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Expression of HPV16 E5 Produces Enlarged Nuclei and Polyploidy through Endoreplication

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Abstract

Anogenital cancers and head and neck cancers are causally-associated with infection by high-risk human papillomavirus (HPV). The mechanism by which high-risk HPVs contribute to oncogenesis is poorly understood. HPV16 encodes three genes (HPV16 E5, E6, and E7) that can transform cells when expressed independently. HPV16 E6 and E7 have well-described roles causing genomic instability and unregulated cell cycle progression. The role of HPV16 E5 in cell transformation remains to be elucidated. Expression of HPV16 E5 results in enlarged, polyploid nuclei that are dependent on the level and duration of HPV16 E5 expression. Live-cell imaging data indicate these changes do not arise from cell-cell fusion or failed cytokinesis. The increase in nuclear size is a continual process that requires DNA synthesis. We conclude HPV16 E5 produces polyploid cells by endoreplication. These findings provide insight into how HPV16 E5 can contribute to cell transformation.

Introduction

Most anogenital (Bosch et al., 2002) and head and neck (Gillison et al., 2000) cancers are causally-linked with infection with certain types of HPV, however, the mechanisms by which this occurs are not fully understood. Understanding how the oncogenes encoded by HPV induce cellular changes during tumorigenesis is critical for developing successful interventions.

There are at least 120 different types of HPV; however, only 13 of these are considered oncogenic (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). Of these 13 HPV types, HPV16 is the most prevalent. It is present in 57% of cervical cancers (Clifford et al., 2003) and 90% of HPV-positive head and neck tumors (Gillison et al., 2008).

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Clues as to how HPV transforms normal cells into a cancerous ones has been shown to be dependent on expression of genes in the early region of the viral genome. HPV16 is an approximately 8000 bp circular DNA virus with eight open reading frames (ORFs). Of those, three are known to encode oncogenes – proteins that can mediate cell transformation when individually transfected into a cell. Of the three HPV16 oncogenes, two (E6 and E7) have been well characterized for their ability to inhibit cell cycle regulators, namely p53 and Rb (Jones, Alani, and Munger, 1997; Werness, Levine, and Howley, 1990). In addition, both oncogenes have been shown to associate with a number of other proteins, and expression of E6 and E7 results in genomic instability, a characteristic of many cancers (Nakamura, Sotozono, and Kinoshita, 2001). Thus, E6 and E7 have well established roles in tumor propagation.

The role of the third oncogene, E5, has been less clear. HPV16 E5 is sufficient to transform mouse fibroblasts and keratinocytes in culture, as assessed by anchorage independent growth and colony formation assays (Leechanachai et al., 1992; Pim, Collins, and Banks, 1992; Straight et al., 1993). Co-expression of E5 with either E6 or E7 enhances the transforming properties of either protein alone (Bouvard et al., 1994; Stoppler et al., 1996; Valle and Banks, 1995).

This *in vitro* analysis has been complemented with studies using transgenic mice. Williams et al. have demonstrated that expression of the HPV16 E5 oncogene [driven by the basal epithelium specific promoter (K14)] is sufficient to cause epidermal hyperplasia and the formation of spontaneous skin tumors (Williams et al., 2005). When the entire HPV16 genome is expressed, the size of the tumor increases as compared to mice expressing only E6 and E7 (Riley et al., 2003). These *in vivo* data provide additional evidence that HPV16 E5 transforms cells independently as well as enhances the transforming properties of other HPV16 oncogenes.

The exact molecular mechanisms by which E5 participates in transformation are unclear. Identifying a molecular role for E5 in infected tissues has been difficult due to its low level of protein expression, rare integration of the E5 gene into the host chromosome, and a lack of reagents, antibodies, and animal models (Conrad et al., 1994; Disbrow, Hanover, and Schlegel, 2005; Oelze et al., 1995; Oetke et al., 2000; Straight, Herman, and McCance, 1995). The diverse functions proposed for E5 include protecting the cell against apoptosis (Kabsch and Alonso, 2002; Zhang, Spandau, and Roman, 2002), interfering with cell-cell communication (Oelze et al., 1995), and inhibition of antigen presentation in infected cells (Zhang et al., 2003). The most commonly accepted model is that the E5 gene product potentiates the signaling of the epidermal growth factor receptor (EGFR) by slowing EGFR endocytic trafficking and degradation (Straight, Herman, and McCance, 1995; Straight et al., 1993; Zhang et al., 2005).

While these proposed mechanisms are reasonable means of promoting the unregulated cell growth that is characteristic of cancer cells, it is difficult to reconcile these proposed functions with the reported profile of E5 expression. Due to a lack a reliable antibodies to the native HPV16 E5 protein, the best estimation of expression of HPV16 E5 comes from monitoring the generation of HPV16 E5 mRNA (Stoler et al., 1992). The levels of HPV16 E5 mRNA are highest immediately after infection and trail off as the infected epithelial cells differentiate. In fact, most HPV16 positive tumors and cell lines derived from cervical cancers do not have detectable levels of E5 mRNA (Pater and Pater, 1985; Schwartz et al., 1985; Stoler et al., 1992). Based on the kinetics of HPV16 E5 expression, it has been a widely held belief that the role of HPV16 E5 is in the initiation of oncogenesis (zur Hausen, 1996; zur Hausen, 2000).

We have recently developed a model system that allows detectable HPV16 E5 to be expressed in large populations of cells. Using this model, we discovered that expression of HPV16 E5 leads to two noteworthy changes in cell morphology: binucleated cells (Hu et al., 2009) and enlarged nuclei. Both of these phenotypes are characteristic of pre-cancerous lesions. Further, these changes are consistent with HPV16 E5 playing a role in initiating oncogenesis.

We have previously reported that the bi-nucleated cells arise through cell-cell fusion (Hu et al., 2009). However, the relationship, if any, between ether cell-cell fusion and enlarged nuclei was unclear. Here we have performed a systematic analysis each cellular change initiated by HPV16 E5 to determine the relationship between these phenotypes and how they might contribute to oncogenesis. Based on our work and the findings of others, HPV16 E5, like many viral proteins, has multiple effects on the cell. These myriad roles may be at the root of why the true pathological role(s) of HPV16 E5 has been so difficult to elucidate.

In this manuscript, we report that expression of HPV16 E5 in human keratinocytes is sufficient to produce enlarged nuclei. The change in nuclear size is accompanied by an increase in cellular DNA content and chromosomal number. Live cell video microscopy indicates these cellular changes are not the result of failed cell division or cell-cell fusion. Rather, the nucleus increases in size gradually and requires DNA synthesis for its growth. From these data, we conclude that HPV16 E5 causes an increase in nuclear size through endoreplication.

Materials and methods

Tissue culture cells

Parental HaCaT, tTA-HaCaT (Hu et al., 2009), and NIH 3T3 cells were maintained in Dulbecco's Minimal Essential Media (DMEM) of 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM glutamine. Stably transfected tTA-HaCaT cells were supplemented with 800 μg/ml G418. All cell lines were maintained at 37°C in 5% CO_2.

Adenovirus generation/expression

Codon-optimized HPV16 E5 was generated as previously described (Disbrow et al., 2003). Hemagglutinin (HA) epitope (MEYDVPDYAH) was engineered on to either the amino (HA-E5) or carboxyl (E5-HA) terminus of the protein by PCR, making the appropriate modification of stop codons. Adenoviruses were generated using Clontech Adeno-X™ Tet-off expression system. All adenoviruses were sequenced at the DNA sequencing facility at the Oklahoma Medical Research Foundation.

Cells were infected with adenoviruses that had been CsCl purified. Cells were infected with ~20 plaque forming units of adenovirus per cell in serum-free DMEM for 2 hours at 37°C. Following incubation, the adenovirus containing media was removed and replaced with growth media. Tetracycline (1 μg/ml) was added during adenoviral infection and recovery.

Plasmids and transfection

HA-tagged, codon optimized HPV16 E5 was generated as described above and subcloned into either the pcDNA3 (Invitrogen) or pCDF1-MCS2-EF2-copGFP (System Biosciences).

The wild type HPV16 genome and E5 frameshift mutation were cloned into a pUC-18 plasmid for the replication and antibiotic selection for purification (Genther et al., 2003). Prior to transfection, CsCl purified cDNA (10 μg) was digested with BamH1 to isolate the

HPV16 genome from the backbone plasmid and the result ~8000 bp DNA fragment was isolated by gel purification. The HPV16 genome (2 μg) along with GFP-neomycin (pEGFP-C1; Clontech) was introduced into 2×10^6 HaCaT cells by nucleofection (Amaxa).

NIH 3T3 cells were transfected with the indicated plasmids using Lipofectamine 2000, according the manufacturer's directions.

Cell lysates and Immunoblotting

Cell lysates were prepared as previously described (Dinneen and Ceresa, 2004). Proteins were resolved on 16% Tris-Tricine gels and transferred to nitrocellulose. Antibodies were obtained from the indicated sources: anti-HA (University of Virginia Lymphocyte Culture Core), α-tubulin (Sigma), HPV16 E7 (Zymed). Proteins were visualized with enhanced chemiluminescence and documented using a UV Products Imaging system.

Indirect immunofluorescence

Indirect immunofluorescence was performed as previously described (Dinneen and Ceresa, 2004). The 12CA5 antibody (University of Virginia Lymphocyte Culture Core) was used at a dilution of 1:1000 as the primary antibody and Alexa 488- or Alexa 568-conjugated goat anti-mouse (Molecular Probes) as the secondary antibody at a dilution of 1:250. Cells were also stained with 1 ng/ml DAPI (Sigma). Images were captured using Olympus AX70 epifluorescent microscope with Q-Capture software.

Fluorescent in situ hybridization *(FISH)*

Cells were fixed and processed for FISH using the AneuVysion probe (Vysis) according to manufacturer's protocol.

FACS analysis

Cells were fixed in 70% ethanol, and incubated with 200 μg/ml propidium iodide (Sigma) for one hour. Cells were analyzed using a FACS Calibur (Benton Dickson). The data from 10,000 cells/experiment were analyzed using Modfit LT 2.0 software (Verity).

Assessment of nuclear size

Images of DAPI stained nuclei were analyzed using Photoshop software to convert pixel length into microns. Where indicated, cells were incubated with 0.5 mM hydroxyurea (Sigma) dissolved in growth media.

Live cell imaging

For monitoring progression through mitosis and cytokinesis, cells were analyzed by video microscopy for twenty-four hours using a Zeiss Axovert 200M microscope equipped with a Hamamatsu ORCA camera.

Mitosis time was calculated and the length of time it took cells to progress from prophase (chromosomal condensation) to separate into two daughter cells. For each condition, at least 10 fields containing 3–8 cells/condition were observed. From those, the rate of mitosis was calculated from 40–53 cells for each condition.

In experiments in which the nuclear size was monitored, HaCaT cells were transfected with red fluorescent protein-tagged histone 2B (RFP-H2B) allowed to recover for 24 hours before infection with adenovirus. After recovery from adenoviral infection, cells were monitored 24 hours with a Nikon microscope equipped with Photometrics Cool Snap HQ camera. Time-

lapse phase-contrast images were collected and processed with NIS-Elements AR 3.0, SP5 Software

Results

HPV16 E5 is necessary and sufficient for the formation of enlarged nuclei

In order to examine the role of HPV16 E5 expression, we generated tetracycline-regulatable adenoviruses that encode a codon-optimized HPV16 E5 that was tagged with an HA-epitope on either the amino- or carboxy-terminus, termed HPV16 HA-E5 or HPV16 E5-HA, respectively. The codon optimization enhances expression of the E5 protein (Disbrow et al., 2003) and the epitope tags permit detection of the protein to which there are no reliable antibodies commercially available. These adenoviruses can only be expressed in cells that express the tetracycline transactivator (tTA); expression of these genes is ablated in the presence of tetracycline. For these experiments, we have stably transfected the tTA into HaCaT cells (tTA-HaCaT cells), a spontaneously immortalized human keratinocyte that is HPV negative (Hu et al., 2009).

When HPV16 HA-E5 is expressed in HaCaT cells, the nuclei become enlarged and increase in size over time (Figure 1A). There is no change in the size of the nucleus of parental HaCaT cells, or cells that have been infected with the HPV16 HA-E5 adenovirus in the presence of tetracycline to ablate protein expression. Over the course of 72 hours of HPV16 HA-E5 expression, the average nucleus size increases ~50% (Figure 1B). Similar changes in nuclear size were observed when HPV16 E5-HA was expressed (data not shown). Protein expression was confirmed by both indirect immunofluorescence (Figure 1A) and immunoblot (Figure 1C).

The increase in nuclear size did not reflect a change in only a few HPV16 HA-E5 expressing cells, but rather a shift in size of the whole population. When the data from over 500 cells from three independent experiments is plotted as a histogram, it is clear that almost the entire population of HPV16 HA-E5 expressing cells has increased its nuclear size while maintaining a Gaussian distribution (Supplemental Figure 1).

Similar increases in nuclear size can be seen in NIH 3T3 cells transfected with HPV16 HA-E5 (Supplemental Figure 2). The smaller increase in nuclear size is likely a reflection of the lower level of protein expression since adenoviral gene transfer is more efficient and produces more protein than transient transfection. Figure 4 demonstrates that the changes mediated by HPV16 E5 expression are dose dependent.

In order to assess whether E5 would cause the nucleus to enlarge in the context of the native virus, we transfected cells with the entire HPV16 genome. As a control for the potential effects of the other HPV16 viral proteins, we transfected cells with a mutant HPV16 with a frameshift mutation in the E5 protein (HPV16 E5 fs) which prevents a functional protein from being produced (Genther et al., 2003). In order to identify transfected cells, we co-transfected the genomic HPV16 contructs with pEGFP, a plasmid that expresses GFP (Figure 2A).

In the original report using this mutant HPV16 genome, the failure to express the E5 gene had no effect on the viral life cycle or on the growth and differentiation of the cells expressing HPV16 E5 fs genome (Genther et al., 2003). Thus, the differences in cell morphology are likely due to the loss of E5 rather, since the mutant HPV16 appears otherwise functional.

Quantification of the nuclei size from 500 cells reveals that expression of the entire HPV16 genome can increase the cell's nuclear size. Cells expressing the HPV E5 fs, had a slight increase in nuclear size as compared to cells transfected with the pEGFP alone, but was less than the wild type HPV16 (Figure 2B). Both the wild type HPV16 and HPV16 E5 fs had comparable levels of E7 expression [within 10% for each experiment as determined by densitometric analysis of non-saturated immunoblots (n=3) – data not shown], indicating the differences were not due to variation in transfection efficiency of the intact virus (Figure 2C). One explanation may be that other HPV16 genes, such as E7, contributes to an increase in nuclear size (Duensing et al., 2009).

Together, these data indicate that HPV16 E5 is necessary and sufficient to induce enlarged nuclei in HaCaT cells. This finding is remarkable because changes in nuclear size and morphology are one of the diagnostic criteria for the presence of precancerous lesions and cancer (Crum and Lee, 2006; Wright et al., 2004).

The HPV16 E5 –mediated increase in nuclear size is associated with doubling of the cell chromosomal content

In order to identify the cause of the enlarged nuclei, we first examined the amount of DNA in each cell. HaCaT cells were uninfected or infected with adenovirus encoding HPV16 HA-E5 in the absence or presence of tetracycline to regulate protein expression. At various times after infection, cells were collected and incubated with propidium iodide to stain the DNA. Cells were analyzed by fluorescent associated cell sorting (FACS). In cells expressing HPV16 HA-E5, there was a time-dependent increase in the percentage of cells that had twice (or more) the normal chromosomal content (4C) (Figure 3A). Uninfected HaCaT cells and cells that did not express HPV16 HA-E5 (HA-E5 + tet) did not show significant increases in the percentage of 4C cells over the course of 72 hours (Figure 3B).

We next wanted to know if the increase in DNA content was a function of the level of HPV16 E5 expression. Taking advantage of the tetracycline-regulatable promoter, we added varying amounts of tetracycline to cells that had been infected with the same amount of HPV16 HA-E5 adenovirus. As shown in Figure 4A, this allowed us to determine the affect of a range of HPV16 E5 concentrations of cellular DNA content. As predicted, higher concentrations of HPV16 HA-E5 resulted in a decrease in the percentage of 2C cells and a concomitant increase in the percentage of 4C cells (Figure 4B and 4C). These data are consistent with the observation that less efficient methods of protein expression (i.e. transfection) produce less robust increases in nuclear size (Figure 2, and Supplemental Figure 2).

To determine whether the enhanced DNA content per cell reflects an increased chromosomal content, HPV16 E5 expressing cells were subjected to Fluorescent *in situ* Hydribization (FISH) analysis, probing with a marker for the X chromosome (Figure 5A). In cells expressing HPV16 HA-E5, there was greater than 15-fold increase in the percentage of cells expressing four copies of the X chromosome as compared to control cells (Figure 5B). The increase in chromosome number was accompanied by an increased nuclear size (Figure 5C). Thus, the observed morphological and biochemical phenotypes correspond to changes in chromosome content.

Molecular basis of enlarged nuclei

Given the strong association of these cellular changes with the development of cancer, we wanted to know how these cellular changes were arising with the long-term goal of specifically inhibiting them. We identified three mechanisms by which cells could have twice the number of chromosomes in one enlarged nuclei: 1) two cells could fuse together

and subsequently their nuclei fuse together, 2) cells could replicate DNA, undergo mitosis but fail to segregate chromosomes successfully or fail cytokinesis, or 3) cells could undergo endoreplication (Storchova and Pellman, 2004). These three possibilities were systematically tested.

We have previously reported that expression of HPV16 E5 causes cell-cell fusion (Hu et al., 2009). We wanted to know if the enlarged polyploidy nuclei in HPV16 E5-expressing cells were the result of nuclear fusion subsequent to cell-cell fusion. To answer this question, we transfected separate populations of tTA-HaCaT cells with either histone 2B tagged with yellow fluorescent protein (YFP) or red fluorescent protein (RFP) that allows for differential labeling of nuclei. These cells were mixed together in a 1:1 ratio and infected with the HPV16 HA-E5 adenovirus. At 24-hour intervals, cells were fixed and processed by indirect immunofluorescence using an anti-HA antibody. We examined over 1000 cells from three different experiments (300–400 cells/experiment) and observed the formation of heterokaryons (Hu and Ceresa, 2009). In all cases, cells with enlarged nuclei expresses only one of the fluorescent proteins (Data not shown). Based on these data, we conclude that cell-cell fusion does not precede the formation of enlarged nuclei.

To determine whether HPV16 HA-E5 expressing cells failed to go through cytokinesis, we expressed HPV16 E5 in HaCaT cells and monitored their rate of cell division using live-cell video microscopy. HPV16 E5-expressing and control cells were monitored for the time between complete nuclear breakdown and separation in to two individual cells. All HPV16 E5-expressing cells were able to divide, albeit with slight differences in the rate of cytokinesis. Surprisingly, there was a trend in which HPV16 E5-expressing cells went through mitosis more rapidly than control cells (Figure 6A).

To be certain we were not selecting for a sub-population of HPV16 E5 positive cell that could undergo cell division, we monitored HPV16 E5 positive cells for chromosomal condensation. Cells were first transfected with red fluorescent protein-tagged histone 2B (RFP-H2B) and subsequently infected with HPV16 E5. RFP-H2B localizes exclusively to the nucleus and provides a clear indication of nuclear size and structure. After recovery for 24 hours, cells were imaged using live-cell video microscopy to monitor the development of enlarged nuclei. This assay allowed us to determine if progression through mitosis and cytokinesis, either normal or aberrant, was a prelude to the formation of enlarged nuclei.

We did not identify any cells that entered mitosis immediately prior to developing an enlarged nuclei. This indicates that the development of enlarged nuclei can occur without progression into mitosis. In addition, live cell imaging provided direct evidence that the enlarged nuclei did not result from two nuclei merging after cell-cell fusion. In contrast, video microscopy provided clear evidence that the enlarged nuclei developed through a steady increase in nuclear size.

Shown are representative micrographs documenting the growth of the nuclei in cells that are HPV16 E5 negative (Figure 6B) and positive (Figure 6C and D) cells over the course of 24 hours (Representative movies are included as Supplemental Movies 1–3). These cells, and the more than 100 other cells that we monitored, exhibited a steady increase in nuclear diameter with no indication of mitotic chromosome condensation prior to the enlargement of the nuclei. Shown in Figure 6E is the time-dependent change in nuclear size of five HPV16 E5 positive cells (solid lines) and five HPV16 E5 negative (dashed lines) cells. In all cells, the nucleus continually increases, but the rate of increase is much faster with higher expression of HPV16 E5. Thus, although HPV16 E5 expressing cells divide (Figure 6A), the development of enlarged nuclei is independent of cell division or of cell-cell fusion (Figure 6B and 6C).

The third possible explanation for how the enlarged nuclei arise is through endoreplication. Endoreplication is broadly defined as multiple rounds of DNA synthesis without cell division. The live-cell imaging data (Figure 6), the increase in DNA content (Figure 3 and 4), and the doubling of chromosomal number (Figure 5) are consistent with the notion that the increased nuclear size arises from multiple rounds of cell division.

We postulated that if the increased nuclear size were due to endoreplication, then the increase in nuclear size would be dependent on DNA synthesis. Therefore, inhibition of DNA synthesis would prevent the increase in nuclear size. To test this hypothesis, we incubated cells with hydroxyurea, a pharmacological agent that reduces the production of deoxyribonucleotides via inhibition of the enzyme ribonucleotide reductase (Sinclair, 1965). In the absence of deoxyribonucleotides, DNA synthesis is terminated. HPV16 E5 expressing cells were incubated with or without hydroxyurea for 24 hour intervals (Schematic in Figure 7A). Cells were fixed, immunostained with an antibody against the HA epitope, the nuclei were stained with DAPI, and the nuclear size was monitored as described previously. Shown in Figure 7B are representative micrographs. In the absence of hydroxyurea, the nuclear size of HPV16 E5-expressing cells grew in a time dependent manner, as shown previously. However, in the absence of DNA synthesis, the growth in nuclear size was arrested (Figure 7C). These data indicate that DNA synthesis is required for the increase in nuclear size.

Discussion

In this study, we show that expression of HPV16 E5 is sufficient to increase nuclear size in both HaCaT and NIH3T3 cells. This phenotype also arises when the E5 gene is expressed in the context of the entire HPV16 genome. The increased nuclear size is dependent both on the level and duration of HPV16 E5 expression and is accompanied by a doubling of the number of chromosomes. We have eliminated cell-cell fusion and failed cytokinesis as potential mechanisms by which these tetraploid cells are arising. Based on our observation that the enlarged nuclei arise through a continual growth and is dependent upon DNA synthesis for that growth, we have concluded these morphological changes occur due to endoreplication.

This finding provides important mechanistic insight to the findings of others. Krawczyk et al. have also reported changes in the nuclear morphology as the result of HPV16 E5 expression (Krawczyk et al., 2008). In that study, the authors examined the synergistic effect of HPV16 E5 and E6 in the development of koilocytosis. Although there are differences in the nuclear morphology in the two reports, it is likely that these difference reflect the level of protein expression and the cell lines examined.

Another study, by Genther et al., demonstrated that in the context of the entire HPV16 E5 genome, the loss of E5 decreased the extent of DNA synthesis (Genther et al., 2003). Using organotypic keratinocyte raft cultures expressing the wild type HPV16 genome or the HPV16 E5 fs mutant, the authors monitored DNA synthesis in cells expressing the virus. In the absence of E5, there was a 50% decrease in BrdU incorporation. The change DNA synthesis is consistent with the idea that E5 is responsible for the changes in nuclear size and chromosome number that we observe.

There have been a number of roles ascribed to HPV16 E5 in mediating oncogenesis. However, most of the previously proposed functions are inconsistent with the kinetics of E5 mRNA expression following HPV16 infection (Stoler et al., 1992) and a role for E5 in cancer initiation (zur Hausen, 1996; zur Hausen, 2000). In contrast to the previously described roles for HPV16 E5, HPV16 E5-mediated endoreplication is consistent with its role in initiating oncogenesis. The ability of HPV16 E5 to promote endoreplication, like

cell-cell fusion (Hu and Ceresa, 2009; Hu et al., 2009), indicates that HPV16 E5 makes fundamental modifications to the host cell's chromosome by inducing polyploidy. It has been demonstrated by others that polyploid cells are a frequent occurrence in precancerous cervical lesion (Olaharski et al., 2006). Further, in tissue culture models, it has been shown that mitosis of polyploid cells frequently produces aneuploid cells (King, 2008). This is thought to be because cells are not equipped to properly segregate twice the number of chromosomes and the subsequent gain or loss of a chromosome(s) and the generation of an aneuploid cell. Aneuploidy is a characteristic of nearly all cancers (Sen, 2000). The gain or loss of chromosomes can result in cellular changes such as an increased rate of cell growth or migration, or resistance to apoptosis.

In most cells, the failure of a cell to have the normal complement of chromosomes results in the induction of apoptosis or quiescence. The presence of the HPV16 E6 and E7 genes increases the possibility that polyploid cells will divide, since they inhibit genes that regulate cell cycle checkpoints. Polyploid cells that develop as the result of E5-mediated enodreplication are a potential initiating event for aneuploidy, and subsequently oncogenesis to occur. However, we can not rule out the possibility that other cellular insults also enhance the transition to oncogenesis.

By their very nature, all viral proteins are introduced into the cell by exogenous expression. It is likely that the exogenous expression system used in our studies produces levels of HPV16 E5 that are greater than those that occur as the result of infection by HPV16. Although in the absence of antibodies against the native E5, that is not known with certainty. However, this methodology allows us to amplify the frequency and magnitude of a phenotype that may be less obvious at lower levels of protein expression. In fact, if infection with HPV16 were to produce such frequent and profound changes in cell morphology as we observe with our expression system, it is likely that the incidence of cancer would be dramatically increased. Fortunately, this is not the case. Only a small percentage of HPV positive individuals develop cancer.

Deciphering the functional role of HPV16 E5 in viral replication and oncogenesis has been difficult. Numerous roles have been ascribed to this protein, many of which appear unrelated. Our own work has revealed two novel phenotypes induced by HPV16 E5 expression: binucleated cells (Hu et al., 2009) and enlarged, polyploid nuclei. Live-cell video microscopy to date has not provided evidence for a direct link between the two phenotypes. However, this does not eliminate the possibility that they are related.

The morphological and chromosomal changes we observe are noteworthy because they frequently accompany the progression of normal cells into cancerous cells. Increased nuclear size, increased DNA content, and tetraploidy are characteristic of Low grade Squamous Intraepithelial Lesions (LSIL) (Mittal, Chan, and Demopoulos, 1990; Prasad et al., 1993). Many of these changes are criteria used in the clinical detection of cervical cancer precursors in screening Pap tests (Crum and Lee, 2006; Wright et al., 2004). Additional characteristics of precancerous lesions have also been reported: the presence of bi-nucleated cells, increased DNA content per cell, and polyploidy (Olaharski et al., 2006). These other morphological changes are not used in the diagnosis of precancerous lesions despite the fact they are less subjective because of the amount of material, time, and expense used to detect them. Our studies complement previous reports describing the oncogenic capacity of HPV E5 (Leechanachai et al., 1992; Straight et al., 1993) and offer mechanistic insights into how E5 expression brings about morphological changes in the cervical epithelium. Further, by identifying endoreplication as the mechanism by which the aberrant nuclei form and increase DNA synthesis arises, there is now a biological process that can be targeted to inhibit oncogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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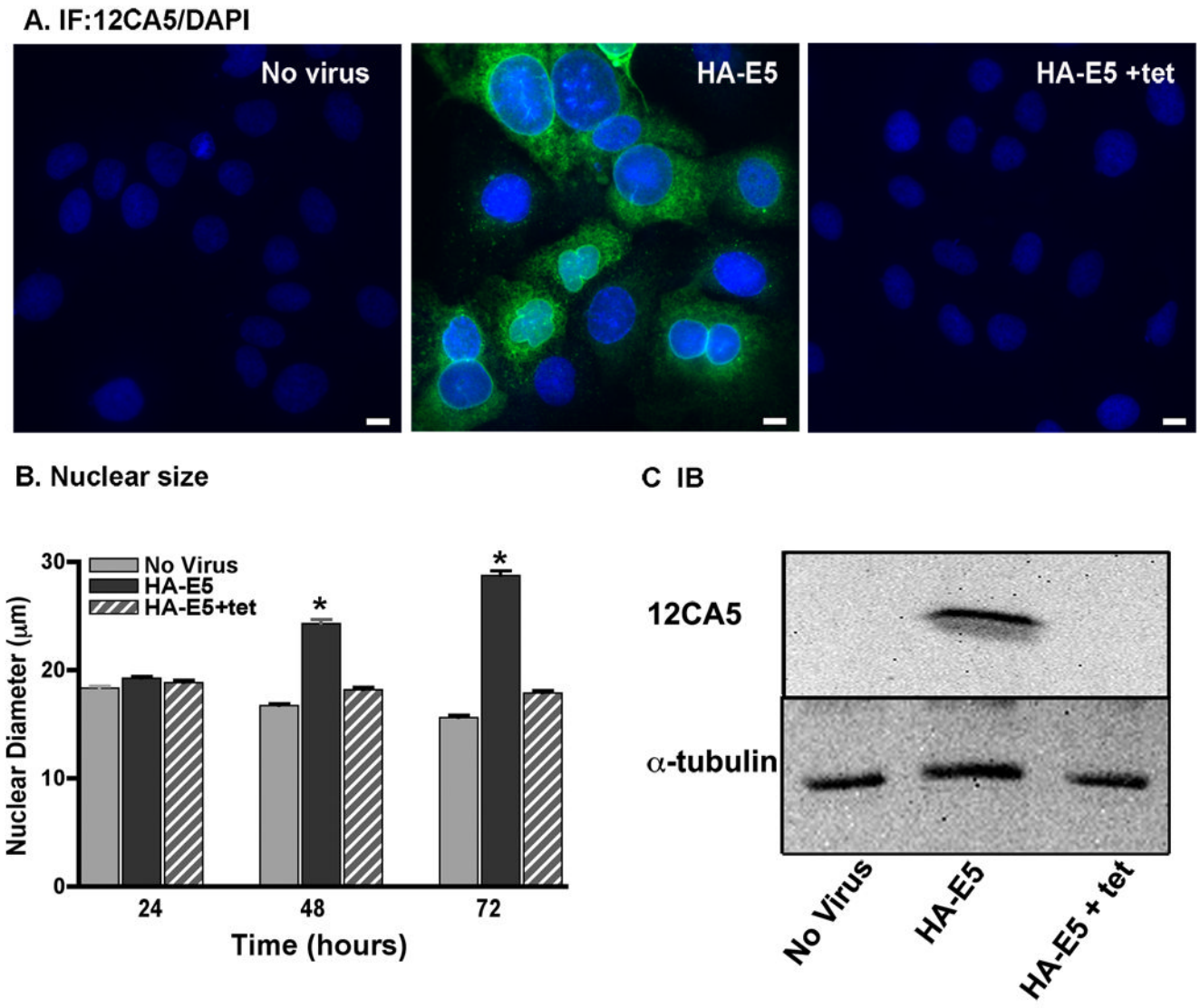

Figure 1. Expression of HPV16 E5 increases nuclear size

A) tTA-HaCaT cells were uninfected or infected with either no adenvirus (No virus), or HPV16 HA-E5 adenoivirus (20 plaque forming units/cell) in the absence (HA-E5) or presence (E5-HA + tet) of tetracycline (1 μg/ml). A) Seventy-two hours after infection, cells were fixed and stained by indirect immunofluorescence with the 12CA5 (anti-HA) mouse monoclonal antibody and nuclei were stained with DAPI. Images were collected using Olympus AX70 epifluorescent microscope with Q-Capture software. Size bar is 10 μm. B) Quantification of the nuclear size in μm. This graph shows the average ± S.E.M. nuclear diameter from more than 150 mono-nucleated cells from three independent experiments. * indicates a p-value of <0.05 (two-tailed, unpaired t-test) as compared to time-matched cells infected in the presence of tetracycline. C) Cell lysates (30 μg) were resolved on a 16% Tris-tricine gel and immunoblotted with the 12CA5 (anti-HA) mouse monoclonal antibody or α-tubulin antibody as a loading control.

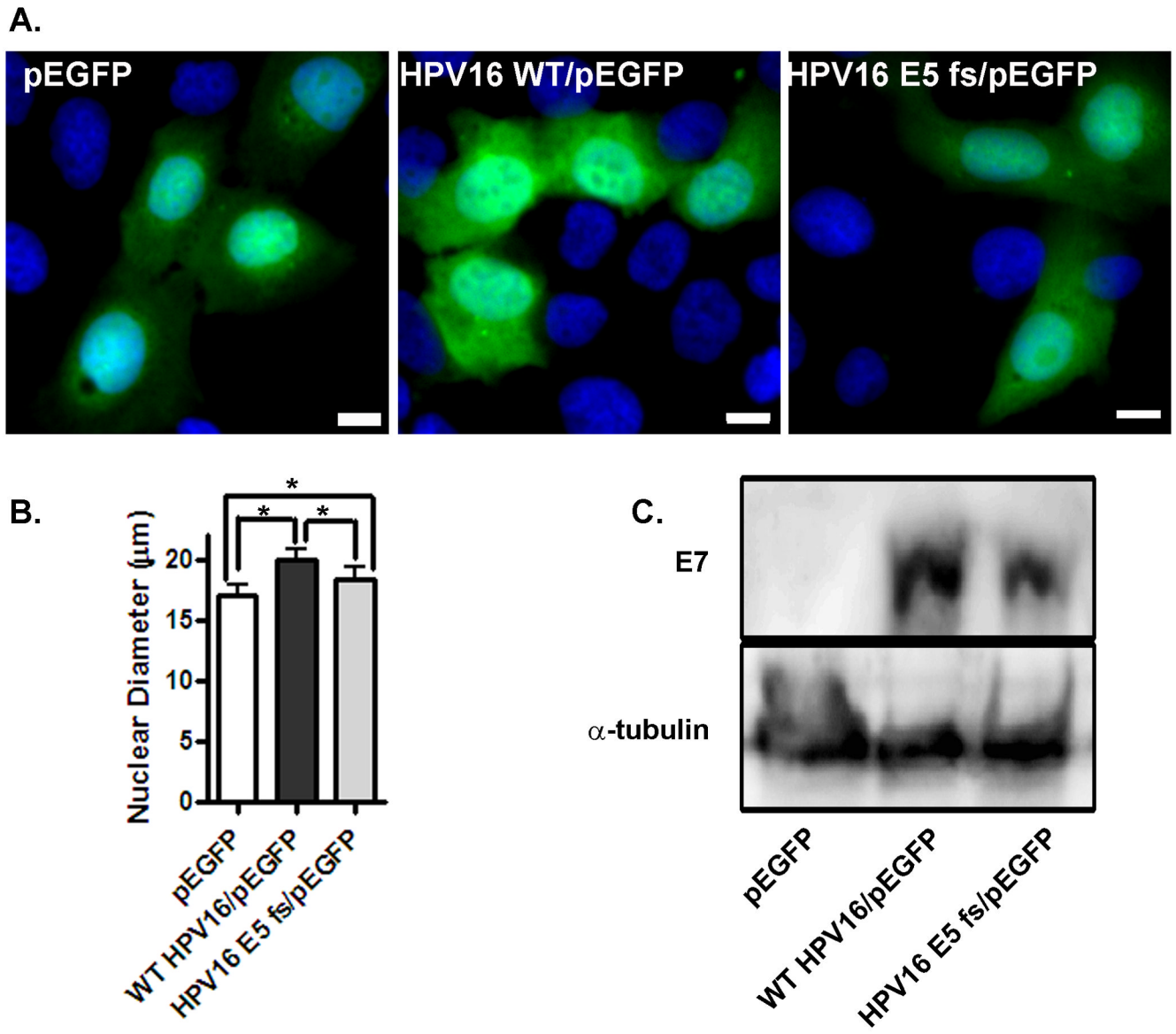

Figure 2. Expression of HPV16 E5 in the context of the entire HPV16 genome causes an increase in nuclear size

HaCaT cells were transfected with pEGFP alone or co-transfected with pEGFP and the wild type HPV16 genome (WT HPV16) or the HPV16 genome with a frameshift mutation in the E5 gene (HPV16 E5 fs). After recovery (72 hours), cells were fixed, and the nuclear size was measured in transfected (GFP-positive) cells. A) Representative micrographs of each transfection condition. B) quantification of > 500 cells (at least 150 cells/experiment; three independent experiments). Data are plotted as the average ± S.E.M. C) Immunoblot of cells treated in parallel to those used in (A). Expression of the wild type and mutant HPV16 genome was confirmed by the presence of the HPV16 E7 oncogene by immunoblot.

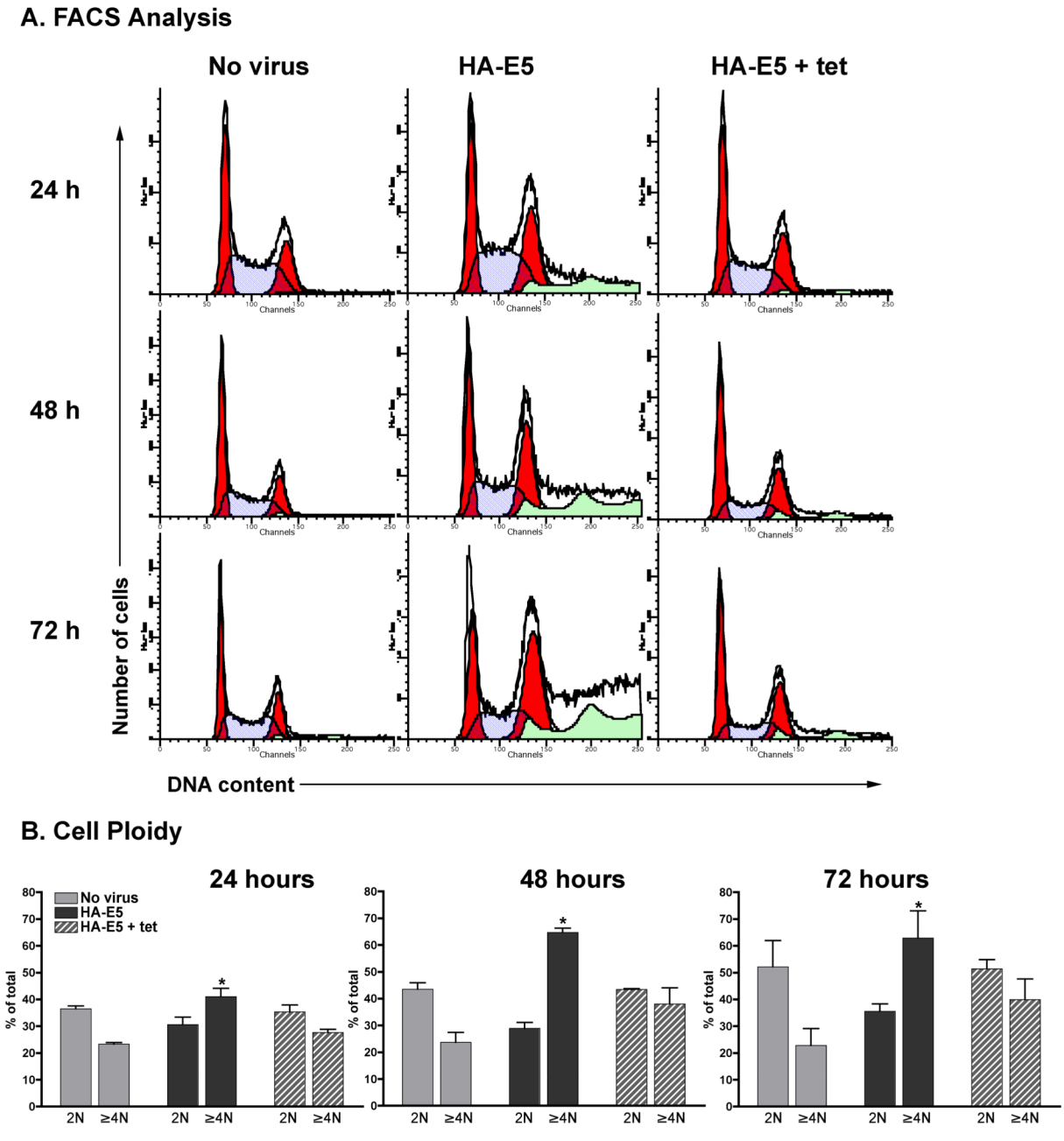

Figure 3. Expression of HPV16 E5 causes an increase in DNA content per cell with time

A) DNA content distribution of HPV16 E5 expressing cells. tTA-HaCaT cells were uninfected or infected with adenovirus encoding for HA-E5 in the absence (HA-E5) or presence of tetracycline (HA-E5 + tet). At the indicated times, cells were fixed, stained with propidium iodide, and analyzed by FACS. A representative analysis is shown with the 2C (left) and 4C (right) cells in red, the S-phase cells in grey, and the cells greater than tetraploid in green. B) Graphical distribution of the cellular DNA content. Data are plotted as the percentage of diploid and polyploidy (tetraploid or greater) cells in each phase. Shown is the average ± S.E.M. (n= 4). * indicates a p-value < 0.05 (two-tailed, unpaired t-test) as compared to cells infected in the presence of tetracycline.

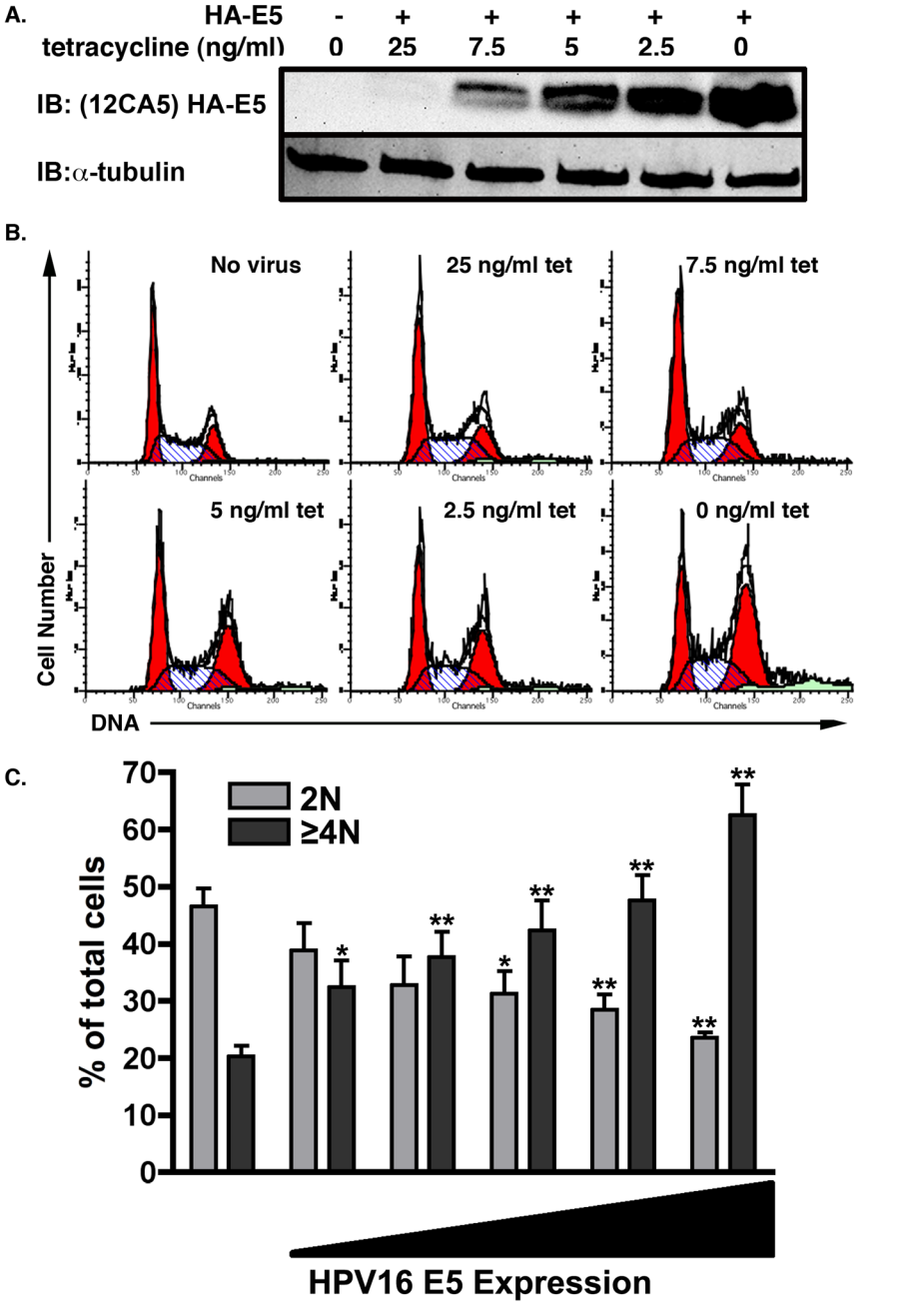

Figure 4. HPV16 E5 mediated increase DNA content per cell is dose dependent

A) tTA-HaCaT cells were infected with or without adenovirus encoding for HA-E5 in the presence of the indicated concentrations of tetracycline (0–25 ng/ml). Following infection (48 hours), cell lysates (80 μg) were prepared, separated on a 16% Tris-tricine gel, and immunoblotted with an anti-HA or an anti-α-tubulin antibody. B) tTA-HaCaT cells from (A) were fixed, propidium iodide stained, and analyzed by FACS. A representative analysis is shown with the 2C (left) and 4C (right) cells in red, the S-phase cells in grey, and the cells greater than tetraploid in green. C) Summary of the DNA content distributions as in (B). Data are plotted as the percentages of 2C, 4C, and ≥4C cells. Shown is the average ± S.E.M.

(n=4). * indicates $p<0.05$; ** indicates $p<0.01$ (two-tailed, unpaired t-test) when compared to no virus control.

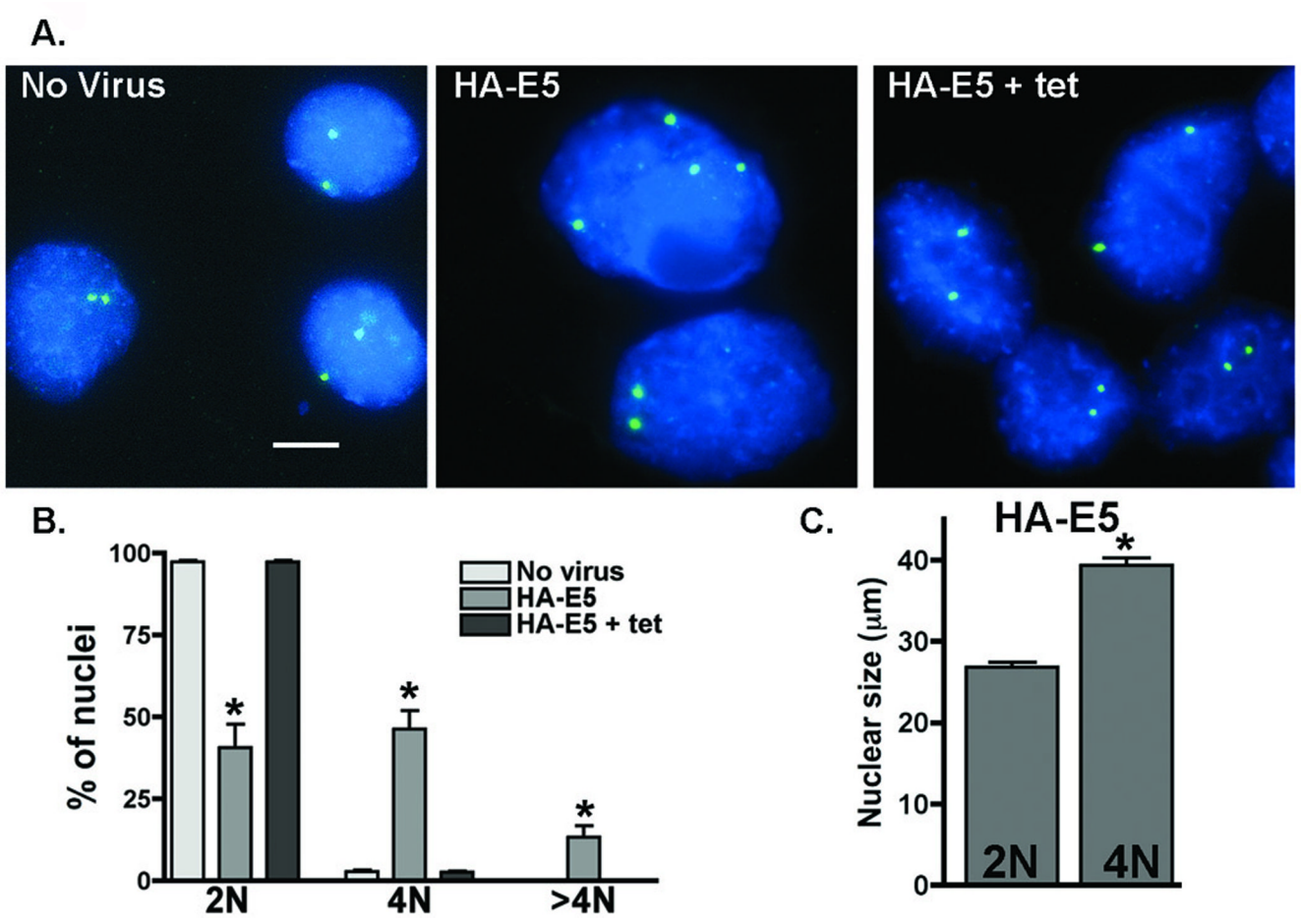

Figure 5. HPV16 E5 increases the percentage of polyploidy cells

A) Images of uninfected tTA-HaCaT cells (No Virus) or cells infected with HPV16 HA-E5 adenovirus in the absence (HA-E5) or presence of tetracycline (HA-E5 +tet). After recovery (72 hours), cells were fixed and processed for Fluorescence In Situ Hybridization (FISH) staining for the X-chromosome (described in Materials and Methods). The X-chromosome stained in green and the nuclei stained blue. Size bar = 10 μm. B) Quantification of the percentage of cells containing 2, 4, or >4 copies of the X-chromosome. Data are plotted as the average percentage ± S.E.M. from three independent experiments (>400 cells per condition). C) Tetraploid cells have a larger nucleus than diploid cells. The average nuclear diameter of HPV16 E5 expressing cell was measured and plotted as a function of the chromosome number. Shown are the average ± S.E.M. for >100 cell for each condition. * indicates $p < 0.05$ (two-tailed, unpaired t-test).

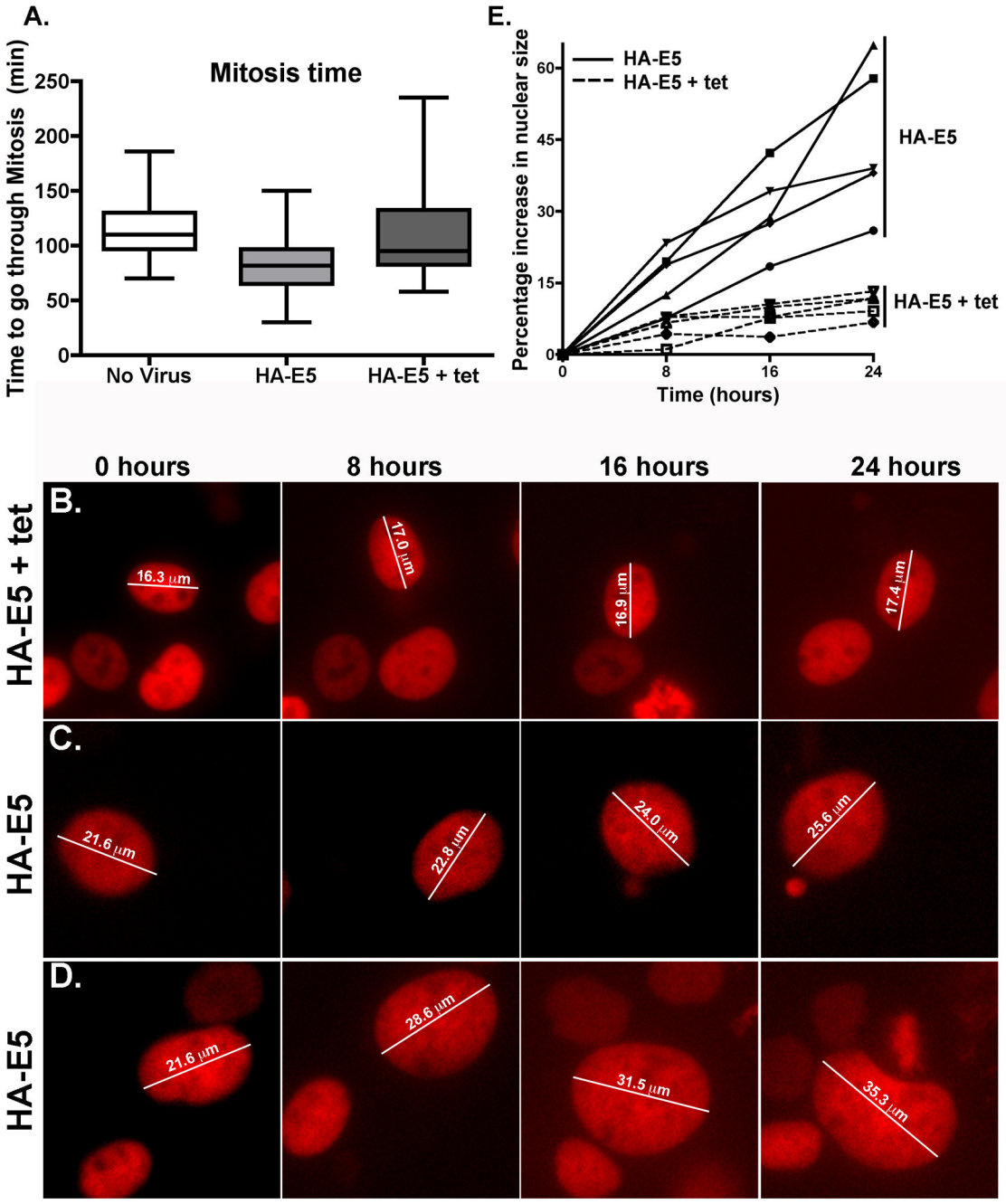

Figure 6. Enlarged nuclei are not the result of failed cytokinesis

A. tTA-HaCaT cells were infected with nothing (no virus) or HPV16 E5 encoding adenovirus in the absence (HPV16 E5) or presence of tetracycline (HA-E5 + tet). Cells were monitored using a Zeiss Axovert 200M microscope equipped with a Hamamatsu ORCA camera. Mitosis times were calculated from more than 40 cells from three different experiments as described in “Materials and Methods”. Data are displayed as a Box and Whisker graph with the SEM. B. tTA-HaCaT cells were transfected with RFP-H2B for 24 hours and then infected with HPV 16 HA-E5 adenoviruses infection for 2 hours, change into growth media after infection. Twenty-four hours after infection, RFP-H2B or YFP-H2B transfected cells were monitored 24 hours under Nikon microscope with Photometrics Cool

Snap HQ camera. Time-lapse phase-contrast images were collected and processed with NIS-Elements AR 3.0, SP5 Software. C. The increase in nuclear size is a continual process. The nuclear size was measured at 0hr, 8hr, 16hr and 24hr. Fig. A showed the represented nuclei. Fig. B shows the change of multiple nuclei size at 8h, 16h and 24h in comparison to 0hr.

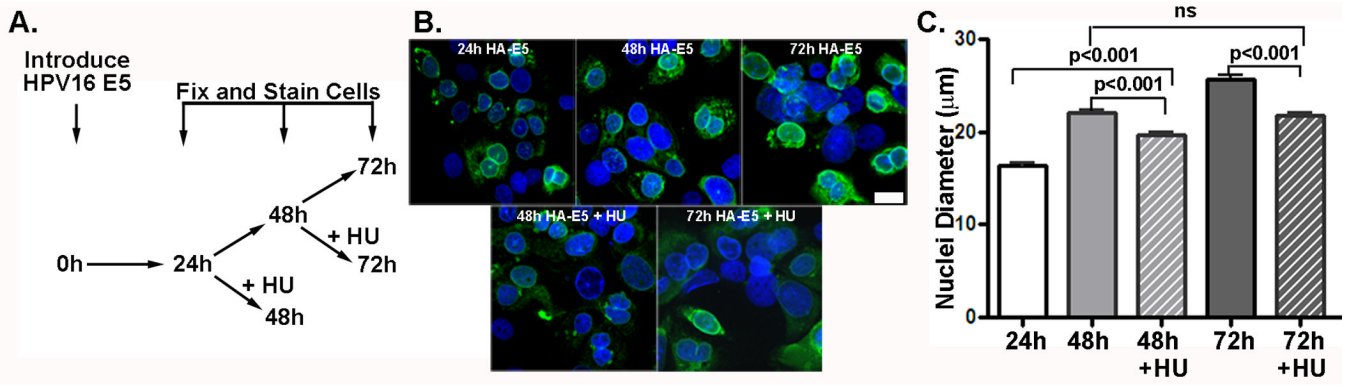

Figure 7. Formation of enlarged nuclei requires DNA synthesis

A) Schematic of treatment of cells. tTA-HaCaT cells were grown on coverslips and infected with HPV16 HA-E5 adenovirus at time = 0 hr. At 24 hour intervals, the media was changed to growth media or growth media supplemented with 0.5 mM hydroxyurea (+HU) and indicated. At 24 hour intervals, cells were fixed, subjected to indirect immunofluorescence using the 12CA5 antibody (Roche) and DAPI staining. B) Representative micrographs showing 12CA5 positive cells (green) and DAPI-stained nuclei (blue). The nuclear size was measured as described in Materials and Methods. C) Nuclear size from each of the 5 conditions. Data are presented as the average ± S.E.M. (n=3) with at least 50 nuclei measured per condition in each experiment. Statistical significance was determined using a student's t-test.