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RNASeq analysis of differentiated keratinocytes reveals a massive response to late events during human papillomavirus type 16 infection, including loss of epithelial barrier function.

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Abstract

The human papillomavirus (HPV) replication cycle is tightly linked to epithelial cell differentiation. To examine HPV-associated changes in the keratinocyte transcriptome, RNAs isolated from undifferentiated and differentiated cell populations of normal, spontaneously immortalised, keratinocytes (NIKS), and NIKS stably transfected with HPV16 episomal genomes (NIKS16), were compared using RNASeq. HPV16 infection altered expression of 2862 cellular genes. Next, to elucidate the role of keratinocyte gene expression in late events during the viral life cycle, RNASeq was carried out on triplicate differentiated populations of NIKS (uninfected) and NIKS16 (infected). Of the top 966 genes altered (>$\log_2$ = 1.8, 3.5-fold change) 670 genes were downregulated and 296 genes were up-regulated. HPV down-regulated many genes involved in epithelial barrier function that involves structural resistance to the environment and immunity to infectious agents. For example, HPV infection repressed expression of the differentiated keratinocyte-specific pattern recognition receptor TLR7, the Langerhans cell chemoattractant, CCL20, and proinflammatory cytokines, IL1A and IL1B. However, IRF1, IFNκ and viral restriction factors (IFIT1, 2, 3, 5, OASL, CD74, RTP4) were up-regulated. HPV infection abrogated gene expression associated with the physical epithelial barrier, including keratinocyte cytoskeleton, intercellular junctions and cell adhesion. qPCR and western blotting confirmed changes in expression of seven of the most significantly altered mRNAs. Expression of three genes showed statistically significant changes during cervical disease progression in clinical samples. Taken together, the data indicate that HPV infection manipulates the differentiating keratinocyte transcriptome to create an environment conducive to productive viral replication and egress.

Importance

Human papillomavirus (HPV) genome amplification and capsid formation takes place in differentiated keratinocytes. The viral life cycle is intimately associated with host cell differentiation. Deep sequencing (RNASeq) of RNA from undifferentiated and differentiated uninfected and HPV16-positive keratinocytes showed that almost 3000 genes were differentially expressed in keratinocyte due to HPV16 infection. Strikingly, the epithelial barrier function of differentiated keratinocytes, comprising keratinocyte immune function and cellular structure, was found to be disrupted. These data provide new insights into virus-host interaction crucial for production of infectious virus and reveal that HPV infection remodels keratinocytes for completion of the virus replication cycle.

Key words: human papillomavirus type 16, epithelial differentiation, keratinocyte transcriptome, cervical disease.

Introduction

Human papillomaviruses (HPVs) infect keratinocytes, causing mainly benign lesions or warts (1). Infection is usually transient and is cleared by the immune system (2). However, persistent infection with “high risk” HPV genotypes (HR-HPV) can cause tumour progression to cervical (3), other anogenital (anal, penile, vulvar and vaginal) (4) and oropharyngeal cancers (5). In the case of the cervix, cervical intraepithelial neoplasia (CIN) generally precedes cervical cancer progression (6). CIN1 is thought to represent a transient HPV infection, while CIN3 represents clinically significant, persistent HPV infection that may, if left untreated, progress to cervical cancer (7).

The pathway of epithelial cell differentiation, from basal to granular layer, is tightly controlled by complex patterns of keratinocyte gene expression (8). The HPV infectious life cycle is tightly linked to epithelial differentiation. HPV infects basal epithelial cells where it begins to express its genome. The viral replication factor E1 and its auxiliary protein, E2, which is also the viral transcription factor, together with the regulatory proteins E6 and E7 are expressed early in infection. E2, E6 and E7 have each been shown to control cellular gene expression (6). Viral gene expression required for vegetative viral genome amplification takes place in differentiating keratinocytes in the mid to upper epithelial layers (9). At this stage other viral regulatory proteins E4 and E5 that can regulate the host cell are expressed (6). Finally, L1 and L2 capsid protein synthesis and virion formation takes place in granular layer keratinocytes and virions are shed from the surface of the epithelium in dead squames (10). The epithelium presents a barrier to the environment and to infectious agents (11). Differentiated keratinocytes possess a dense filamentous network comprised of keratins and other molecules such as filaggrin. Moreover, keratinocytes have an important role in innate and adaptive immunity, and cytokines, chemokines and other immune signalling molecules released by these cells are essential for epithelial homeostasis (12). HPVs have evolved to modulate the epithelium to allow infection, virion formation and egress (6), and many means by which HPV evades the immune response have been documented (13). Elucidating the

interactions between HPV and the infected keratinocyte is key to understanding the HPV life cycle and how persistent infection may facilitate development of cervical disease.

A number of previous studies have used a microarray approach to further our understanding of the HPV infectious life cycle and cancer progression. The first compared gene expression in normal keratinocytes with that in HPV31-infected keratinocytes (14). Two subsequent studies examined gene expression changes during tumour progression in HPV18-infected (15) or HPV33-infected keratinocytes (16). A recent study investigated undifferentiated keratinocytes containing HPV16 or HPV18 episomal genomes. However, no studies have analysed how cellular gene expression is altered in differentiating keratinocytes supporting the productive phase of the viral life cycle (17). Here we used Next Generation Sequencing (RNASeq) to examine global changes in the keratinocyte transcriptome due to epithelial differentiation and HPV infection. Our study reveals that HPV infection induces massive changes in the transcriptome during keratinocyte differentiation. In particular, changes in many genes encoding the keratinocyte structural barrier and immune function were altered. Key statistically highly significant changes in gene expression were confirmed by RT-qPCR and western blotting and investigated in clinical samples representing the cervical disease spectrum. These data can be used to understand late events in the viral life cycle and the mechanisms behind cervical disease progression.

Results

The HPV E2 transcription factor (18) and the viral oncoproteins E6 (19), E7 (20) and E5 (21) can all play a role in controlling cellular gene expression, and HPV infection is known to have a significant effect on keratinocyte growth and differentiation (6). In order to elucidate how cellular gene expression is altered during HPV infection we examined changes in the keratinocyte transcriptome during differentiation and HPV16 infection using normal, spontaneously immortalised keratinocytes (NIKS) and the same cells stably transfected with HPV16 genomes (NIKS16). NIKS16 clone 2L maintains ~100 episomal HPV16 genomes per cell (if cultured at low passage (<13)) and forms a CIN1-like (low grade cervical disease) stratified epithelium upon raft culture, suggesting that these cells represent a transient HPV16 infection (22). We also examined a second HPV16 infection model, W12 cells, which are HPV16-infected basal cervical epithelial cells isolated from a patient with a low grade cervical lesion (23). W12 clone 20863 (W12E) cells (if cultured at low passage (<17)) also maintain ~100 episomal HPV16 genomes (24). Both cell lines are capable of differentiation. We used the differentiation protocol from Jeon et al (1995) where cells are induced to differentiation by culturing to high density in 1.2 mM Ca^{++}. Differentiated NIKS16 and W12E cell populations expressed involucrin, loricrin and keratin 10 proteins, key markers of keratinocyte differentiation, (Figure 1A). NIKS16 cells (and W12 cells (25)) expressed viral late proteins E2, E4 and L1 (Figure 1A, B). A time course of NIKS and NIKS16 differentiation over a 13 day period is shown in Figure 1C. As expected, NIKS cells (Figure 1C lanes 1-4) expressed more involucrin over the time course than NIKS16 cells (Figure 1C lanes 5-8) because HPV infection impairs epithelial differentiation (6). Absolute quantification of viral genome copies by PCR showed that differentiated W12 cells had an average of 15,250 genome copies while there was an average of 9937 copies NIKS16 cells (Figure 1D). Viral late mRNA levels, as measured by L1 open reading frame detection, the common reading frame in all late mRNAs, was increased 16.1-fold in W12 cells and 12.6-fold in NIKS16 cells

upon differentiation (Figure 1E). These data indicate that NIKS16 and W12 cells can be differentiated in monolayer culture.

Global changes in the transcriptome of HPV16-infected keratinocytes.

RNASeq was carried out using RNA prepared from undifferentiated and differentiated NIKS and NIKS16 populations. Comparing undifferentiated with differentiated uninfected NIKS, 809 mRNAs were up-regulated while 422 mRNAs were down-regulated (Figure 2A). In contrast, comparing undifferentiated to differentiated HPV16-infected NIKS16 keratinocytes, 2041 genes were up-regulated while 2052 genes were down-regulated (Figure 2B). Because NIKS16 cells are derived directly from NIKS (22) and were differentiated using the same protocol, the 2862 additional changes observed upon differentiation of HPV16-positive keratinocytes are likely attributable to HPV infection. A similar number of gene expression changes to that for NIKS16 cells were observed between undifferentiated and differentiated W12E cells (data not shown). There is no HPV-negative equivalent to W12 cells but we compared the overlap of RNASeq changes in the transcriptome of differentiated W12 cells compared to NIKS cells and NIKS16 cells compared to the parent NIKS cells. Despite the fact that these cells are of different origin, W12 is a female HPV-immortalised mucosal epithelial cell line while NIKS is a spontaneously male cutaneous epithelial cell line, there was a 41% overlap in upregulated genes (Figure 2C) and a 38% overlap in downregulated genes (Figure 2D). These data suggest that the effects of HPV infection and the differentiation process is somewhat similar both cell types.

HPV16 infection abrogates differentiation and epithelial barrier formation

We are interested in elucidating the link between keratinocyte differentiation and late events during HPV replication. Therefore, we compared the transcriptome of differentiated NIKS to NIKS16 cells. Three, replicate, single-end sequencing experiments were carried out and changes that gave a p-value>0.05 across three replicates were discarded to achieve

significance. Supplementary Table S1 lists the top 966 changes in gene expression ($p<0.05$, $\log_2>1.8$, 3.5-fold change). 670 genes were downregulated while 296 were upregulated, with a range of 184-fold downregulation to 87-fold up-regulated. The data in Figure 3 shows the mean of the results of three separate RNASeq experiments. As expected, key epithelial differentiation markers were down-regulated in NIKS16 cells (Figure 3A). Suprabasal layer keratins were also down-regulated. Keratin 12, which is usually only expressed in the corneal epithelium (26), was the only keratin whose levels were increased in NIKS16 cells (Figure 3B). Expression of cell junction proteins that are key to epithelial barrier function was significantly altered. Desmosome cell-cell junction proteins required for cell adhesion (Figure 3C) (27), and gap junction connexin (Cx) proteins 26, 30 and 32, that allow transfer of small molecules between differentiating epithelial cells (28), were down-regulated (Figure 3D). Claudin proteins control tight junctions, and CLDN3, 10 and 22 were up-regulated while CLDN11 and 17 were down-regulated (Figure 3E). Claudin upregulation can still have a negative impact on the function of tight junctions in a phenomenon referred to as “leaky claudins” (29). Several adherens junction-associated cadherins (27) were also down-regulated (Figure 3F). Small proline-rich repeat protein (SPRR) family members that contribute to barrier formation by forming the cornified layer in differentiated epithelial cells (30) were down-regulated (Figure 3G). The calcium gradient in the epithelium is altered upon loss of barrier formation (31) and levels of RNAs encoding a range of calcium ion-binding proteins (e.g. S100A8/A9 calgranulin complex, DSG1, matrix Gla protein (MGP), calcium/calmodulin kinase 2B (CAMK2B)) were reduced (Supplementary Table S3). Taken together, the data suggest that HPV infection inhibits epithelial barrier formation and epithelial integrity.

The epithelial barrier also involves immune signalling and significant changes in expression of many genes whose products are involved in intrinsic and innate immunity were also observed (Table 1). Previously, a microarray study revealed that HR-HPV repressed activation of the immune response in undifferentiated epithelial cells through IL-1β. Similarly,

in HPV-infected differentiated cells we found IL1B gene expression was down-regulated. IL1A was also down-regulated, as were IL32G and IL36B that activate keratinocyte immune functions. The Langerhans cell chemoattractant CCL20 was down-regulated in the presence of HPV16. However, CCL28 that controls T-cell homing in mucosal epithelia, E6/E7-regulated CXCL12 and CX3CL1 were all up-regulated. The type 1 IFN regulator, IRF1 and the epithelial IFNκ were up-regulated, an unexpected finding since HPVE6 and E7 have been shown to inhibit their expression (32-34). We found a 6-fold down-regulation of the viral DNA pattern recognition receptor TLR7, which is expressed specifically in differentiated keratinocytes (35), together with up-regulation of viral restriction factors APOBEC3B, IFIT1, 2, 3 and 5, CD74, OASL and RTP4 (Table 1). These data indicate that the keratinocyte-mediated immune response is under controlled of HPV16 in the upper epithelial layers, and that there are significant differences to HPV-regulation of immune signalling in differentiated, compared to basal, epithelial cells (17)

Cellular networks involved in the immune response and keratinocyte structure and metabolism are altered by HPV16 infection.

Following adjustment of the data set to exclude any changes where the triplicate values gave a p-value of >0.05, gene ontology network pathway analysis of the top 1000 up or down-regulated genes was carried out. Analysis revealed distinct gene classes whose expression was altered by HPV16-infection (Figure 4). Response to type 1 interferon was up-regulated but cytokine and chemokine expression was repressed. Cell matrix adhesion was up-regulated while cell-cell adhesion was down-regulated (reported by the Cytoscape programme as negative regulation of up-regulated leukocyte genes) (Figure 4A). Other significantly down-regulated pathways included keratinization, arachidonic acid metabolism, reactive oxygen and nitric oxide biosynthesis, VEGF and temperature homeostasis (Figure 4B). Network analysis indicated that pathways related to the type 1 interferon response were

strongly connected (Figure 4C) while down-regulated genes were associated through cytokine/chemokine/VEGF pathways (Figure 4D). A value of $\log_2$ change>2.5 was chosen to construct a wider pathway linkage diagram. IRF1 and KDR were major HPV-up-regulated genes encoding hub proteins that connected a number of cell growth and apoptosis signalling pathways. IL-1B and REL, an NFkB family transcriptional co-activator, linked HPV-down-regulated cytokine and VEGF (Figure 5, Supplementary Table S3).

Verification of gene expression changes due to HPV16 infection

Six genes from among the most statistically highly significant changes (Table 2: padj values are shown where p=0.05 in the triplicate data set is given the value of 1) were selected for further study (negative: DSG1, SERPINB3, KRT10, positive: VTCN1, KDR, AZGP1). Although IL1B had a padj =1 (actual p-value=0.05) it was also included because expression of this important cytokine was found to be a key gene network hub in both undifferentiated (17) and differentiated HPV-infected cells (Figure 5). These genes all encode proteins with known metabolic or immune/inflammatory roles in the normal epithelium. KRT10 is a differentiation-specific keratinocyte filament protein. DSG1 is a calcium-binding desmosome regulator. KDR (vascular endothelial growth factor receptor 2, VEGFR-2) has an autocrine function in cell proliferation, adhesion and migration (36). IL1B “node” cytokine activates adaptive immunity. VTCN1 is a T-cell activation inhibitor. SERPINB3 controls epithelial inflammatory responses and AZGP1 is induced by IFNγ in keratinocytes (37). mRNA expression in NIKS versus NIK16 cells and W12 cells was validated by qRT-PCR (Table 2).

Protein levels encoded by these mRNAs were examined in undifferentiated and differentiated NIKS, NIKS16 and W12 cells (Figure 6). Levels of AZGP1, KDR, DSG1,KRT10, and involucrin increased upon NIKS16 and W12 cell differentiation, while SERPINB3 levels decreased and VTCN1 levels did not change. Compared to differentiated NIKS cells, there were higher levels of VTCN1, AZGP1 and KDR in NIKS16 cells but KRT10

and DSG1 levels were much lower in differentiated NIKS16, compare to NIKS cells, as expected. SERPINB3 levels were greatly reduced following differentiation of NIKS16, but not NIKS cells. There is no HPV-negative W12 cell equivalent to NIKS cells so it is not possible to be sure if the changes in protein expression in W12 cells upon differentiation are due to HPV infection. These data confirm that selected keratinocyte transcriptomic changes due to HPV16 infection are reflected in protein levels.

HPV16 infection-regulated mRNA as biomarkers of cervical disease

It could be argued that the NIKS16 model of the HPV16 life cycle may not directly relate to cervical HPV infection because NIKS16 cells are foreskin, not cervical, keratinocytes, However, NIKS16 cells appeared to represent a low grade cervical lesion when grown in raft culture (22) and the organisation of the HPV life cycle at different anatomical sites is quite similar (38). HPV16-associated gene expression changes in keratinocytes could be related to the productive life cycle but could equally be associated with cervical disease progression. Therefore, to test whether any of the HPV-related changes in keratinocyte gene expression we detected could have potential as HPV-associated cervical disease biomarkers, we quantified levels of expression of three up- and three-down-regulated genes (two regulators of the inflammatory response (IL1B, SERPINB3), two proteins involved in cell signalling (KDR, VTCN1), and two involved in barrier function (KRT10, DSG)) by RT-qPCR in liquid based cytology (LBC, Pap smear) samples. Apart from choice due to gene function, IL1B RNA was chosen for analysis because it encoded a hub in the interactome (Figure 5), VTCN1 and DSG1 were chosen as representative of very highly significantly altered RNAs, KRT10 was chosen as a differentiation marker, KDR was chosen as an RNA potentially involved in cancer formation and SERPINB3 was chosen because it was an early-identified cervical cancer marker (39). Due to lack of mRNA we were unable to test AZGP1. A control cDNA from differentiated W12E cervical keratinocytes was included in each qPCR plate as a

standard and absolute levels of RNA in the LBC samples (normalised against GAPDH) were calculated using the Pfaffl standard curve method (40, 41). KRT17 was analysed as a known biomarker of cervical disease progression (42). Figure 6 shows the mean and range of values for each mRNA in 7 no detectable disease (NDD), 10 low grade cervical lesion (CIN1) and 10 high grade cervical lesion (CIN3) samples. Although we analysed 10 samples graded as NDD, once HPV typing status was revealed, 3 of these were HPV-positive. We decided to exclude these from the analysis in order to compare HPV-negative with HPV-positive clinical samples. KRT10 mRNA levels were very low making analysis of significance difficult, and there was high variability in levels of IL1B and VTCN1. However, very high levels of IL1B mRNA were detected in all patient samples, regardless of disease stage. DSG1 was significantly increased between no detectable disease (NDD) and low grade disease but significantly decreased between low grade and high grade disease. KDR and SERPINB3 levels were significantly up-regulated between low grade and high grade disease, similar to the positive control, KRT17. These data suggest that RNASeq analysis has potential to uncover novel biomarkers of cervical disease.

Discussion

The aim of our work is to examine how human papillomavirus replication is linked to keratinocyte differentiation. In particular we are interested in how differentiating keratinocytes respond to HPV infection during the late, productive phase of the viral life cycle. As a model to compare HPV-negative to HPV-positive keratinocytes, we used NIKS and NIKS16 cells. NIKS are spontaneously immortalised neonatal foreskin keratinocytes that have no alterations in differentiation or apoptosis (43). NIKS16 cells were derived directly from NIKS cells by stable transfection of the HPV16 genome isolated from W12 cells (22). We have shown that the NIKS16 cells adequately supported the infectious viral life cycle (as previously reported (22)) because several key markers of keratinocyte differentiation and viral life cycle completion: viral genome amplification, viral late mRNA induction and capsid protein production, were detected. Moreover, because there was repression of VEGF pathways, reduced expression of HOX and MMP proteins, and no general up-regulation of EMT markers, these cells are likely not undergoing tumour progression. Because NIKS cells are foreskin keratinocytes, they will likely have a number of differences in their gene expression profile compared to cervical keratinocytes. We did not have access to spontaneously immortalised HPV-negative cervical keratinocytes but we compared changes in W12 gene expression with NIKS cells. There was around 40% identity in the up- and down-regulated genes between NIKS16 and W12 cells. W12 cells are naturally infected, female, mucosal epithelial cells while NIKS16 cells are male cutaneous epithelial cells and spontaneously immortalised, and these significant differences likely account for the remaining 60% of non-overlapping genes. Therefore, NIKS16 is potentially a more robust model for HPV16-associated penile lesions than cervical lesions and it will be interesting in future to compare these data sets with similar sets from differentiated uninfected and infected cervical keratinocytes. 3D raft culture would undoubtedly provide a superior approach for examining keratinocyte differentiation and HPV infection. However, for analysis of late events in the viral life cycle in differentiated keratinocytes, this is technically

challenging, and difficult to reproduce, because RNA isolation from multiple, microdissected, upper epithelial layer sections would be required for triplicate RNASeq experiments. Our current dataset should provide an important basis for subsequent analysis of raft culture models.

Many transcriptomic studies have analysed cellular changes during HPV-associated tumour progression or due to overexpression of viral proteins (14, 15, 18, 21, 44-51). Of the microarray studies investigating changes due to HPV infection, as opposed to tumourigenesis, one compared expression of HPV31-positive and negative cervical keratinocytes (14), a second examined HPV33-negative and positive vaginal keratinocytes (16) while another compared undifferentiated anogenital keratinocytes with or without episomal HPV16 and 18 genomes (17). All of these studies focused on the effect of HPV on basal keratinocytes, the site of viral entry, and initial replication. No studies to date have examined keratinocyte responses to late events in the viral replication cycle. Moreover, the previous studies used microarray analysis which does not provide the unparalleled depth of information available from RNASeq. To our knowledge, this is the first report comparing the transcriptome of uninfected to HPV-infected differentiated keratinocytes using RNASeq. HPV infection induced massive changes (2862 additional expression changes compared to HPV-negative NIKS cells) in the keratinocyte transcriptome. Desmosomes, adherens, tight and gap junction classes were all down-regulated in the presence of HPV16, likely due to HPV16 E6/E7 reactivation of the cell cycle and decreased keratinocyte differentiation (52) as has been reported previously (17). Together with high level down-regulation of SPRRS, altered arachidonic acid metabolism and changes in mucins (Supplementary Table S3), one can conclude that HR-HPV infection results in a broad abrogation of epithelial barrier function and epithelial integrity. Reduced barrier function could result in increased fragility of cells in the upper epithelial layers to facilitate viral egress.

Keratinocytes are key players in the immune response, and they produce a panoply of molecules involved in host defence against pathogens. In differentiated NIKS16 keratinocytes, HPV infection altered gene expression related to innate immunity, including reduced expression of TLR7, IL1A, IL1B, NLRP3, IL36B, and IL32G. TLR7, a pattern recognition receptor for viral nucleic acids, is upregulated upon keratinocyte differentiation (35) and activates proinflammatory cytokines, and other molecules involved in the adaptive immune response. There was a 6-fold down-regulation of TLR7 in the presence of HPV16 suggesting that the virus represses pattern recognition during vegetative viral genome amplification, but by a different mechanism to that used in undifferentiated keratinocytes where infection suppresses TLR9 (17). There was a corresponding reduction in NFκB-regulated CCL20, known to be regulated by HPV E7 (53), and required to recruit Langerhans cells. Indeed, NFκB signalling was affected and the NFκB family member, REL, was a major HPV-regulated control node in the pathway analysis of negatively regulated genes (Supplementary Figure 1). Surprisingly, we discovered that the epithelial-specific IFNκ, and IRF1 that controls type 1 IFNs, were up-regulated by HPV16 in differentiated keratinocytes. Previously, HPV16 E7 or HPV38 E6E7 were shown to inhibit IRF1 expression (32, 33), while HPV16 E6 was shown to repress IFNk transcription through promoter methylation (54). However, these studies used overexpression of the viral oncoproteins. The levels of E6 or E7 proteins may be much lower in differentiated keratinocytes compared to that in the undifferentiated epithelial cells or cervical cancer cells used in these studies. In contrast to E6 and E7, E5 can stimulate IRF1 expression in HaCaT cells (55). Changes due to expression of the entire virus genome may be more complex and quite different to that seen with expression of individual viral proteins. Up-regulation of IFITs corresponded with the observed activation of the type 1 interferon response. Only IFIT1 has been shown to inhibit HPV replication (56, 57) therefore, the roles of other IFITs in inhibiting HPV infection remain to be determined. APOBEC3B was up-regulated however, we found no changes in expression of APOBEC3A, a known HPV restriction factor, but its expression may be differentially regulated only in less differentiated keratinocytes (58). The observed up-

371 regulation of CXCR6 and CXCL12 is in agreement with CXCL12 detection in HPV-induced
372 lesions and its role in the productive HPV life cycle (59). We also detected changes in some
373 SERPINs (e.g. SERPINB3) that are involved in the inflammatory/immune response (60). We
374 did not detected changes in STAT1 that has been shown to be controlled by E6 and E7 (61).
375 It is possible that it undergoes changes of less than the cut-off of >3.5-fold considered here.
376 However, STAT1 controls IRF1 expression, which was upregulated 4-fold and STAT1 was a
377 central node connecting gene pathways regulated by HPV16 (Supplementary Figure 1). Of
378 course, because we used an immortal cell line, immortalisation could account for some of
379 the changes we observed. It will be important to analyse innate immune regulators in
380 differentiated primary cervical keratinocytes in future studies. Our data reveal that HPV
381 suppression of intrinsic and innate immunity takes place not only in infected basal epithelial
382 cells (17) but also in keratinocytes harbouring late events in the HPV life cycle, and that a
383 differentiation stage-specific set of events may be relevant to this life cycle stage. The
384 stimulation of the IFN response and viral restriction factors in differentiated HPV-infected
385 cells requires further study. Production of progeny viral genomes and virions may stimulate
386 the IFN response and lead to apoptosis and this could aid release and dissemination of virus
387 particles.
388
389 The E5, E6, E7 and E2 proteins of HPV 16 are known to control cellular gene expression. E6
390 and E7 control keratinocyte cell cycle and apoptosis and abrogate differentiation. Many of
391 the changes in gene expression we have observed can be attributed to these functions of
392 the viral oncoproteins. These changes are clearly important for the replicative life cycle of
393 HPV16 but could also contribute to HPV persistence and development of neoplasia (6).
394 Similar to data from one overexpression study of HPV16 E6 in human foreskin keratinocytes
395 (50), the differentiation marker involucrin, vimentin that is expressed upon epithelial stress,
396 and signal transduction proteins MEST and H19, were up-regulated in our analysis.
397 However, we detected none of the other changes affecting cell cycle, proliferation, DNA
398 damage, metabolism or signalling that have previously been reported (50). We discovered

only 7 genes (Semaphorin 5A (SEMA5A), CXCL1, ENTPOT, Follistatir (FST), Cytochrome P450 (CYP) 24A1, Pleckstrin homology-like domain A1 (PHLDA1) and ribosomal proteins S27-like (RPS27L)) out of a total of 99 altered in another study using siRNA depletion of E6 in HPV-positive tumour cells (47). Compared to a study of W12 cells with integrated HPV16 genomes expressing different levels of E6 and E7, we detected E6-regulated loricrin (LOR) and cytochrome P450 (CYP) 1B1, and E7-regulated FABP4, SERPINA3, SLURP1 out of the top 20 genes up-regulated by each protein (62). Only one out of 12 master regulators of E6 or E7 function defined by Smith et al (62) was in common with our study. This was downregulation of PRDM1 (BLIMP-1) which acts as a repressor of IFN-β gene expression. E5 overexpression in HaCaT keratinocytes yielded 61 mRNAs with significant changes (21) but only two of these (Keratin 8, MMP16) were in common with our RNASeq data. In a microarray study of E2 overexpression in U2OS cancer cells where 74 genes were found to be regulated, only 3 of these (heterotrimeric G-complex protein 11 (GNG11) involved in cell signalling**,** histamine N-methyltranferase (HNMT) involved in methylation of histamine and SERPINA3 which is up-regulated in response to decreased transglutaminase activity) were altered in our study. Increased viral oncoprotein expression levels in HPV-positive cancer cells, or in cells overexpressing viral proteins, compared to the model we have used, i.e. keratinocytes supporting expression of all viral proteins from the intact HPV16 genome where expression levels are much lower (3), could explain the fact that we did not detect many of these changes. Moreover, we have only considered expression changes >3.5-fold, while these other studies considered 2-fold changes. RNASeq analysis of the W12 tumour progression series (63) would help to delineate infection versus cancer-related changes.

Liquid based cytology samples (LBCs, Pap smear samples) contain cells scraped from the top of the cervical epithelium and thus contain HPV-infected differentiated keratinocytes. Therefore, some of the mRNA changes we have detailed could be biomarkers of cervical disease. Very high levels of IL1B mRNA were detected in all patient samples, regardless of disease stage, likely due to inflammation commonly observed in diseased cervix. Statistically

significant changes in KDR and SERPINB3 expression, like the known biomarker KRT17, indicate their potential in identifying high grade cervical disease. DSG1 was significantly increased between no detectable disease (NDD) and low grade disease but significantly decreased between low grade and high grade disease. This is in contrast to the clear down-regulation of DSG1 expression due to HPV16 infection of NIKS and suggests either that NIKS16 cells may not represent a low grade HPV16-positive lesion or that the levels of DSG1 in cervical keratinocytes is very different to that in foreskin keratinocytes.

In conclusion, we report for the first time RNASeq analysis of changes in the keratinocyte transcriptome caused by HR-HPV infection. Infection caused massive changes in epithelial gene expression. These changes showed mainly a profile expected of viral infection, rather than tumour progression. The large dataset we have developed opens up the possibility of a deeper understanding of late events in the HPV replication cycle in response to keratinocyte differentiation. As well as shedding light on late events during the HPV16 life cycle, the RNASeq data could uncover potential biomarkers of HPV-associated anogenital disease progression. From our analysis, DSG1, KDR and SERPINB3 expression may have potential as robust markers that can risk-stratify cervical disease, i.e. identify cervical disease cases that have a high probability of regression, and this would be of significant clinical value. However, further longitudinal studies where biomarker status is linked to clinical outcomes would be required to validate any biomarkers for such an application.

Materials and Methods

Clinical sample panel underlying pathology and HPV status

Anonymised, cervical liquid based cytology samples were obtained from the Scottish National HPV archive which holds Generic Scotland A Research Ethics Committee approval for Research Tissue banks (REC Ref 11/AL/0174) for provision of samples for HPV related research after approval from an independent steering committee. The Scottish HPV Archive also comes under the auspice of the NHS Lothian Bioresource. The panel comprised HPV negative/cytology negatives samples (no disease, n=7) samples with low-grade cytological abnormalities with histological confirmation of cervical intraepithelial neoplasia (CIN) 1 (low-grade disease, n=10) and samples with high-grade cytological abnormalities with histological confirmation of CIN2 or worse, including cancer (high-grade disease, n=10). Cytology grades were reported according to the British Society for Clinical Cytopathology (BSCC) classification (64-66). HPV testing was performed by the Optiplex HPV genotyping Assay (Diamex, Heidelberg, Germany) according to manufacturer's instructions. The Optiplex test is a PCR based assay which uses a luminex platform for the detection of 24 individual HPV types including all established as high-risk according to the International Agency on Research on Cancer. For the purposes of this panel, the main function of the genotyping was for the annotation of no disease "controls". Women with negative cytology and HPV negative status are at a very low risk of underlying disease (negative predictive value for a high grade lesion of >95% (67)) All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

Cell lines

W12E (24), NIKS (43), and NIKS16 cells (22) were co-cultured in E-medium with mitomycin C-treated J2 3T3 fibroblast feeder cells as previously described (24). Differentiation was induced by growth to high density in 1.2 mM Ca^{++} (24). 3T3 cells were grown in DMEM with

475 10% donor calf serum. Prior to harvesting, 3T3 cells were removed by trypsinisation and
476 cells layers washed twice with PBS. All cells were maintained under humidified 5% CO_2 95%
477 air at 37°C.

478 ***RNA isolation – cell lines***

479 Protocols followed the manufacturer's instructions. Total RNA was prepared using Qiagen
480 RNeasy kit. RNA was quantified by measuring the ratio of absorbance at 260 and 280 nm
481 using a Nanodrop ND-1000 spectrophotometer (ThermoScientific). Polyadenylated RNA was
482 prepared using an oligo-dT-based mRNA extraction kit (Oligotex, Qiagen).

483 ***RNA isolation – clinical samples***

484 LBC cells in 4ml in PreservCyt collection medium (Cytyc Corporation) were pelleted by
485 centrifugation in a Beckman GPR bench top centrifuge at 1500g for 10 min. The cell pellet
486 washed with sterile PBS. RNA extraction was carried out using the RNeasy miRNA
487 preparation kit (Qiagen). RNA was quantified and purity assessed by measuring the ratio of
488 absorbance at 260 and 280 nm using a Nanodrop ND-1000 spectrophotometer.

489 ***qRT-PCR***

490 For cell line and clinical samples, DNA was removed using Maxima DNase and treated RNA
491 was reverse transcribed using Maxima First Strand cDNA synthesis kit according to
492 manufacturer's instructions (ThermoScientific). Standard curves were generated as
493 recommended (Applied Biosystems instruction manual). Triplicate amplification reactions
494 containing 100 ng cDNA each were carried out. GAPDH and β-actin were used as the
495 internal standard controls. Probes and primers are: GAPDH F: 5'-
496 GAAGGTGAAGGTCGGAGT-3', GAPDH R: 5'- GAAGATGGTGATGGGATTTC-3', GAPDH
497 Probe: 5'-CAAGCTTCGTTCTCAGCC. KRT10F: 5'- TGGTTCTTGCCTCAGAAGAGCTGA-
498 3', KRT10 R: 5'- AGTACACGGTGGTGTCTGTGTCAT-3', KRT10 Probe:
499 TGTGTCCACTGGTGATGGGAATGTGG-3'. DSG1 F: 5'- ACGTTCACGATAACCGACCAGC

500 AT-3', DSG1 R: 5'- ATTCCATGCAAATCACGGCCAGAG-3', DSG1 Probe: 5'- AACGTGGT
501 AGTGACAGAGAGAGTGGT-3'. KDR F: 5'- TGGTTCTTGCCTCAGAAGAGCTGA-3', KDR
502 R: 5'- AGTACACGGTGGTGTCTGTGTCAT-3', KDR Probe: 5'-
503 TGGCATCTGAAAGCTCAAACC
504 AGACA-3'. IL1B F: 5'- TCTGTACCTGTCCTGCGTGTTGAA-3', IL1B R: 5'- TGCTTGAGAGG
505 TGCTGATGTACCA-3', IL1B Probe: 5'- CAAGCTGGAATTTGAGTCTGCCCAGT-3'. VTCN1
506 F: 5'-CACCAGGATAACATCTCTCAGTGAA-3', VTCN1 R: 5'- TGGCTTGCAGGGTAGAATG
507 A-3', VTCN1 Probe: 5'- AAGCTGAAGATAATCCCATCAGGCAT-3'. SERPINB3 F: 5'-
508 GCTGC
509 CAAATGAAATCGATGGTCTCC-3', SERPINB3 R :5'- TTCCCATGGTTCTCAACGTGTCCT-
510 3', SERPINB3 Probe: 5'-AACTCGGTTCAAAGTGGAAGAGAGCT-3'. KRT17 F: 5'- GATGC
511 GTGACCAGTATGAGAAG-3', KRT17 R: 5'- CGGTTCAGTTCCTCTGTCTTG-3', KRT17
512 Probe: 5'- ATGGCAGAGAAGAACCGCAAGGAT-3'. Reaction mixes (25 µl) contained 1x
513 Mastermix (Stratagene), 900 nM primers, 100 nM probe, 300 nM reference dye
514 (Stratagene). qPCR reactions were performed and analysed on an Applied Biosystems
515 7500 Fast System. Graphing and statistical analyses were performed using GraphPad Prism
516 7. Statistical analysis (all three groups were compared to each other) was performed by
517 Kruskal-Wallis test and data analysed by one way ANOVA with Tukey's post-test. A
518 significance level of $p<0.05$ was used.

519 ***Western blot analysis***

520 Cells were lysed in 2 x protein loading buffer (125 mM Tris (pH 6.8), 4% SDS, 20% glycerol,
521 10% mercaptoethanol and 0.006% bromophenol blue with fresh protein inhibitor cocktail
522 (Roche, UK)). Protein extracts were syringe-passaged through a 22-gauge needle 15 times
523 then sonicated in a Sonibath for three 30 sec pulses to break up DNA strands. The samples
524 were boiled at 100°C for 5 min before loading on a 12% Novex gel (Invitrogen) and
525 electrophoresed at 150V for 1 hour in 1X MES buffer. Gels were transferred to a
526 nitrocellulose membrane using the iBlot transfer kit and iBlot Gel Transfer Stacks (Invitrogen)

527 as per the manufacturer's instructions. Membranes were blocked in 5% milk powder in PBST
528 at room temperature for at least one hour. Membranes were washed 3 times in PBST for 5
529 minutes each then incubated with primary antibody. Mouse monoclonal antibodies, GAPDH
530 (Meridian, 6C5), involucrin (Sigma, I9018), loricirn (Abcam, ab85679), serpinB3 (Sigma,
531 2F5) and keratin 10 (Abcam, ab9026) were used at a dilution of 1:1000. HPV16 E2 antibody
532 (Santa Cruz, TVG261) was used at 1:500 dilution. HPV16 L1 antibody (Dako, K1H8) was
533 used at 1:400 dilution. HPV16 E4 antibody (Gift of J. Doorbar, Cambridge, UK, clone B11)
534 was used at a dilution of 1:50. Rabbit polyclonal antibodies DSG1 (Abcam, ab133662),
535 VEGFR2 (KDR) (Abcam, ab39256). AZGP1 (Invitrogen PA5-44912) were used at 1:1,000
536 dilution. VTCN1 (Proteintech, 12080-1-AP) was used a a dilution of 1:500. The blots were
537 incubated in their respective antibody for 1 hour at room temperature or overnight at 4°C.
538 After 1 hour, the blots were washed 3 times in PBS-T for 5 min. They were then placed in
539 secondary antibody for 1 hour (HRP-linked goat anti-mouse or goat anti-rabbit (Pierce) were
540 used at 1:2000 dilution. Blots were washed 3 times in PBST for 5 minutes before being
541 incubated with ECL western blot substrate. The blots were exposed to X-ray film
542 (ThermoScientific) and processed in an X-Omat processor.

543

544 ***Illumina sequencing***

545 Integrity of RNAs was assessed using an Agilent 2100 Bioanalyser. cDNA was synthesised
546 using reagents from the TruSeq RNA Sample Preparation kit (Illumina) according to the
547 manufacturer's instructions. cDNA libraries were sequenced with a 73 base single-end read
548 on an Illumina Genome Analyser IIx at the Glasgow Polyomics facility at the University of
549 Glasgow. Samples have been submitted to SRA@ncbi.nih.gov. STUDY: PRJNA379358
550 (SRP104232). SAMPLE: NIKS16_D11_Mar17 (SRS2131727), EXPERIMENT: Differentiated
551 NIKS16 cells (SRX2745325) RUN: NIKS_HPV16_D11_Mar17.fq.gz (SRR5457256).
552 SAMPLE: NIKS16_D5_Mar17 (SRS2131728), EXPERIMENT: Undifferentiated NIKS16 cells

(SRX2745326), RUN: NIKS_HPV16_D5_Mar17.fq.gz (SRR5457258). SAMPLE: NIKS_D11_Mar17 (SRS2131729), EXPERIMENT: Differentiated NIKS cells (SRX2745327), RUN: NIKS_D11_Mar17.fq.gz (SRR5457259). SAMPLE: NIKS_D5_Mar17 (SRS2131730), EXPERIMENT: Undifferentiated NIKS cells (SRX2745328), RUN: NIKS_D5_Mar17.fq.gz (SRR5457260)

Computational analysis

Datasets were cleaned of reads with runs > 12Ns. Alignment to the human cDNA set (145,786 cDNAs – downloaded on 28th November 2011) was performed using Bowtie version 0.12.7. Further alignment to an updated human cDNA set (180,654 cDNAs downloaded April 30th 2012) was carried out using BWA 0.7.12-r1039 . DESeq implemented in BioConductor (68) was used to select cellular genes whose expression was up or down-regulated by HPV in NIKS16 compared to NIKS cells implemented in the R environment. The raw read counts were normalised using (RPKM). DESeq uses a negative binomial error distribution to model transcript abundance and determine the differential expression. The significance of differential expression was estimated for each gene and then corrected for multiple comparisons (Padj). The top 1000 differentially expressed genes based on log-fold change (Log_2FoldChange) of >1.8 (3.5-fold change) are listed in Supplementary Table S3.

Functional analysis of differentially expressed genes

GO (69) and KEGG (70) enrichment analyses were performed using Cytoscape (http://cytoscape.org/) with ClueGO (Version 2.3.2) (71). The statistical test used for the enrichment was based on a two-sided hypergeometric option with a Bonferroni step-down correction, a *P*-value less than 0.05 and a kappa score of 0.4.

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Declaration of Conflicts of Interest

We declare no conflicts of interest.

589 Table 1. Changes in expression of immune regulatory molecules and viral restriction factors.

Gene ID	**Category**	**Negative fold change**	**Positive fold change**
TLR7	PRR	6-fold	
NLRP3	Inflammasome component	7-fold	
IL1A	Cytokine	7-fold	
IL1B	Cytokine	4-fold	
IL32G	Cytokine	17-fold	
IL36B	Cytokine	6-fold	
CCL20	Chemokine	7-fold	
CCL28	Chemokine		5-fold
CXCL12	Chemokine		4-fold
CX3CL1	Chemokine		32-fold
APOBEC3C	Restriction factor		4-fold
IFIT1	Restriction factor		6-fold
IFIT2	Restriction factor		7-fold
IFIT3	Restriction factor		7-fold
IFIT5	Restriction factor		4-fold
CD74	Restriction factor		4-fold
AOSL	Restriction factor		4-fold
RTP4	Restriction factor		13-fold
IRF1	IFN regulatory transcription factor		4-fold
IFNκ	Interferon kappa		8-fold

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592 PRR: pattern recognition receptor. IFN, interferon.

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Table 2. RNASeq expression changes in mRNAs of statistical significance ($p<0.025$) verified by qPCR

Gene ID	**Padj**	**NIKS16/NIKS-fold change RNASeq**	**NIKS16/NIKS-fold change qPCR**	**W12/NIKS-fold change qPCR**	**Gene Function**
DSG1	2.05 x10^{-5}	-19.95	-4.20	-3.52	Desmoglein1: calcium-binding desmosome regulator
IL1B	1	-8.68	-5.65	-7.73	Interleukin 1b: inflammatory response regulator
SERPINB3	0.008	-8.40	-4.28	-4.410	Intracellular protease inhibitor, inhibits active inflammatory response
KRT10	0.021	-7.07	-10.26	-3.70	Keratin10: epithelial cytofilament
KDR	0.025	10.21	10.10	4.30	VEGFR-2, tyrosine kinase receptor
VTCN1	1.4 x 10^{-9}	46.12	8.94	10.56	V-set domain-containing T-cell activation inhibitor-1
AZGP1	2.05 x10^{-5}	12.64	7.49	8.31	Zinc alpha-2 glycoprotein: lipid metabolism
GAPDH		1	1	1	Glyceraldehyde-3-phosphate dehydrogenase (control)
Beta-actin		1	1	1	Actin (control)

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Figure Legends

Figure 1. Characterisation of the HPV16 life cycle in NIKS16 and W12 cells. A. Expression levels of keratinocyte protein differentiation markers and viral L1 protein in undifferentiated (U = monolayer culture for 5 days) and differentiated (D = monolayer culture for 13 days) W12 and NIKS16 cells. GAPDH is shown as a loading control. B. Expression levels of viral E2 and E4 proteins at 8 (mid differentiation phase) and 13 (differentiated) days of a time course of NIKS16 differentiation in monolayer culture. C. Time course of involucrin protein expression over a 13 day differentiation period (monolayer cells are mostly undifferentiated after 5 days culture and fully differentiated after 13 days of culture) for NIKS and NIKS16 cells. invol, involucrin. D. Absolute quantification by qPCR of L1 gene copies, as a measure of viral genomes, in differentiated W12 and NIKS16 cells. E. Viral late mRNA levels quantified by detecting L1-containing mRNAs by qRT-PCR in undifferentiated and differentiated W12 and NIKS16 cells. Invol, involucrin. K10, Keratin 10.

Figure 2. HPV16 infection induces massive changes in the keratinocyte transcriptome. A. mRNA numbers expressed versus the level of expression of each individual mRNA in undifferentiated (U) versus differentiated (D) NIKS (HPV-negative) cells. B. mRNA numbers expressed versus the level of expression of each individual mRNA in undifferentiated (U) versus differentiated (D) NIKS16 (HPV-positive) cells. C. Venn diagram showing the percentage identity between upregulated genes of NIKS and NIKS16 cells compared to NIKS and W12 cells. D. Venn diagram showing the percentage identity between downregulated genes of NIKS and NIKS16 cells compared to NIKS and W12 cells. Identity was determined using the GFOLD tool to calculate the differential fold changes of the two comparisons.

Figure 3. Keratinocyte differentiation and epithelial barrier function is altered by HPV infection. Significant changes in expression (>$\log_2$=1.8; 3.5-fold) of proteins involved in

keratinocyte differentiation and epithelial barrier function comparing HPV16-infected, differentiated NIKS keratinocytes to uninfected, differentiated NIKS keratinocytes. These are the mean values from three separate RNASeq experiments A. Markers of differentiation (filaggrin, loricrin, involucrin and transglutaminase (TGM1)). B. Keratins (K). C. Desomosomal proteins, desmogleins (DSG) 1 and 4 and desmocoilin (DSC). D. Gap junction proteins, connexins (Cx) 26, 30.2 and 32. E. Claudins. F. Cadherins. G. small proline rich proteins (SPRRs).

Figure 4. ClueGO analysis of significantly up-and down-regulated genes in HPV16-infected, differentiated NIKS keratinocytes compared to uninfected, differentiated NIKS keratinocytes. We used CluePedia, which extends ClueGO (71) functionality down to genes, and visualizes the statistical dependencies (correlation) for markers of interest from the experimental data. A. Gene ontology (GO) pathway terms specific for up-regulated genes. B. GO pathway terms specific for down-regulated genes. The bars represent the numbers of genes associated with the term on the left hand side. The percentage of altered genes is shown above each bar. Red asterisks refer to significance. C. Functionally grouped network for up-regulated genes. D. Functionally grouped networks for down-regulated genes. Only the label of the most significant term per group is shown. The size of the nodes reflects the degree of enrichment of the terms. The network was automatically laid out using the organic layout algorithm in Cytoscape. Only functional groups represented by their most significant term were visualized in the network. Padj< 0.05 changes were analysed.

Figure 5. Interactome of negatively and positively changed genes comparing differentiated NIKS with differentiated NIKS16 cells. Interactome of genes linked through statistical correlation of A. up-regulated and B. down-regulated genes from the experimental data (p-value <0.05). Grey lettering, and diamonds indicates genes identified in the RNASeq data set. Black lettering indicates linked genes. Nodes for genes identified

in the data set are indicated by black box outlines. Dots/lines surrounding nodes indicate the numbers of linked pathways. The pathways analysis was produced using Cluepedia (http://apps.cytoscape.org/apps/cluepedia).

Figure 6. Western blot analysis of proteins levels encoded by selected, significantly altered mRNAs (Table 2). Protein extracts were prepared from undifferentiated and differentiated HPV-negative NIKS and HPV16-positive NIKS16 and W12 cell populations. Much greater levels of involucrin (invol) were detected in the differentiated, compared to the undifferentiated cell populations indicating differentiation was achieved. GAPDH was used a protein loading control. A. Protein levels corresponding to significantly up-regulated mRNAs. B. Protein levels corresponding to significantly down-regulated mRNAs. U, undifferentiated. D, differentiated.

Figure 7. Expression levels of selected, significantly altered mRNAs in different grades of HPV-associated pre-neoplastic cervical disease. mRNA expression levels were calculated from qPCR data using GAPDH and beta-actin as the internal controls and expressed relative to levels in a single sample of differentiated, HPV16-positive W12 cell RNA that was included in every PCR run. NDD, no detectable disease/borderline, all HPV-negative. Low grade, cervical intraepithelial neoplasia 1 (CIN1), all HPV-positive. High grade disease, cervical intraepithelial neoplasia 1 (CIN3), all HPV-positive.

Figure 1 KlymenkoNGS

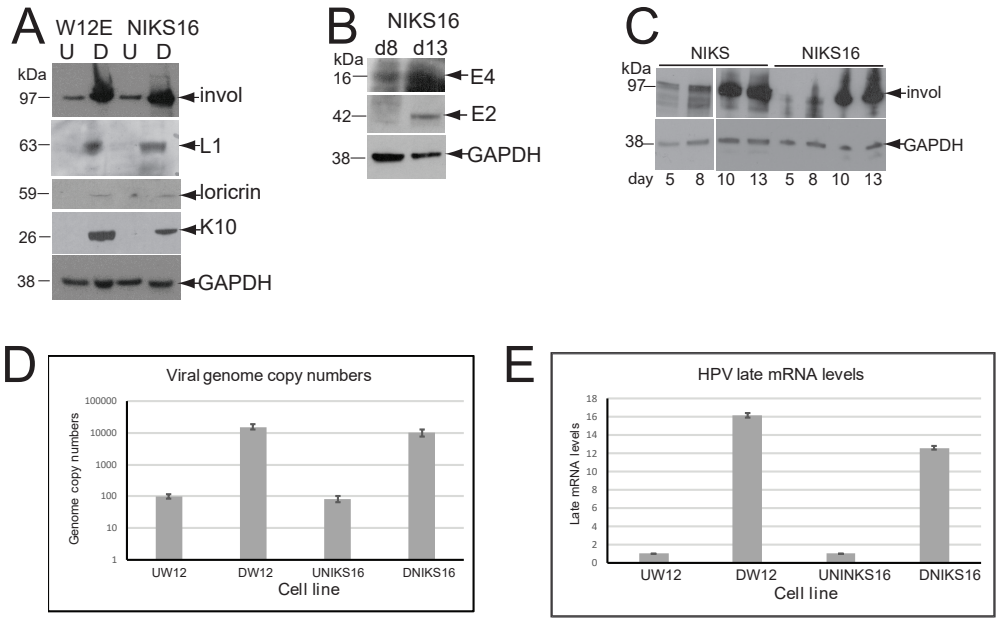


Figure 1 KlymenkoNGS

Figure 2 KlymenkoNGS

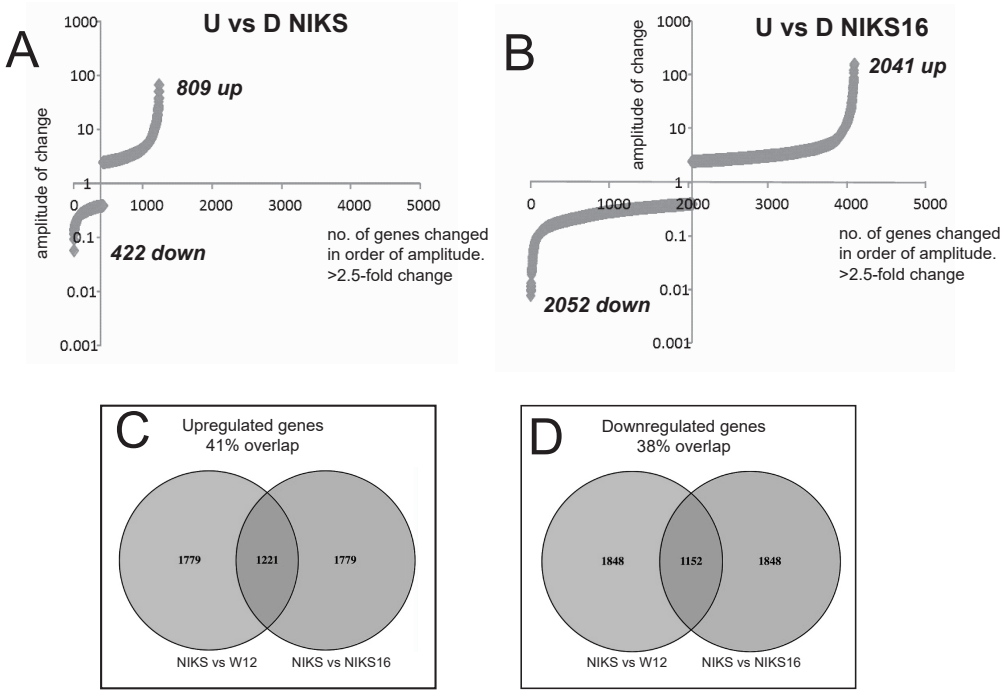


Figure 3 Klymenko et al.

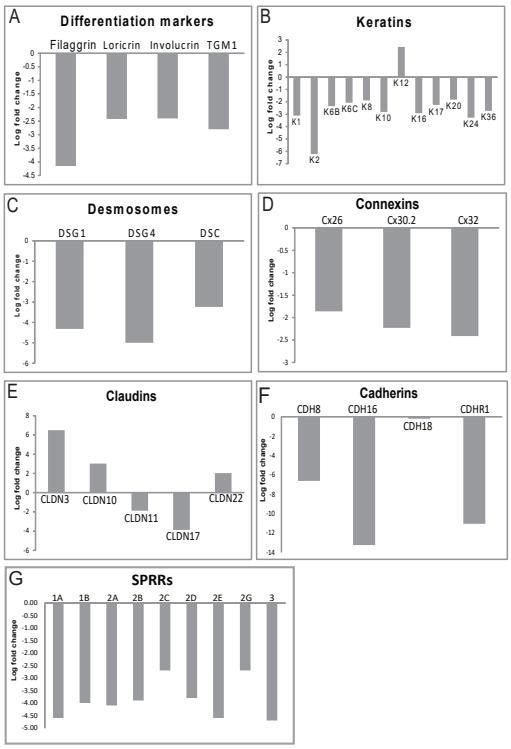
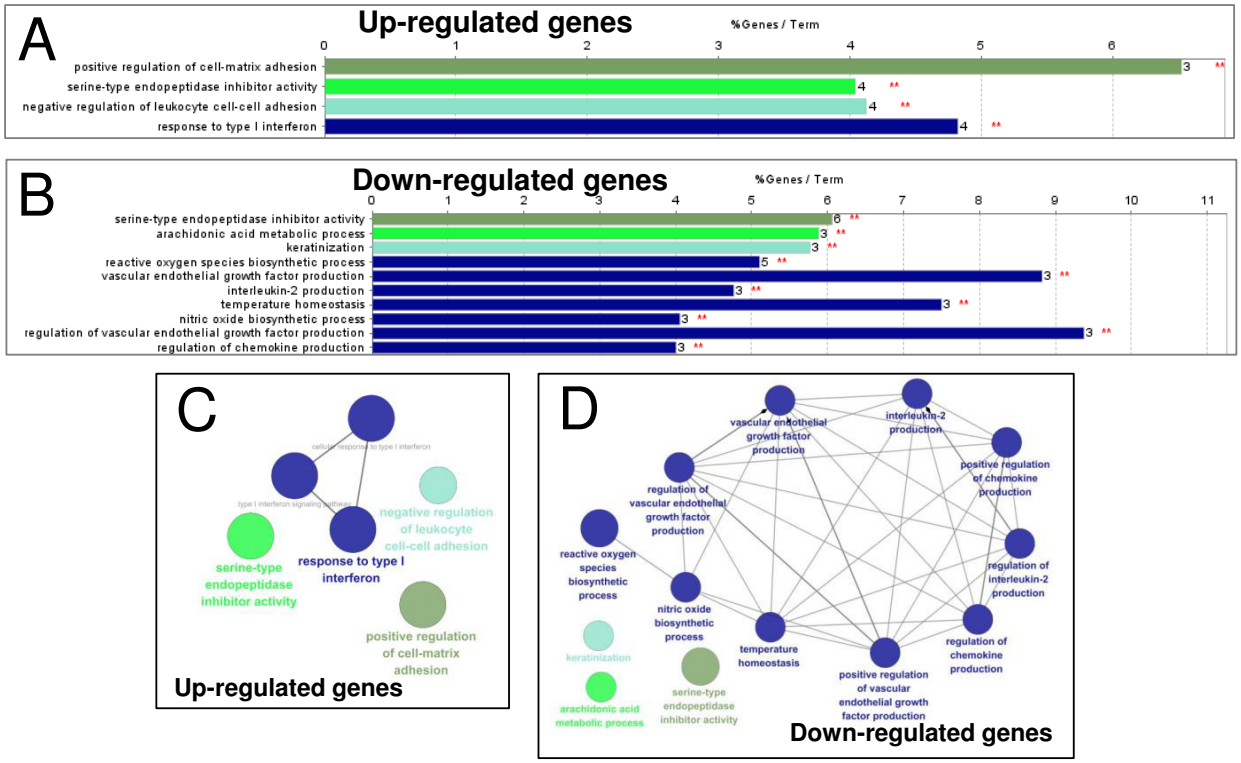
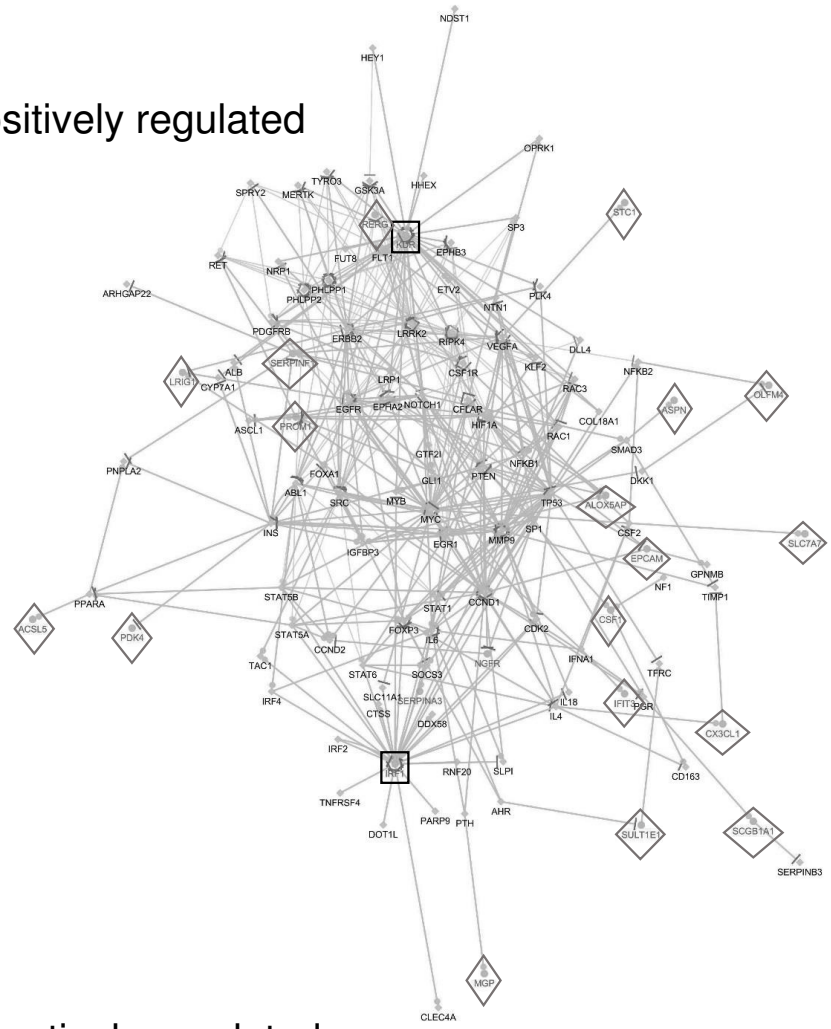


Figure 3 Klymenko et al.

Figure 4 Klymenko et al.



A positively regulated



B negatively regulated

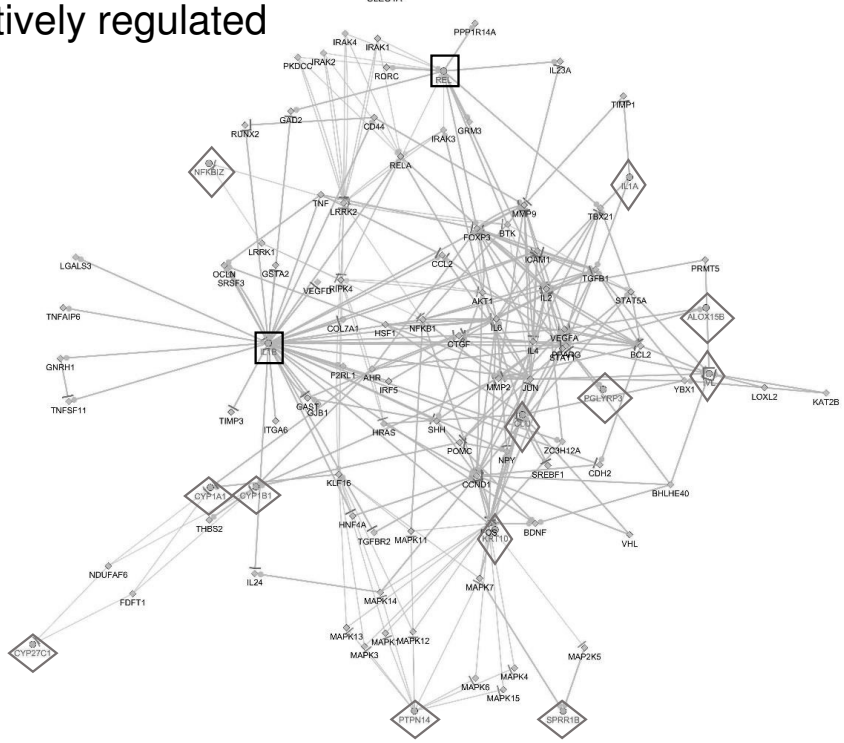


Figure 6 Klymenko et al.

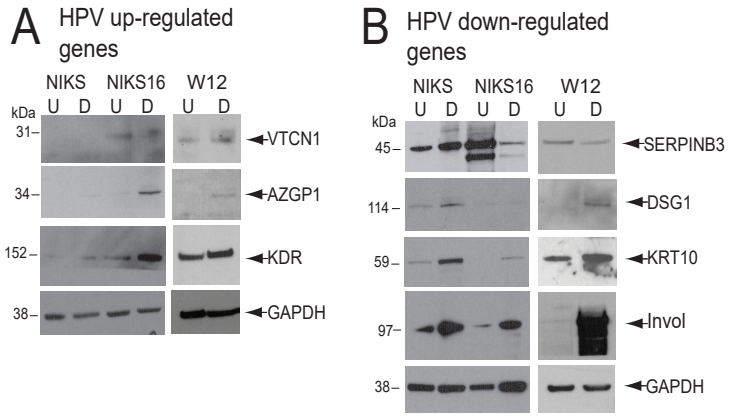


Figure 6 Klymenko et al.

Klymenko et al. Figure 7