein association network was created with genes from 32 GO terms related to cell cycle (Fig. 3A, Table S4). Genes that are not known to be associated with any other gene in this group were excluded from the figure for simplified visualization. The protein association analysis revealed 15 genes that are central to the network as highlighted in red in Fig. 3A. These genes include

nucleoporins (*NUP 43, 85, 107*), centromere proteins (*CENP E, F, P*), and kinetochore-associated proteins (*KNTC1, SKA2*). This suggests that HPV infection drives the cell cycle and increases the expression of structural proteins involved in cell cycle and cell division. Amongst these genes, *NUP43*, *NUP85*, *NUP107*, *CENPP*, and *SKA2* have not yet been reported to be associated with HPV infection by previous gene expression studies.

Similarly, a protein association network was created with genes from 42 GO terms related to DNA metabolism (Fig. 3B, Table S5). Genes in this network include minichromosome maintenance complex components (*MCM 3, 6, 7*), replication factors (*RFC 2, 3, 5*), and DNA polymerase subunits (*POLA1, POLE, POLE2, POLD1*). Amongst these genes, *POLA1*, *POLE*, and *POLE2* have not yet been reported to be associated with HPV infection by previous gene expression studies. Previous studies have shown that overexpression of MCM genes are correlated to cervical carcinogenesis and specifically, *MCM7* has been shown to interact with HPV18 E6 oncoprotein (49, 50). These results are consistent with upregulation of cell cycle genes since cell division requires DNA replication and proteins associated with DNA metabolism. Additionally, our result suggests that the upregulation of MCM genes is initiated at early stages of HPV infection and sustained throughout carcinogenesis.

Downregulation of skin development, immune response, and cell death. Within the downregulated gene sets, we combined genes from 7 GO terms in the protein association analysis for skin development, which gave 4 small networks (Fig. 3C, Table S6) including a network of late cornified envelope genes (*LCE 1D, 1E, 1F, 3C, 4A, 5A*) and a network of laminins (*LAMA3*, *LAMB3*, *LAMC2*). All eight genes in the

network of LCEs were included in the GO term “epithelial cell differentiation” while the three laminin genes were included in the GO term “epidermis development.” The LCE genes encode stratum corneum proteins of the epidermis, and are all located in the same region of chromosome 1 (1q21.3) suggesting that epigenetic modifications might be suppressing the expression of this region as a whole. Our study shows for the first time the downregulation of a network of LCE genes in HPV infection. The two previous studies that analyzed global gene expression changes in early stage HPV infection may not have observed changes in LCE genes since they used monolayer cultures that do not allow formation of the four layers of differentiating keratinocytes in the epidermis (22, 23). The three laminin genes *LAMA3*, *LAMB3*, and *LAMC2* encode the three subunits that make up laminin 5, which plays an important role in wound healing, keratinocyte adhesion, motility, and proliferation (51, 52).

Genes from 16 GO terms were included in the protein association analysis for inflammation (Fig. 3D, Table S7). Ubiquitin C (*UBC*), which is downregulated 2.48-fold with HPV16 infection, is at the center of this network with the most associations with other genes related to inflammation. *UBC* is one of the two polyubiquitin genes that are involved in various cellular processes including protein degradation, protein trafficking, cell-cycle regulation, DNA repair, and apoptosis (53). Other ubiquitin-related genes that were significantly downregulated in our microarray analysis includes proteins that are involved in ubiquitin conjugation (*UBE2G1*, *UBE2F*, *UBR4*, *UBTD1*), whereas two of the four ubiquitin-related genes that were significantly upregulated are involved in deubiquitination (*USP1*, *USP13*). This suggests that a decrease in ubiquitination may be important in the HPV16 life-cycle, and that the virus is trying to achieve this by

292 decreasing ubiquitination and increasing deubiquitination. In previous studies, UBR4
293 has been shown to be a cellular target of HPV16 E7 oncoprotein (54, 55), and we have
294 shown that HPV16 upregulates deubiquitinase UCHL1 in order to escape host immunity
295 (56). Since ubiquitins are involved in many cellular processes, it is hard to identify
296 which specific pathway is being affected by the decrease in ubiquitination. It is possible
297 that the virus is preventing degradation of proteins that are targeted by ubiquitin. Also,
298 since our results suggest that HPV infection drives cell cycle and downregulates cell
299 death, it is possible that the downregulation of ubiquitination is involved in these
300 processes. The network also included cytokines (*IL1A*, *IL1B*, *IL36B*), MAP kinases
301 (*MAPK13*, *MAP2K4*, *MAP3K9*), proteases (*CTSD*, *CTSC*, *KLK7*, *FURIN*), serine
302 protease inhibitors (*SERPINB1*, *SERPINE1*), and antimicrobial genes (*S100A7*,
303 *RNASE7*, *PRSS3*, *HIST1H2BC*, *HIST1H2BE*, *HIST1H2BG*, *LCN2*). Amongst these
304 genes, *MAP3K9*, *CTSD*, *CTSC*, *SERPINE1*, *HIST1H2BC*, *HIST1H2BE*, and
305 *HIST1H2BG* have not yet been reported to be associated with HPV infection by
306 previous gene expression studies. Of note, RNASE7 is a broad-spectrum antimicrobial
307 protein that we have previously shown to be downregulated by HPV infection (57). In
308 terms of specific signaling pathways, regulation of I-kappaB kinase (IKK) and NF-κB
309 signaling was significantly represented in the network (Fig. 3D, highlighted in red),
310 which is consistent with our previous study that showed suppression of NF-κB activation
311 by HPV16 (58). Most of these genes were shown to interact with UBC (Fig. 3D), and it
312 is known that ubiquitination and proteolytic degradation of NF-κB inhibitor IκB can lead
313 to NF-κB activation (59). This suggests that HPV16 is evading the immune system by

314 suppressing the NF-κB pathway, and that this suppression may be mediated by
315 downregulation of UBC.

316 Lastly, genes from 4 GO terms were included in the protein association analysis
317 for cell death and gave 5 small networks (Fig. 3E, Table S8). The networks include
318 genes from the JNK signaling pathway (*TIAM1*, *IL1B*, *VANGL2*, *CTGF*, *WNT7B*)
319 suggesting that HPV16 may be suppressing cell death, and promoting transformation
320 and tumorigenesis through this pathway. This is consistent with our previous study that
321 showed downregulation of cell death with HPV infection (60). Both NF-κB and JNK
322 pathways are downstream of TNF signaling, and TNF ligands and receptors
323 (*TNFRSF19*, *LITAF*, *TNFSF9*) were downregulated in the microarray. This suggests
324 that HPV16 is downregulating NF-κB and JNK pathways via TNF signaling
325 downregulation. Amongst these genes, *TIAM1*, *VANGL2*, *WNT7B*, *TNFRSF19*, and
326 *LITAF* have not yet been reported to be associated with HPV infection by previous gene
327 expression studies.

328 **Gene transcription changes correlate with changes in protein expression.**
329 Of the numerous biological processes that were identified with GO analysis, we wanted
330 to focus on processes and pathways that we felt were unique and most relevant to HPV
331 infection and life-cycle. Therefore, four genes that were modulated at least 10-fold were
332 selected for validation from processes involving epidermal development and
333 differentiation (*KLK8*, *RPTN*, *KRT23*), and immune response (*SERPINB4*). Additionally,
334 *UBC* was selected for validation since it was shown to be central to the protein
335 association network of inflammation (Fig. 3D), and cyclin-dependent kinase 2 (*CDK2*)
336 was included for analysis as a marker of proliferation. In our microarray analysis *KLK8*,

RPTN, *KRT23*, *SERPINB4*, and *UBC* were downregulated 25.8, 57.5, 11.1, 48, 2.48-fold, respectively, and *CDK2* was upregulated 1.74-fold with HPV16 infection. KRT23 is a structural protein in epithelial cells, whereas KLK8 and RPTN are involved in skin barrier proteolytic cascade and cornified cell envelope formation, respectively (61, 62). Recently, studies have reported that KRT23 may be involved in other cellular processes including cell cycle regulation and apoptosis (63), which are key processes that are modulated by HPV infection in our study. KRT23 has not yet been reported by any other gene expression studies of HPV infection and, therefore, could be developed into a novel biomarker of productive HPV infection. In a recent gene expression profiling study, SERPINB4 was shown to be downregulated in early stage HPV infection consistent with our analysis (23). SERPINB4 is a serine protease inhibitor that is overexpressed in inflammatory skin diseases and various cancers including squamous cell carcinomas, and may play a critical role in the immune response against HPV replication and virion production as it has been shown that increased SERPINB4 expression can activate NF-κB (64–70). Similarly, KLK8 has been shown to be overexpressed in cervical cancer, ovarian cancer, and oral squamous cell carcinoma (71–74). So far, not much is known about these proteins in the context of pre-neoplastic HPV infections, and therefore, they could potentially become biomarkers or therapeutic targets of HPV infection at its early stages. In particular, overexpression of SERPINB4 and KLK8 in cancers prominently contrasts our microarray data that includes the two genes amongst the top downregulated genes. This contrast highlights the different microenvironments of the precancerous and cancerous states of HPV infection, and understanding the role of SERPINB4 and KLK8 may provide critical

360 insight into the mechanism of HPV-induced carcinogenesis. For the validation
361 experiments, six new raft cultures were put up with uninfected keratinocytes isolated
362 from different cervical biopsies and six 16HCK raft cultures were put up from three
363 16HCK cell lines in duplicates. In order to validate the microarray results in new tissue
364 samples, all of the raft cultures were set up with new cell lines except for one set of
365 16HCK rafts.

366 Downregulation of *KLK8*, *RPTN*, *SERPINB4*, and upregulation of *CDK2* at the
367 transcriptional level was observed with RT-qPCR consistent with the microarray
368 analysis (Fig. 4). However, transcription of *UBC* didn't show statistically significant
369 modulation with HPV16 infection. We then further validated downregulation of KLK8,
370 RPTN, KRT23, and SERPINB4 at the translational level with western blot (Fig. 5A).
371 Although KLK8 expression was visibly downregulated with HPV16 infection in western
372 blot, statistical significance was not reached. To measure UBC protein expression, we
373 used an anti-ubiquitin antibody as a proxy for measuring UBC translation since UBC is
374 simply a polyubiquitin protein that accounts for the majority of basal level ubiquitin in
375 cells (53, 75). Western blot against ubiquitin showed differential ubiquitination of
376 various proteins with HPV16 infection and downregulation of monoubiquitin (Fig. 5B).
377 Lastly, downregulation of RPTN, KRT23, SERPINB4, and Ubiquitin were validated with
378 immunofluorescence staining (Fig. 6). In particular, RPTN, KRT23, and SERPINB4 are
379 minimally expressed in the basal layers of both infected and uninfected controls with no
380 significant difference in expression between the two groups. In contrast, the three
381 proteins are strongly expressed in the upper layers of uninfected tissues and this

382 expression is significantly decreased with HPV16 infection. The downregulation of the
383 proteins may be attributed to the loss of the cornified layer in infected raft tissues.

384

385 **Discussion**

386 Despite widespread screening for cervical cancer and development of HPV
387 vaccines in recent decades, the burden of cervical cancer remains to be one of the
388 highest amongst female cancers worldwide. In an attempt to understand HPV infection
389 and its progression to cancer at a holistic level, many studies have investigated global
390 gene expression changes that occur with HPV infection. Most of these studies focus on
391 neoplastic lesions and cancerous lesions (13–21). This is because early stages of HPV
392 infection, which typically lasts years, is often cytologically and clinically asymptomatic
393 until it reaches neoplastic stages of infection and, therefore, the majority of clinical
394 samples are limited to these late cancerous stages of infection. As a result, there is a
395 gap in knowledge in global gene expression at pre-neoplastic stages of HPV infection.
396 In two previous studies, early passage HPV16-immortalized human keratinocytes (22)
397 and spontaneously immortalized human keratinocytes transfected with the HPV16
398 genome (23, 76) were used to measure global gene expression changes at
399 precancerous stages of HPV infection. However, these two studies used keratinocytes
400 derived from foreskin, which is not ideal for modeling HPV infection in cervical tissue
401 since the virus may have tissue-specific effects (24). Moreover, these studies used
402 monolayer cell cultures that do not allow the virus to progress through its entire life-
403 cycle. Amongst the genes that were reported to be modulated in the two studies, only
404 28.1% and 1.6% of them matched our results, and moreover, some of the genes were

405 modulated in the opposite direction (22, 23). The stark discrepancies between our
406 study and the two previous studies can be attributed to our use of organotypic raft
407 culture system instead of monolayer cell cultures. The HPV life-cycle is dependent on
408 the various stages of keratinocyte differentiation that occur in the epidermis of the skin.
409 Since the HPV life-cycle spans all layers of the epidermis (basale, spinosum,
410 granulosum, corneum), monolayer cell cultures cannot produce progeny virus particles
411 and, therefore, are limited models of HPV infection. In our study, we overcome these
412 limitations by using human cervical keratinocytes and by creating organotypic raft
413 cultures that allow the full progression of the HPV life cycle. Raft cultures were also
414 made from uninfected primary HCKs to serve as control. Viral titers were measured on
415 all 16HCK raft tissues to check for high levels of viral particle production (Table 1),
416 which confirms that the viral genome is maintained episomally allowing productive
417 infection. Integration of viral genome into the host genome, and subsequent reduction
418 in viral particle production is a hallmark event in the progression of precancerous to
419 cancerous lesions and thus, high levels of viral particle production indicates that the
420 infection is in its earlier precancerous stages. Our study presents for the first time
421 global gene expression changes in cervical tissue with productive HPV infection.

422 Our microarray data showed that the majority of the modulated genes are
423 downregulated (Fig. 1). Gene ontology analysis of the microarray data identified gene
424 categories that were significantly represented including cell cycle progression and DNA
425 metabolism in the upregulated genes, and skin development, immune response, cell
426 death in the downregulated genes (Fig. 2). The upregulation of cell cycle and DNA
427 metabolism genes, and downregulation of cell death genes reflect the proliferative

nature of persistent HPV16 infection and is likely the result of viral oncogenes E6 and E7 inhibiting the cell cycle regulatory genes *TP53* and *RB1*, respectively. Downregulation of immune response and skin development genes can be understood in the context of the virus modulating the host environment to achieve efficient replication and virion production. The trends of modulation in these five gene categories are consistent with previously reported studies of global gene expression (13, 16–19, 22, 23, 27, 28, 31, 35–37).

Several genes were selected for validation at the transcription and translational levels based on the degree of fold-change, relevance to the HPV life-cycle, and protein association network analysis. *KLK8* and *RPTN* were selected for validation as they were amongst the top downregulated genes and are both involved in epithelium development. It is not surprising to see many genes involved in epithelium development to be affected by HPV infection since the virus infects, replicates, and assembles in the epithelium. In particular, HPV is not a lytic virus and is released from the epidermis via desquamation. Repetin was downregulated 57.5-fold with HPV16 infection in our microarray analysis and this downregulation was validated with qPCR, western blot, and IF staining. Repetin is a component of the epidermal differentiation complex, and is involved in the formation of the cornified cell envelope (CE) (62). The CE is an insoluble matrix of covalently linked proteins formed beneath the plasma membrane of differentiating keratinocytes and plays an important role in the skin's function as a protective physical barrier against the external environment (77). In the context of HPV infection, the CE may hinder virion release because of its function as a physical barrier. Additionally, a previous study has shown that CEs of epithelial tissue infected with

451 HPV11 are thinner and more fragile compared to those of healthy tissue (78).
452 Therefore, it can be speculated that downregulation of Repetin by HPV may be a
453 strategy to weaken the CE and increase the efficiency of virion release.

454 *KLK8* was downregulated 25.8-fold in our microarray analysis, which was
455 validated with qPCR and western blot. *KLK8* was the most significantly downregulated
456 gene of the seven KLK genes that were downregulated with HPV16 infection: *KLK3*,
457 *KLK5*, *KLK6*, *KLK7*, *KLK10*, *KLK13* were downregulated 3.6, 4, 22.1, 8.6, 7.5, 21.5-fold,
458 respectively. A previous gene expression study has also identified a cluster of KLK
459 genes (*KLKs 5, 6, 7, 10, 11*) that are downregulated at early stages of HPV16 infection
460 (22). KLKs are a family of 15 serine proteases that are clustered on chromosome
461 19q13.4 and one of their main functions is cleaving corneodesmosomal adhesion
462 molecules in the cornified layer of the epidermis, which allows regulated desquamation
463 of keratinocytes (61, 79–81). It is counterintuitive that HPV16 infection downregulates
464 KLKs since the virus is released via desquamation. We speculate that the rate at which
465 normal epithelium desquamates is faster than the rate at which HPV virions mature in
466 the cornified layer, and therefore, the virus may be downregulating KLKs in order to
467 impede desquamation and allow virions to adequately mature before being released to
468 the surrounding environment. *KLK8* also plays a role in activation of the antimicrobial
469 peptide LL-37 and thus, HPV16 may be downregulating the protein in order to prevent
470 antimicrobial reaction (61, 82).

471 SERPINB4, also known as squamous cell carcinoma antigen 2 (SCCA2), is a
472 member of the serpin family of serine protease inhibitors and was downregulated 48-
473 fold in our microarray analysis. The downregulation in microarray analysis was

validated with qPCR, western blot, and IF staining. SERPINB4 along with SERPINB3 (squamous cell carcinoma antigen 2; SCCA1) have been shown to be overexpressed in various types of cancers including cervical, esophageal, lung, breast, and liver cancers (65, 67–69, 83–85). One of the mechanisms through which SERPINB4 contributes to tumor maintenance is the inhibition of granzyme M-induced cell death(86). Additionally, SERPINB4 overexpression is associated with inflammatory diseases including psoriasis and atopic dermatitis (64, 66, 87–89). Remarkably, *KLK8* shares the same expression pattern in these diseases: while our microarray analysis shows significant downregulation during productive HPV16 infection, the overexpression of *KLK8* has also been associated with both squamous cell carcinomas and inflammatory skin diseases, such as psoriasis and atopic dermatitis (71–74, 90). We speculate that the virus downregulates *SERPINB4* and *KLK8* during productive infection as part of a broad effort to dampen the inflammatory response. Of particular note is that the two genes are overexpressed in various cancers while in our study they are amongst the top downregulated genes during productive HPV infection. Additionally, we have identified nine other genes that are significantly downregulated in our microarray analysis, but have shown to be overexpressed or contribute to disease progression in various types of cancers (Table 4). This highlights the fact that early productive stages of HPV infection present a vastly different microenvironment and disease state from the cancerous stages of infection when virion production is significantly decreased. This suggests that KLK8 and SERPINB4 may interfere with the HPV life-cycle or contribute to the immune surveillance against the virus, and therefore, are downregulated during productive infection. In contrast, the two proteins may be necessary for tumor

497 maintenance, and therefore, overexpressed during the cancerous stages of infection.
498 However, since we did not measure levels of expression of these proteins at cancerous
499 stages we cannot definitively compare the protein levels between precancerous and
500 cancerous stages. A direct comparison would require creating organotypic raft cultures
501 with cervical cancer cell lines and measuring SERPINB4 and KLK8 protein levels. In
502 future studies, we aim to investigate the mechanism of *KLK8* and *SERPINB4*
503 downregulation, and how the two genes affect HPV entry, intracellular trafficking,
504 replication, and assembly.

505 UBC was the central protein in the network of inflammatory genes that were
506 significantly downregulated with persistent HPV16 infection (Fig. 3D). Although qPCR
507 did not show a statistically significant modulation of *UBC* expression, western blot of
508 ubiquitin showed downregulation of monoubiquitin and differential pattern of protein
509 ubiquitination while IF staining showed downregulation of the protein with persistent
510 infection (Fig. 5, Fig. 6). Since ubiquitin is involved in numerous biological processes it
511 is hard to conclude which specific pathways are affected with HPV16 infection.
512 However, many proteins that are associated with UBC in the protein association
513 network (Fig. 3D) are a part of the NFKB pathway, and we speculate that UBC plays a
514 central role in downregulating this pathway especially since it has been shown that
515 ubiquitin-proteasome pathway plays a role in NF-κB activation. In future studies, we
516 would like to investigate the role of *UBC* in relation to top downregulated genes
517 associated with inflammation, such as *SERPINB4* and *KLK8*.

518 In conclusion, our study shows for the first time global gene expression changes
519 with productive HPV16 infection in an organotypic raft culture model. With gene

ontology analysis, broad gene categories were identified that were significantly modulated with persistent HPV16 infection, and these results were largely consistent with what previous studies have reported. In particular, we identified top downregulated genes that have not yet been extensively studied in the context of HPV infection, and that have potential to be developed as therapeutic targets or biomarkers. Moreover, expression patterns of *SERPINB4* and *KLK8* highlighted that precancerous and cancerous stages of HPV infection are two distinct disease states. Although some of the observed gene modulations were consistent with what was expected from results of previous studies, we also observed novel changes that have not been reported before. We attribute these new findings to our unique model of organotypic raft cultures at early stage HPV16 infection, which allows the virus to go through its complete life-cycle. Future studies investigating how the regulation of *SERPINB4* and *KLK8* changes throughout the different stages of infection may shed light on unidentified mechanisms of HPV persistence and tumorigenesis.

Materials and Methods

Creating cervical cell lines and organotypic raft cultures. Primary human cervical keratinocytes (HCK) were isolated from cervical biopsies as previously described (91). The Human Subjects Protection Office of the Institutional Review Board at Penn State University College of Medicine screened our study design for exempt status according to the policies of this institution and the provisions of applicable federal regulations and, as submitted, found not to require formal IRB review because no human participants are involved as defined by the federal regulations.

HCK cell lines persistently infected with HPV16 (16HCK) were produced by electroporating primary HCK with HPV16 plasmid DNA as previously described (92, 93). The electroporated cells were cultured with mitomycin-C-treated (Enzo Life Sciences) J2 3T3 feeder cells as previously described (91).

Organotypic raft cultures were grown as previously described (93, 94) at first or second passage for primary HCK and sixth to ninth passage for 16HCK. The raft tissues were harvested after 10 days for microarray analysis and 20 days for qPCR, Western blot, and immunofluorescence staining. Viral gene expression has been shown to peak between 10 and 12 days (95) while virion maturity reaches maximum stability around 20 days (96).

Microarray analysis. Raft tissue from primary HCK and 16HCK were harvested at 10 days and RNA was extracted using the RNeasy Fibrous Tissue Midi Kit (Qiagen). The experiment was conducted with three primary HCK and three 16HCK samples in duplicates. Microarray analysis was performed using the Illumina HumanHT-12 v4 Expression Beadchip (Illumina, San Diego, CA), which targets over 31,000 annotated genes with more than 47,000 probes derived from the National Center for Biotechnology Information (NCBI) RefSeq Release 38 (November 7, 2009) and other sources. RNA quality and concentration were assessed using an Agilent 2100 Bioanalyzer with RNA Nano LabChip (Agilent, Santa Clara, CA). cRNA was synthesized by TotalPrep Amplification (Ambion, Austin, TX) from 500 ng of RNA according to manufacturer's instructions. T7 oligo (dT) primed reverse transcription was used to produce first strand cDNA. cDNA then underwent second strand synthesis and RNA degradation by DNA Polymerase and RNase H, followed by filtration clean up. In vitro transcription (IVT)

566 was employed to generate multiple copies of biotinylated cRNA. The labeled cRNA was
567 purified using filtration, quantified by NanoDrop, and volume-adjusted for a total of 750
568 ng/sample. Samples were fragmented, and denatured before hybridization for 18
569 hours at 58°C. Following hybridization, the beadchips were washed and fluorescently
570 labeled. Beadchips were scanned with a BeadArray Reader (Illumina, San Diego, CA).

571 The CLC Genomics Workbench 4.8 package
572 (https://www.qiagenbioinformatics.com/) was used to determine the significantly
573 differentially expressed genes of the HPV16+ versus primary tissue. For each
574 comparison, quantile normalization was performed followed by pairwise homogeneous
575 t-test resulting in normalized fold changes and p-values. Significantly differentially
576 expressed genes were considered to be those with $p < 0.05$ and absolute fold change
577 >= 1.5.

578 **Gene ontology analysis and protein association network.** In order to
579 categorize the significant gene expression changes into gene ontology (GO) groups the
580 GOrilla package was used (http://cbl-gorilla.cs.technion.ac.il/) (29). Two unranked lists
581 of genes, target (significantly modulated genes) and background (all genes in the
582 microarray), were used to identify significantly enriched GO terms. We focused on the
583 sub-ontology Biological Processes for our analysis. REVIGO was used to further
584 summarize the redundancy in the GO analysis (http://revigo.irb.hr/) (30). In our analysis,
585 we used the similarity coefficient of 0.7 (medium size list) to summarize the GO list.

586 In order to identify protein-protein associations amongst the upregulated and
587 downregulated genes, the online tool STRING (https://string-db.org/) was used (48).
588 Genes from similar GO categories were pooled together to form protein association

networks of “cell cycle” and “DNA metabolism” for the upregulated genes, and “skin development,” “immune response,” and “cell death” for the downregulated genes.

Viral Titers (DNA encapsidation assay). Viral titers of each raft experiment were measured with the qPCR-based DNA encapsidation assay as previously described (96, 97).

RT-qPCR. RT-qPCR was used in order to measure levels of transcription of SERPINB4, KLK8, RPTN, CDK2, and UBC. The experiment was conducted with three primary HCK and three 16HCK samples in duplicates. For *SERPINB4*, forward primer 5’-ATTTCCTGATGGGACTATTGGCAATG-3’, reverse primer 5’-CAGCAGCACAATCATGCTTAGA-3’, and probe 5’-/56-FAM/ACGACACTG/ZEN/GTTCTTGTGAACGCA/3IABkFQ/-3’ was used. For *KLK8*, forward primer 5’-TGGGTCCGAATCAGTAGGT-3’, reverse primer 5’-GCAGGAACATCCACGTCTT-3’, and probe 5’-/56-FAM/CCCTGGATT/ZEN/CTGGAAGACCTCACC/3IABkFQ/-3’ was used. For *RPTN*, forward primer 5’-CCACAAATATGCCAAAGGGAATG-3’, reverse primer 5’-GTCATTTGGTCTCTGGAGGATG-3’, and probe 5’-/56-FAM/ACTGCTCTT/ZEN/GGCTGAGTTTGGAGA/3IABkFQ/-3’ was used. For *CDK2*, forward primer 5’-GCCTGATTACAAGCCAAGTTTC-3’, reverse primer 5’-CGCTTGTTAGGGTCGTAGTG-3’, and probe 5’-/56-FAM/AGATGGACG/ZEN/GAGCTTGTTATCGCA/3IABkFQ/-3’ was used. For *UBC*, forward primer 5’-GGATTTGGGTCGCAGTTCTT-3’, reverse primer 5’-TGGATCTTTGCCTTGACATTCT-3’, and probe 5’-/56-FAM/AGGTTGAGC/ZEN/CCAGTGACACCATC/3IABkFQ/-3’ was used. TATA-binding

protein (TBP) was used as control for which forward primer 5'-CACGGCACTGATTTTCAGTTCT-3', reverse primer 5'-TTCTTGC TGCCAGTCTGGACT-3', and probe 5'-HEX-TGTGCACAGGAGCCAAGAGTGAAGA-BHQ-1-3' was used. All primers and probes were synthesized by Integrated DNA Technologies, and QuantiTect Probe RT-PCR Kit (Qiagen) was used for the PCR reactions. All RT-PCR reactions were performed using the C1000 Thermal Cycler (Bio-Rad). The thermal cycler was programmed for 30 minutes at 50 °C, then 15 minutes at 95 °C, then 42 cycles of 15 seconds at 94 °C and 1 minute at 54.5 °C.

Western blot. Raft tissue were harvested at 20 days and used to prepare total protein extracts as previously described (98). The experiment was conducted with three primary HCK and three 16HCK samples in duplicates. Total protein concentrations were measured using the Peterson protein assay as previously described (99). The total protein extracts were applied to sodium dodecyl sulfate polyacrylamide gel (8-10%) and transferred to nitrocellulose membrane, then incubated overnight at 4 °C with antibodies against SERPINB4 (Lifespan Biosciences, LS-C172681, 1:2000 dilution), KLK8 (Abnova, H00011202-M01, 1:2000 dilution), RPTN (Lifespan Biosciences, LS-B17, 1:2000 dilution), KRT23 (Abcam, ab117590, 1:2000 dilution), and ubiquitin (Cell Signaling, 3933S, 1:2000 dilution). GAPDH antibody (Santa Cruz, sc-47724, 1:1000 dilution) was used as control. The membranes were then washed and incubated with horseradish peroxidase-linked secondary antibody (GE Healthcare, NA931VS/NA934VS) and developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare). Densitometry analysis was conducted by normalizing the protein expression levels to GAPDH.

635 **Immunohistochemistry and Immunofluorescence Staining.** Raft cultures
636 were grown for 20 days and fixed in 10% buffered formalin, embedded in paraffin, and
637 4-µm cross-sections were prepared. A section from each sample was stained with
638 hematoxylin and eosin as previously described (26).

639 For Immunofluorescence staining, the slides were submerged in xylenes for
640 deparaffinization, and then were rehydrated. Antigen retrieval was achieved by
641 submerging the slides in Tris-EDTA buffer (pH 9) in a 90 °C water bath for 10 minutes.
642 The slides were then rinsed with TBS-Tween and blocked with Background Sniper
643 (Biocare Medical). The slides were then stained with the primary antibody overnight at
644 4 °C. Each sample was stained with antibodies against SERPINB4 (Lifespan
645 Biosciences, LS-C172681, 1:2000 dilution), RPTN (Lifespan Biosciences, LS-B17,
646 1:1000 dilution), KRT23 (Abcam, ab117590, 1:500 dilution), and Ub (Cell Signaling,
647 3933S, 1:750 dilution). The slides were then rinsed with TBS-Tween 3 times and
648 stained with secondary antibody (Life Technologies, Alexa Fluor 488) diluted 1:200 for 1
649 hour at room temperature. Next, the slides were stained with Hoechst nuclear stain
650 (1:5000 dilution) for 15 minutes and rinsed with TBS-Tween twice. All antibodies were
651 diluted in Da Vinci Green diluent (Biocare Medical). The experiment was conducted
652 with three primary HCK and three 16HCK samples in duplicates. A Nikon Eclipse 80i
653 microscope and NIS Elements version 4.4 software was used to acquire images.

654 **Statistical analysis.** In order to establish statistical significance in qPCR data
655 and Western blot densitometry analysis, t-test was used with a p-value cutoff of $p < 0.05$.

656 **Accession number.** The GEO accession number for our microarray data is
657 GSE109039.

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1068 Table 1. Viral titers of organotypic rafts

Raft Identification	Viral particles per raft
16HCK-1, replicate 1	9.38×10^7
16HCK-1, replicate 2	1.18×10^7
16HCK-2, replicate 1	5.25×10^8
16HCK-2, replicate 2	6.5×10^9
16HCK-3, replicate 1	5.81×10^7
16HCK-3, replicate 2	1.6×10^9

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1071 Table 2. Top 50 upregulated genes with productive HPV16 infection

GENE	FUNCTION	FOLD CHANGE
MT1G	Metallothioneins have a high content of cysteine residues that bind various heavy metals; these proteins are transcriptionally regulated by both heavy metals and glucocorticoids.	12.032

MT1H	Mineral absorption/ metabolism	7.679
TGFBR3	Binds to TGF-beta. Could be involved in capturing and retaining TGF-beta for presentation to the signaling receptors	7.295
KLHL35	Kelch-like family member. Kelch-repeat β-propellers are generally involved in protein-protein interactions	5.814
DLK2	Calcium ion binding and protein dimerization activity	5.057
FBLN1	Cell adhesion/ migration/ ECM architecture organization	5.056
ASS1	Arginine biosynthetic pathway	4.446
GPER	G Protein coupled estrogen receptor; cAMP signaling, calcium mobilization	4.446
TDRD9	Probable ATP binding RNA helicase	4.393
TWIST1	Transcription factor, role in cell lineage determination and differentiation	4.208
FANCE	Member of the Fanconi anemia complementation group E	4.183
JAM3	Cell to cell adhesion in epithelial and endothelial cells	4.169
CDKN2A	Cell cycle regulation	4.122
TSPAN4	Cell surface protein that mediate cell development,	3.733

	activation, growth and motility	
LTBP4	Involved in assembly, targeting and activation of TGFB1	3.718
ERAP2	Aminopeptidase involved in peptide trimming, MHC class I presentation	3.551
LOC341230	Similar to argininosuccinate synthetase	3.454
MXRA5	Matrix remodeling associated proteins	3.281
RPS23	Component of the 40S ribosomal subunit	3.23
C14orf132	Putative uncharacterized protein	3.165
CRIP2	Putative transcription factor with two LIM zinc binding domains	3.148
ANGPTL2	Member of the vascular endothelial growth factor family	3.061
GOLPH4	Involved in endosome to Golgi protein trafficking	3.058
DPYSL2	Cytoskeletal remodeling, endocytosis	3.013
CDC7	Checkpoint control kinase critical for G1/S transition	2.981
FAM134B	ER anchored autophagy receptor	2.947
NASP	Histone binding protein with a role in cell division, cell cycle progression and proliferation	2.885
HEG1	Calcium ion binding receptor component, may act	2.866

	through stabilization of endothelial cell junctions	
ZCWPW1	Zinc finger domain containing protein	2.8
MTE	Metallothioneins have a high content of cysteine residues that bind various heavy metals.	2.786
MDC1	Required for checkpoint mediated cell cycle arrest in response to DNA damage	2.783
CDH13	Calcium dependent cell adhesion proteins	2.775
OLFML2A	Extracellular matrix binding protein	2.762
LOC392871	Undetermined gene ortholog	2.728
CYBRD1	Ferric chelate reductase	2.726
RASIP1	Ras interacting protein required for the formation of vascular structures	2.721
LOC729137	Undetermined gene ortholog	2.7
GPNMB	Type 1 transmembrane glycoprotein	2.693
MOBKL2B	May regulate the activity of kinases	2.672
LOC388494	Undetermined gene ortholog	2.645
C10orf54	Putative uncharacterized protein	2.644
E2F2	E2F family of transcription factors	2.572

LFNG	Transferase enzyme	2.571
LOC100133866	Undetermined gene ortholog	2.568
NFIX	DNA binding protein, capable of activating transcription and replication	2.56
P4HTM	Prolyl hydroxylases	2.553
RELL1	Receptor expressed in lymphoid tissue like 1	2.551
FOXQ1	FOX genes involved in gene development, cell proliferation and tissue specific gene expression	2.53
TJAP1	Tight junction associated protein	2.527
HOXA11AS	Non-coding RNA gene	2.525
FN3KRP	Phosphorylates psicosamines and ribulosamines	2.514

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1074 Table 3. Top 50 downregulated genes with productive HPV16 infection

GENE	**FUNCTION**	**FOLD CHANGE**
RPTN	Involved in the cornified cell envelope formation. Multifunctional epidermal matrix protein. Reversibly binds calcium.	-57.487

SERPINB4	May act as a protease inhibitor to modulate the host immune response against tumor cells	-48.016
TCN1	Binds Vit B12 and protects it from the acidic environment of the stomach	-34.735
CST6	Active cysteine protease inhibitors. Loss of function is associated with progression of primary tumor to a metastatic phenotype	-27.758
KLK8	Serine proteases with diverse physiological function	-25.804
CEACAM6	GPI anchored glycoprotein and plays a role in cell adhesion	-24.469
KLK6	Serine proteases regulated by steroids	-22.088
KLK13	Expression regulated by steroid hormones and important marker for breast cancer	-21.464
RNASE7	Pancreatic ribosomal protein; broad spectrum antimicrobial activity against bacteria and fungi	-20.029
PRSS3	Digestive protease specialized for the degradation of trypsin inhibitors. In the ileum, may be involved in defensin processing, including DEFA5	-19.968
LCE1D	Precursors of the cornified layers of the stratum corneum	-17.868
MMP9	Zinc dependent endopeptidases and major proteases	-17.142

	involved in the degradation of the ECM	
FLJ22662	Weak phospholipase activity. May act as an amidase or peptidase	-14.394
TMEM45B	Transmembrane protein 45B	-13.469
DMKN	Expressed in differentiated layers of the skin. Upregulated in inflammatory disease	-13.186
C6orf15	Uncharacterized protein coding gene	-12.641
TMEM45A	Paralog of TMEM45B	-12.629
PNLIPRP3	Triglyceride lipase activity	-11.232
KRT23	intermediate filament protein	-11.074
ANXA9	Calcium dependent phospholipid binding protein; binds ECM proteins	-10.728
LCE3C	Precursors of the cornified layers of the stratum corneum	-10.044
S100A7	Calcium binding proteins involved in cell cycle progression and cellular differentiation	-9.88
EPS8L1	Substrate for the Epidermal growth factor receptor	-9.659
RORA	Member of NR1 family of nuclear hormone receptors	-9.136
LCN2	Iron trafficking protein involved in apoptosis, innate immunity and renal development	-9.134

LCE1E	Precursors of the cornified layers of the stratum corneum	-9.067
SPNS2	Sphingolipid transporter with critical function in cardiovascular, immunological and neuronal development	-8.787
FAM3D	Related to cytokine activity	-8.734
KLK7	Member of the kallikrein family of serine proteases	-8.61
HMOX1	Heme oxygenase, an essential enzyme in heme catabolism	-8.548
RNF39	Role in early phase of synaptic plasticity	-8.5
SH3BGRL2	Protein disulphide oxidoreductase activity	-8.478
C1orf68	Uncharacterized protein coding gene	-8.417
POF1B	Key role in organization of the epithelial monolayers by regulating the actin cytoskeleton	-8.233
ATP6V1C2	Subunit of the peripheral V1 complex of the vacuolar ATPase	-8.232
MMP10	Zinc dependent endopeptidases and major proteases involved in the degradation of the ECM	-7.953
LOC730833	Undetermined gene ortholog	-7.935
CAPNS2	Calcium regulated thiol protease, role in tissue remodeling and signal transduction	-7.64

KLK10	Member of the kallikrein family of serine proteases	-7.553
IL1RN	Inhibits the activity of Interleukin-1	-7.499
ABCG1	Intracellular lipid transport	-7.408
THEM5	Role in mitochondrial fatty acid metabolism	-7.287
CYP2J2	Role in arachidonic acid metabolism	-7.259
PRSS2	Serine protease involved in defensin processing	-7.239
LOC645869	Undetermined gene ortholog	-6.803
PSG4	May play a role in regulation of innate immune system	-6.559
FAM63A	Role in protein turnover, deubiquitinase activity	-6.52
MAP2	Stabilizing/stiffening of microtubules	-6.474
SLC5A1	Na^+ /glucose cotransporter	-6.468
IL1F7	Interleukin 1 related gene	-6.392
EPB41L3	Tumor suppressor that inhibits cell proliferation and promotes apoptosis	-6.356

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1077 Table 4. Differential gene expression between productive HPV16 infection and cancers.

Gene	**Expression fold-**	**Cancers in which gene expression**

	change in productive HPV infection	**increases and/or promotes disease progression**
SERPINB4	-48.0	HNSCC (65), cervical (67, 69), esophageal (68), lung (100), liver (85), breast (84)
KLK5	-3.2	Bladder (101), ovarian (102), lung (103), breast (104), oral squamous cell carcinoma (OSCC) (73)
KLK6	-22.1	Bladder (101), ovarian (102), colorectal (105), head and neck squamous cell carcinoma (HNSCC) (106), pancreatic (107)
KLK8	-25.8	Bladder (101), ovarian (102), salivary gland (108), cervical (74), OSCC (73), colorectal (107)
KLK10	-7.6	Ovarian (102), OSCC (73), pancreatic (107), colorectal (107)
KLK13	-21.5	Lung (109), ovarian (110)
MMP9	-17.1	Cervical (38), breast (111), HNSCC (112), prostate (113)
MMP10	-8.0	OSCC (114), HNSCC (115), esophageal

		(116)
LCN2	-9.1	Breast (40, 41), esophageal (42), pancreatic (43), ovarian (44), colorectal (45), thyroid (46)
CTSD	-1.5	Breast (117), ovarian (118)
CTSV	-2.8	Breast (119), colorectal (119)

Figure Legends

FIG 1. Heat map of significantly modified genes. Raft experiments were set up with three primary HCK and 16HCK cell lines in duplicates, and the tissue were harvested at 10 days of culture for RNA extraction and microarray analysis. The heat map shows genes that were significantly modulated ($p < 0.05$) at least 5-fold with HPV16 infection.

FIG 2. Enriched GO terms with lowest p-values. GOrilla was used to perform initial GO analysis of our microarray data, and REVIGO was used to summarize the enriched GO genesets from the upregulated (A) and downregulated (B) list of genes. The entire list of GO terms can be found in Table S2 and Table S3. Met. proc.: metabolic process.

FIG 3. Protein association networks. Online tool STRING was used to create protein functional association networks of upregulated (A-B) and downregulated (C-E) genes with >1.5-fold modulation ($p<0.05$). (A) Genes from GO categories related to cell cycle

regulation were combined to create the network. (B) Genes from GO categories related to DNA metabolism were combined to create the network. (C) Genes from GO categories related to skin development and differentiation were combined to create the network. (D) Genes from GO categories related to inflammation and immune response were combined to create the network. (E) Genes from GO categories related to apoptosis and cell death were combined to create the network. Thicker and darker lines represent greater confidence in protein interaction based on supporting data. Genes that had no connection to any other gene within the network were excluded from the diagram.

FIG 4. RT-PCR of genes of interest. Raft experiments were set up with three 16HCK cell lines in duplicates and six primary HCK cell lines. The raft tissues were harvested at 20 days of culture. RNA was extracted from the tissues and RT-PCR was performed to measure transcription levels of SERPINB4, KLK8, RPTN, CDK2, and UBC. Transcription levels of TATA-binding protein (TBP) was used as control to normalize each measurement (statistical significance $p<0.05$).

FIG 5. Western blot analysis of raft tissue. (A) Protein expression of SERPINB4, KLK8, RPTN, and KRT23 were tested with Western blots in primary HCK and 16HCK raft tissue harvested at 20 days of culture. Densitometry analysis was conducted on the Western blots (statistical significance $p<0.05$). (B) Protein expression of Ubiquitin was tested with Western blot in primary HCK and 16HCK raft tissue harvested at 20 days of culture. Experiments were conducted in three 16HCK cell lines in duplicates and six primary HCK cell lines. GAPDH was used as control. Images were acquired with Bio-Rad ChemiDoc MP Imaging System and Image Lab Version 6.0.0 software.

FIG 6. Immunofluorescence staining of raft tissue. The spatial protein expression of SERPINB4, RPTN, KRT23, and Ubiquitin was observed by performing immunofluorescence staining of primary HCK and 16HCK raft tissue fixed at 20 days of culture (green: target protein, blue: nuclear staining). Experiments were conducted in three 16HCK cell lines in duplicates and six primary HCK cell lines. Magnification 200×. H&E: hematoxylin and eosin staining. Images were acquired with Nikon Eclipse 80i microscope and NIS Elements version 4.4 software.

Supplemental Material

Table S1. Genes significantly modulated with HPV16 infection in human cervical tissue.

Table S2. GO analysis of genes upregulated with HPV16 infection in human cervical epithelium. GO analysis performed with GOrilla and summarized with REVIGO.

Table S3. GO analysis of genes downregulated with HPV16 infection in human cervical epithelium. GO analysis performed with GOrilla and summarized with REVIGO.

Table S4. Upregulated genes: GO terms related to cell cycle.

Table S5. Upregulated genes: GO terms related to DNA metabolism.

Table S6. Downregulated genes: GO terms related to skin development.

Table S7. Downregulated genes: GO terms related to inflammation.

Table S8. Downregulated genes: GO terms related to cell death.

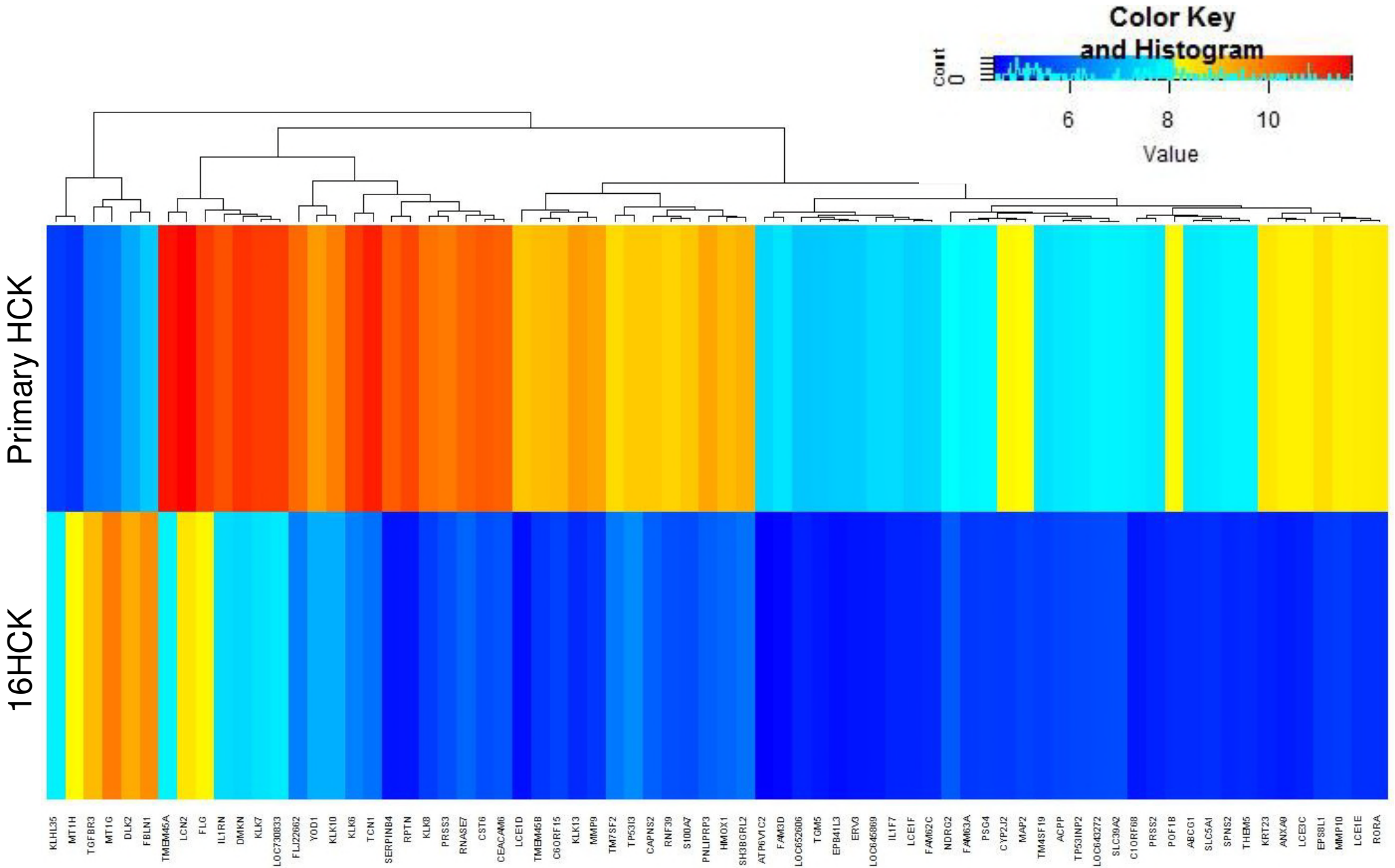


FIG 1. Heat map of significantly modified genes. Raft experiments were set up with three primary HCK and 16HCK cell lines in duplicates, and the tissue were harvested at 10 days of culture for RNA extraction and microarray analysis. The heat map shows genes that were significantly modulated ($p < 0.05$) at least 5-fold with HPV16 infection.

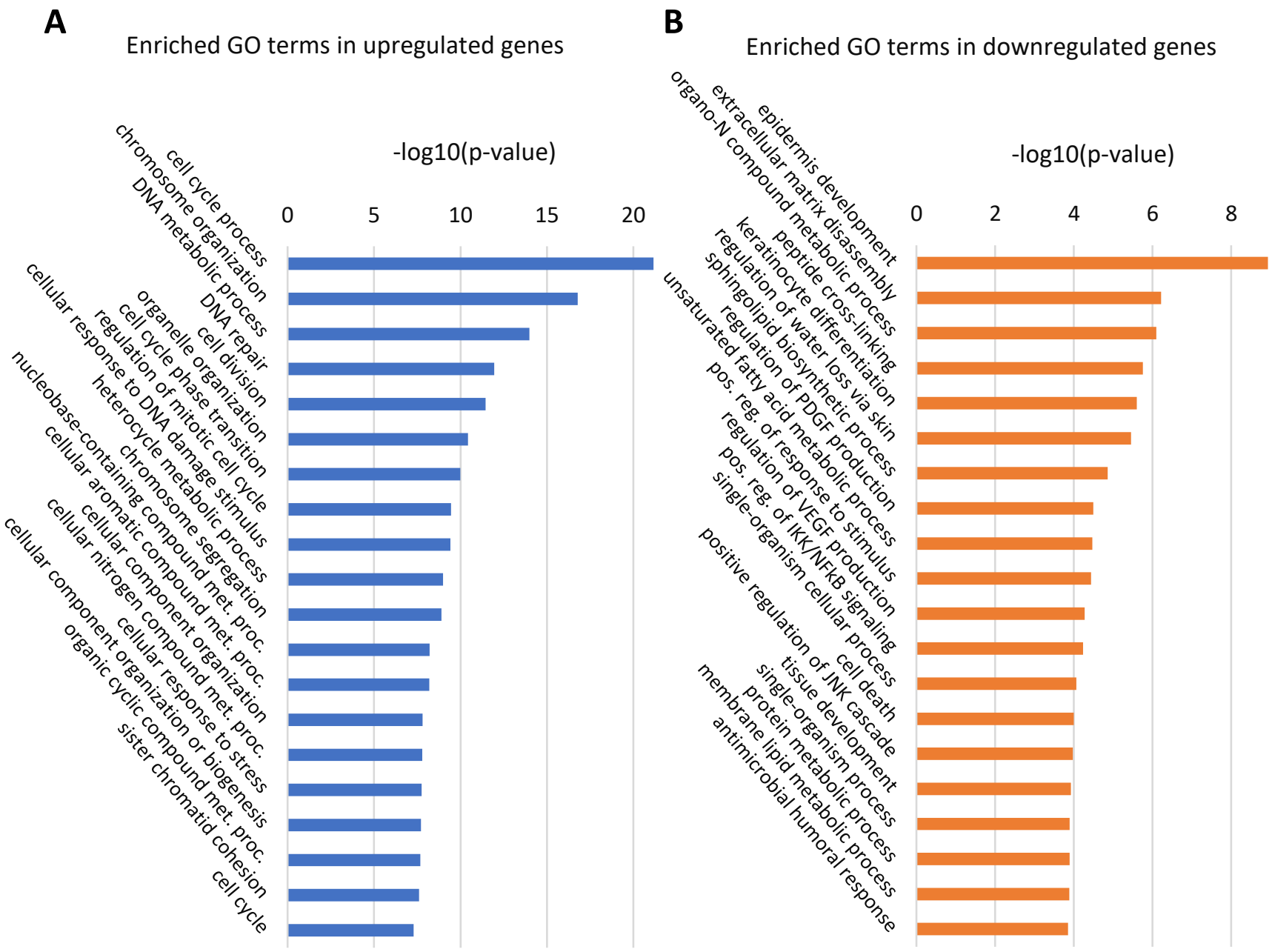


FIG 2. Enriched GO terms with lowest p-values. GOrilla was used to perform initial GO analysis of our microarray data, and REVIGO was used to summarize the enriched GO genesets from the upregulated (A) and downregulated (B) list of genes. The entire list of GO terms can be found in Supplementary Table 2 and Supplementary Table 3. Met. proc.: metabolic process.

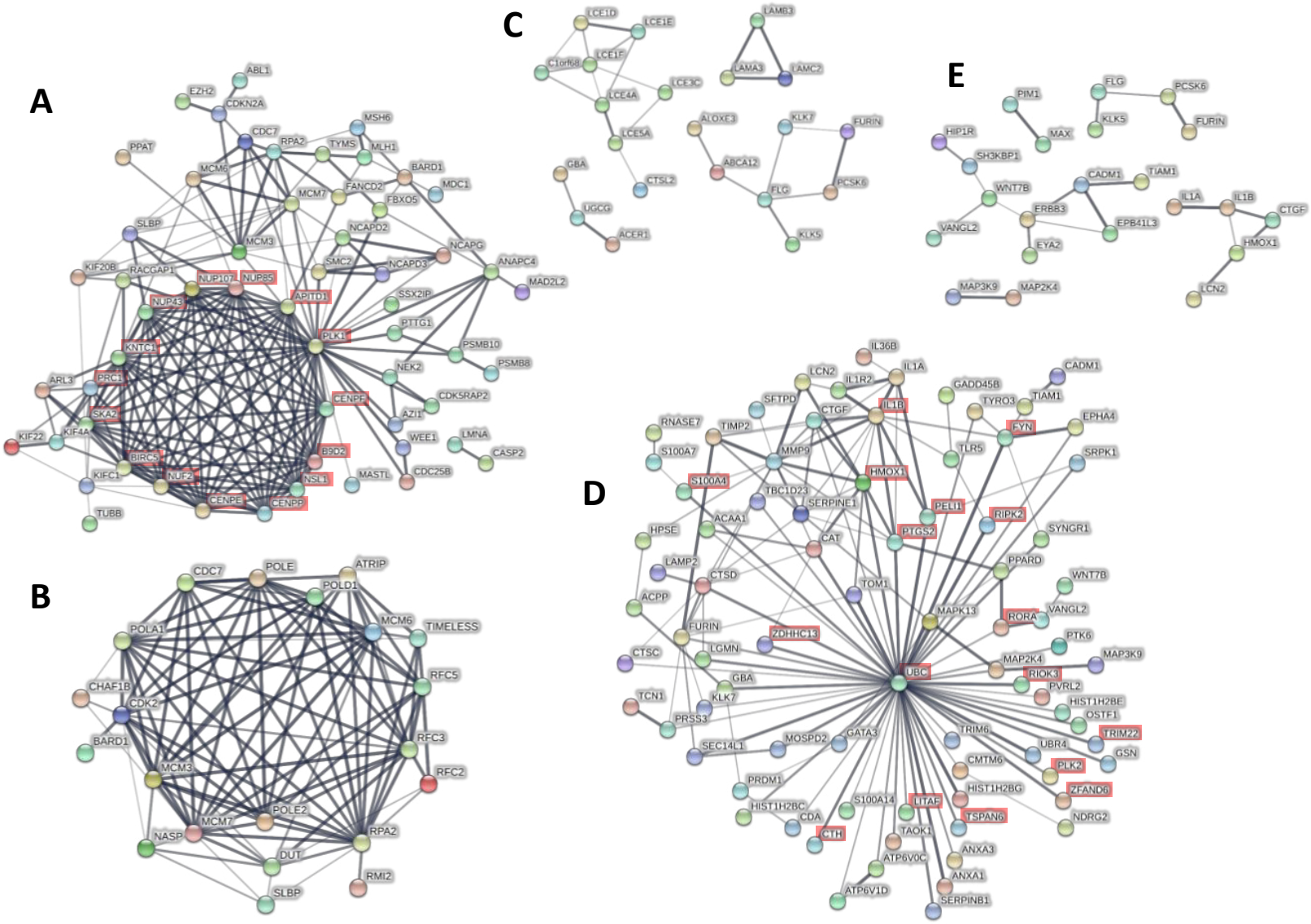
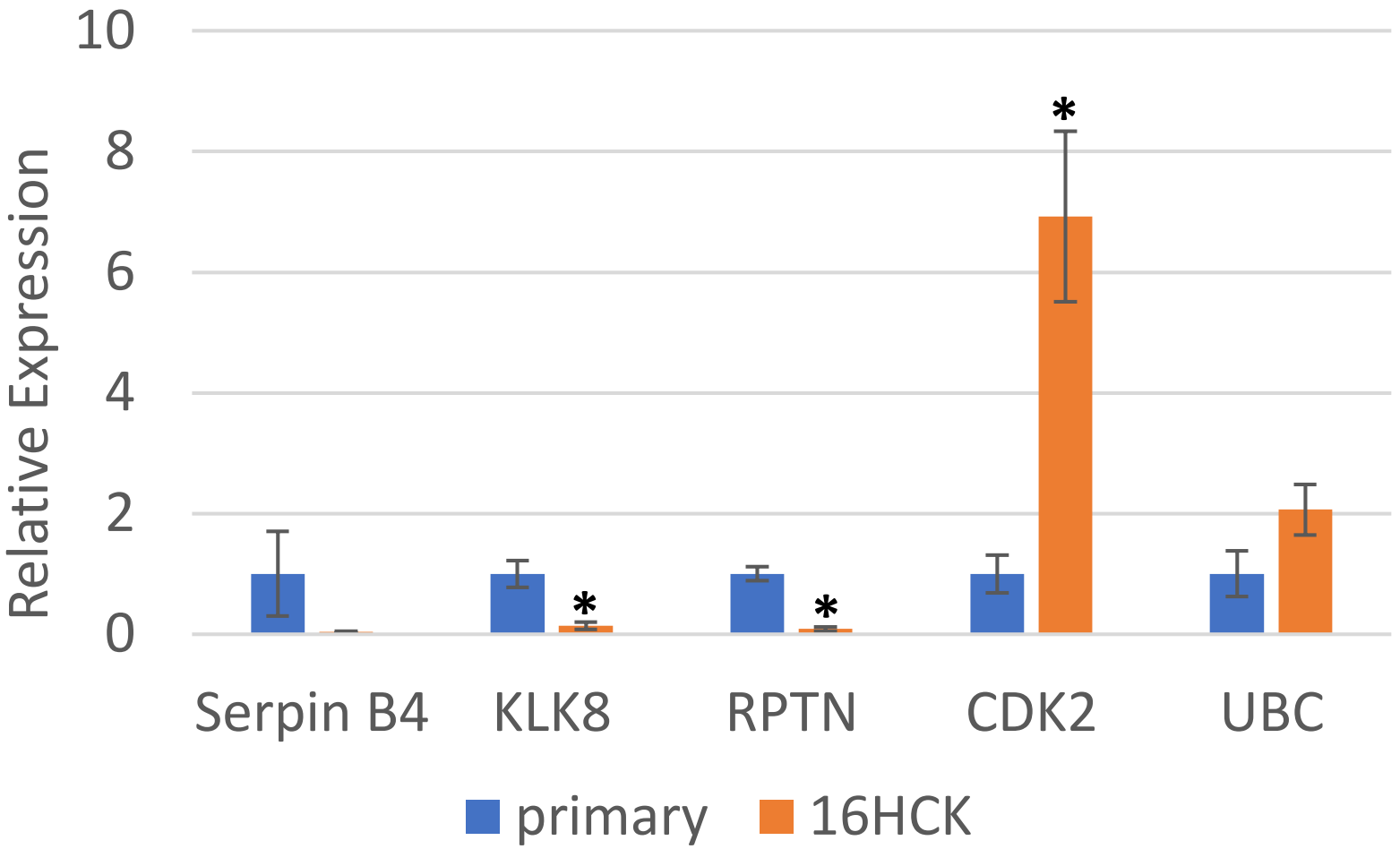


FIG 3. Protein association networks. Online tool STRING was used to create protein functional association networks of upregulated (A-B) and downregulated (C-E) genes with >1.5-fold modulation ($p<0.05$). (A) Genes from GO categories related to cell cycle regulation were combined to create the network. (B) Genes from GO categories related to DNA metabolism were combined to create the network. (C) Genes from GO categories related to skin development and differentiation were combined to create the network. (D) Genes from GO categories related to inflammation and immune response were combined to create the network. (E) Genes from GO categories related to apoptosis and cell death were combined to create the network. Thicker and darker lines represent greater confidence in protein interaction based on supporting data. Genes that had no connection to any other gene within the network were excluded from the diagram.



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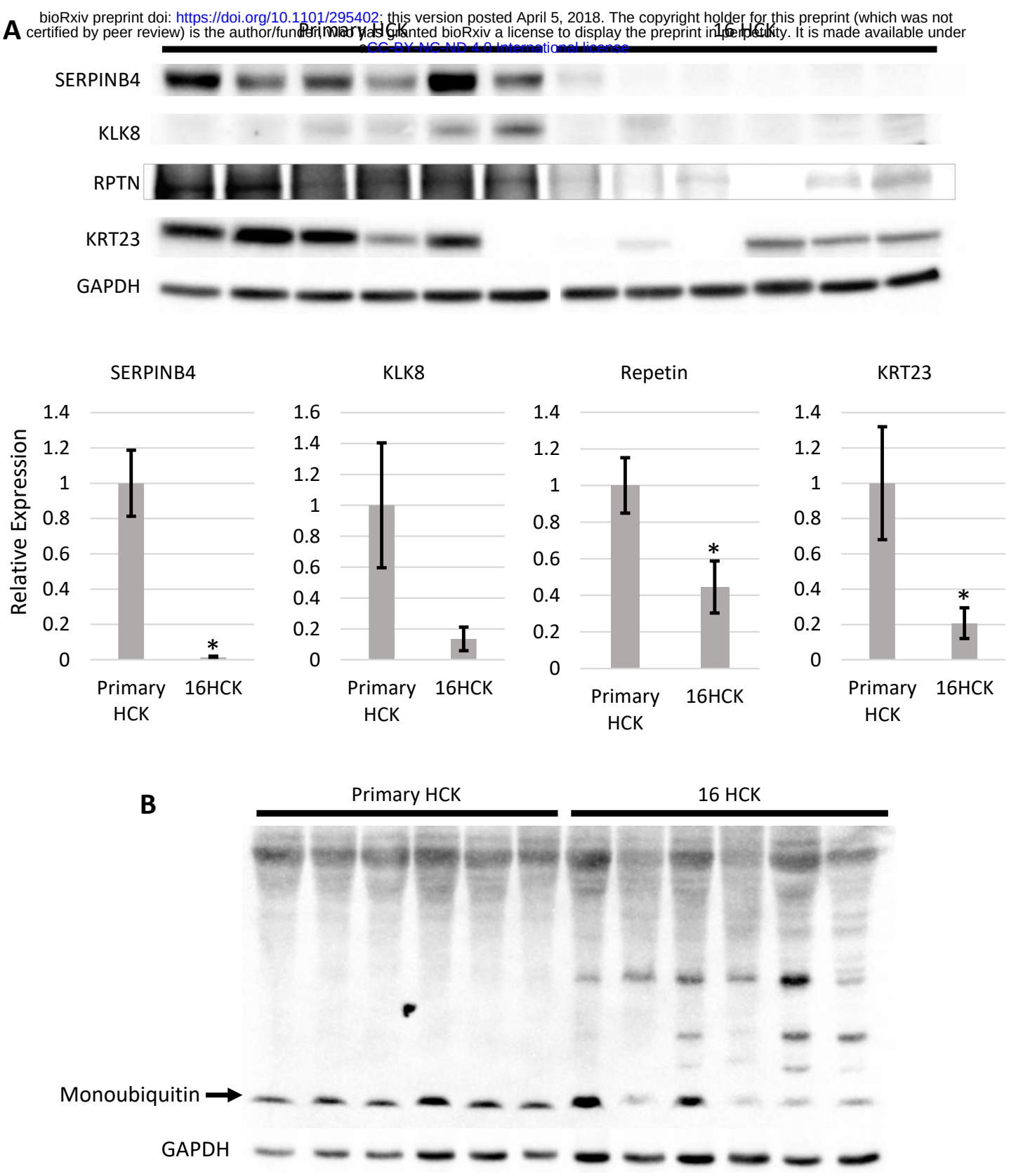

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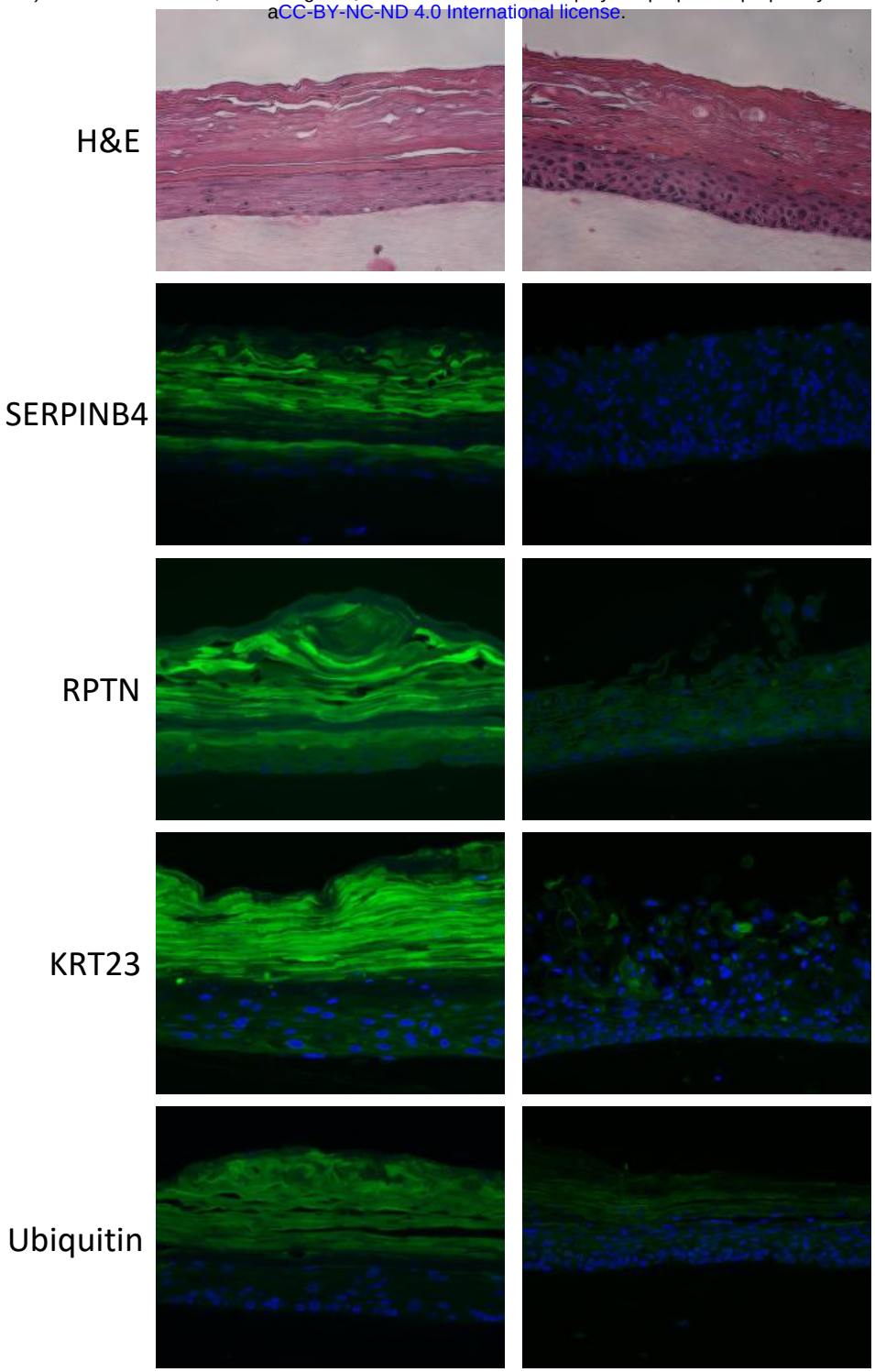

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