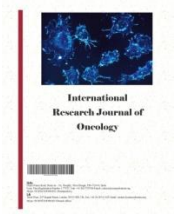

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HPV-16 and HPV-18 Affects Viability and Proliferation in Normal Breast Tissue Cells

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Authors' contributions

This work was carried out in collaboration among all authors. Author KK designed the study, performed the statistical analysis and wrote the protocol and first draft of the manuscript. Authors MLH and TM performed all experiments. All authors read and approved the final manuscript.

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ABSTRACT

Introduction and Aims: Recently HPV-16 and 18 have been found in the oral cavity and have been significantly linked as causative agents of oral cancer. Research has also shown the effects of HPV on breast cancer cells. More recently, HPV-16 and 18 have been found in normal breast tissue. The carcinogenic effects of HPV on oral and breast tissues have been demonstrated; however, the effect of HPV on non-cancerous breast tissue cells has not yet been studied. Based upon this information, the objective of this study was to evaluate the effects of HPV on normal breast tissue.
Study Design: This is an observational laboratory-based study of human cell cultures.
Place and Duration of Study: This study was conducted in the Department of Biomedical Sciences at the University of Nevada, Las Vegas – School of Dental Medicine between May 2017 and May 2019.
Methodology: HPV16 and HPV18 strains were used to infect normal, non-cancerous breast tissue cell lines Bst-Hs578 and 18485 *in vitro*. Cellular growth and viability was evaluated to

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determine if HPV mediated any of these cellular phenotypes. Cells were plated into 96-well assay plates to measure proliferation. Viability was measured using a BioRad TC20 automated cell counter.

Results: The addition of HPV-16 and HPV-18 had significant effects on cellular phenotypes in both Bst-Hs578 and 184B5 cells. For example, cell viability increased between 21% and 41% in Bst-Hs578 cells and 23% to 53% in 184B5 cells over three days. In addition, cellular proliferation was also significantly altered with both HPV strains in each of the cell lines, increasing approximately two-fold at each of the three-day assay time points. Each of these phenotypes was sustained over the course of one week and three week assays.

Conclusion: Although studies have demonstrated that HPV can modulate oral and breast cancer cells, no studies to date have demonstrated that HPV has the potential to mediate the growth or viability of normal, non-cancerous breast tissue. This study may be among the first to demonstrate that HPV is capable of modulating these phenotypes in normal, non-cancerous breast tissue – which will be important for dentists, oral healthcare professionals and epidemiologists who are interested in HPV prevention and vaccination.

Keywords: Human papillomavirus (HPV); breast; normal tissue; growth.

ABBREVIATIONS

HPV : Human papillomavirus
DNA : Deoxyribonucleic acid
CIN : Cervical intraepithelial neoplasia
ATCC : American Type Culture Collection
RPMI : Roswell Park Memorial Institute
PABA : Para-aminobenzoic acid
MEM : Minimal Essential Media
DMEM : Dulbecco's Modified Eagle's Medium

1. INTRODUCTION

The Human Papillomavirus (HPV) family encompasses a group of closely related non-enveloped DNA viruses that are known to infect skin and mucosal tissues [1]. The induction of early viral promotion genes are known oncoproteins that concomitantly inactivate two of the key critical tumor suppressor pathways linked with p53 and retinoblastoma (Rb) - first elucidated in cervical intraepithelial neoplasia (CIN) [2,3]. This knowledge has led to the widespread adoption of HPV-related screening to increase early treatment and prevention of CIN [4].

As screening and research into HPV-associated pathology and tumorigenesis in cervical cancers advanced, research uncovered many additional forms of HPV-associated cancers in mucosal and epithelial tissues [5]. This research has mainly focused on anogenital cancers, such as penile and anal tumors [6-8]. However, more recently HPV-16 and 18 have been found in the oral cavity and have been significantly linked as causative agents of oral cancer – as well as other cancers of the head and neck [9-11].

Most recently, evidence has emerged linking HPV infection with breast cancer [12,13]. Most studies of this phenomenon have traditionally focused on whether HPV may be an ancillary factor that modulates already developing breast tumors rather than answering the question if it may, in fact, be oncogenic in these tissues [14,15]. Much of this research has found contradictory and conflicting evidence, which has contributed to the urgency for more focused and targeted research in this area [16-18]. Because most of the studies to date have focused on the effects of HPV in already developing or developed breast cancers, far less is known about the effects of HPV on non-cancerous breast tissue cells [19]. Based upon this information, the objective of this study was to evaluate the effects of HPV on normal breast tissue.

2. METHODOLOGY

2.1 Cell Culture

Normal, non-cancerous breast tissue cell lines Bst-Hs578 (HTB-125) and 184B5 (CRL-8799) were obtained from American Type Culture Collection (ATCC). Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media, which has been deemed suitable for many mammalian epithelial cell lines due to high concentrations of biotin, vitamin B_{12} and para-aminobenzoic acid (PABA) – constituents not found in Minimal Essential Media (MEM) or Dulbecco's Modified Eagle's Medium (DMEM) [19,20]. Cell culture media was supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin and cells were maintained in tissue culture treated flasks at

37°C and 5% CO_2 in a humidified tissue culture chamber [21,22]. All cells used in these experiments were early passage (Passage 4-6), which was well within the manufacturer recommendation for use of non-immortalized, non-cancerous normal cells prior to passage twenty (P20), where some cells may have begun reaching replicative senescence or the Hayflick limit [21,22].

2.2 HPV Transient Transfection

Bst-Hs578 and 184B5 cells were seeded into tissue culture-treated flasks with the appropriate media until approximately 70% confluence was achieved. Transient transfection was accomplished using the Stratagene Mammalian Transfection Kit and protocol for $CaPO_4$ transfection, as previously described [19,23-25]. The full length HPV type 16 (ATCC#45113) and HPV type 18 (ATCC#4152) cloned into pBluescript SK- (suitable for transient transfection) were added at 1.0 ug/mL to each cell line, as previously described [23-25].

The mock transfection or negative controls were established by performing the same protocol but without the addition of the full length HPV virus. In brief, Bst-Hs578 and 184B5 cells were seeded into tissue culture-treated flasks with the appropriate media until approximately 70% confluence was achieved. Mock transfection was accomplished using the Stratagene Mammalian Transfection Kit and protocol for $CaPO_4$ transfection without the addition of any plasmid vector, as previously described [19,23-25].

2.3 Cellular Viability

Cellular viability was measured using the Trypan Blue exclusion assay. In brief, Trypan Blue 0.4% solution (Gibco) was added to control and experimental cells to create a 1:1 dilution, which was incubated at room temperature. Each plate was placed on a hemocytometer grid on a Zeiss Axiovert inverted microscope. Four to six grids (1.0 mm^2) were counted and averaged to determine cell number. To verify these results control and experimental cells were also trypsinized and diluted 1:1 with trypan blue for viability using a BioRad TC20 automated cell counter.

2.4 Cellular Proliferation

Cells were plated in 96-well flat bottom tissue-culture treated assay plates from Corning Costar (Fisher Scientific) at a concentration of 1.2 x 10^4 cells per well. In brief, cells were fixed at several time points, including 24 hours (1 day), 48 hours (2 days), 72 hours (3 days), 168 hours (1 week) and 504 hours (3 weeks) using 10% buffered formalin. After 24 hours of fixation, formalin was aspirated and cells were stained with Gentian Violet 2% aqueous solution (Fisher Scientific) and washed with 1x Phosphate Buffered Saline (PBS). Proliferation of cells was measured as absorbance in each of the assay plates using a BioTek ELx808 microplate reader at 595 nm.

2.5 Statistical Analysis

All experiments were performed in triplicate, with all data in figures presented using standard deviation (SD). Differences between control (no transfection), negative control (mock transfection) and experimental (transfected) cells were measured using two-tailed t-tests, which are appropriate for parametric biological data. Significance was set at alpha α=0.05.

3. RESULTS

The transfection of HPV-16 and HPV-18 was sufficient to induce significant effects on Bst-Hs578 cells (Fig. 1). More specifically, the addition of HPV-16 was sufficient to significantly increase viability ranging between 31.6% and 49.9% over three days. This represented an increase from the baseline control (non-transfected) cell viability of 19-22% to 25%-31%, $p<0.05$. This increase was sustained over one week (1.95-fold increase to 39%) extending to three weeks (3-fold increase to 54%), $p<0.05$. Similar results were observed with HPV-18, demonstrating an increase in viability between 21.1% and 38.1% (23%-29%) over three days – peaking with a two-fold increase at weeks one and three to 40%-41%, $p<0.05$.

The transfection of HPV-16 and HPV-18 was also sufficient to induce significant effects on 184B5 cells (Fig. 2). More specifically, the addition of HPV-16 was sufficient to significantly increased viability between 31.2% and 46.7% over three days. This represented an increase from the baseline control (non-transfected) cell viability of 15-17% to 21%-32%, $p<0.05$. This increase was sustained over one week (61% increase to 29%) extending to three weeks (72.2% increase to 31%), $p<0.05$. Furthermore, the addition of HPV-18 induced an increase in viability between 23.5% and 53.3% (21%-23%) over three days, which was sustained over one week (66.7% increase to 30%) to week three (55.6% increase to 28% viability), $p<0.05$.

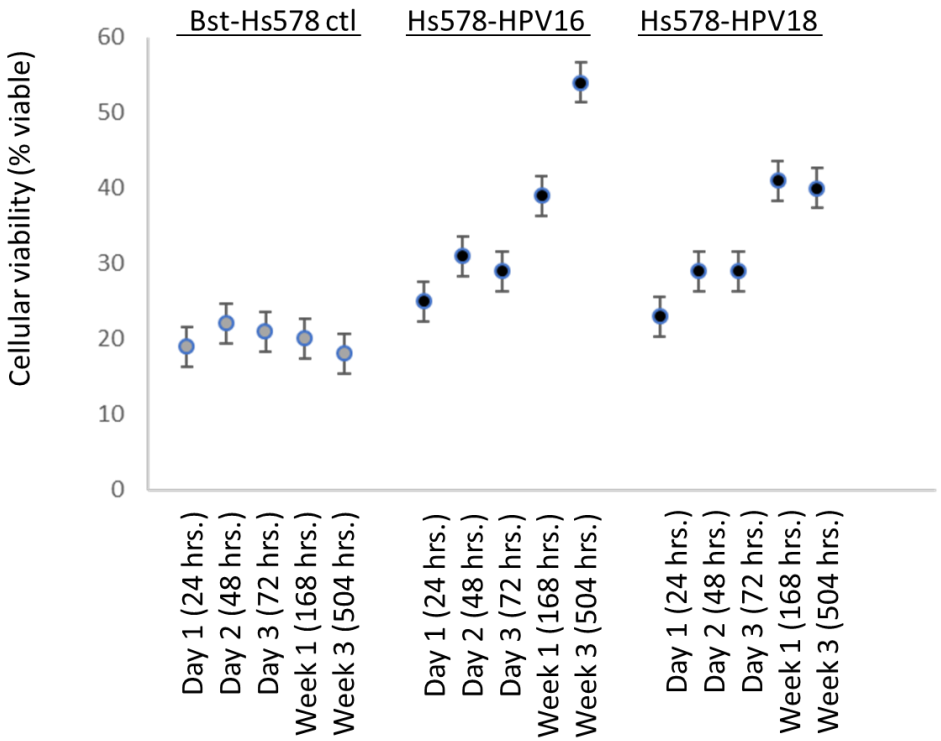

Fig. 1. Changes in cellular viability in Bst-Hs578 cells following HPV transfection. The addition of high-risk HPV-16 and HPV-18 strains increased viability over three days between 21 and 41%. Sustained increases in viability were observed at Week 1 (2-fold increase) and Week 3 (3- and 2-fold increase, respectively)

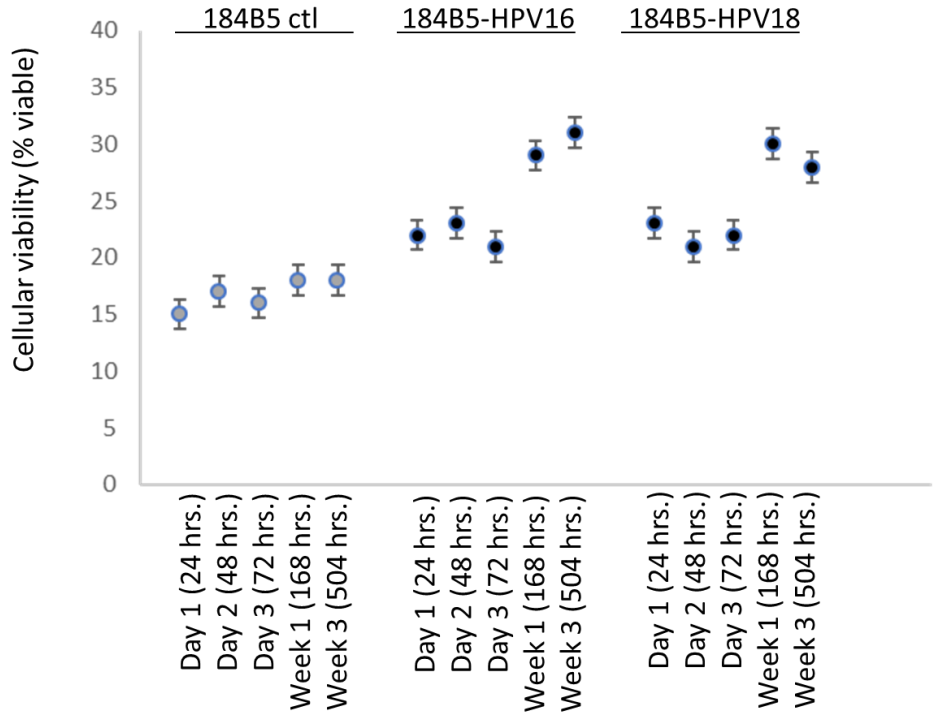

Fig. 2. Changes in cellular viability in 184B5 cells following HPV transfection. The addition of high-risk HPV-16 and HPV-18 strains increased viability over three days between 23% and 53%. Sustained increases in viability were observed at Week 1 (67% increase) and Week 3 (56% increase)

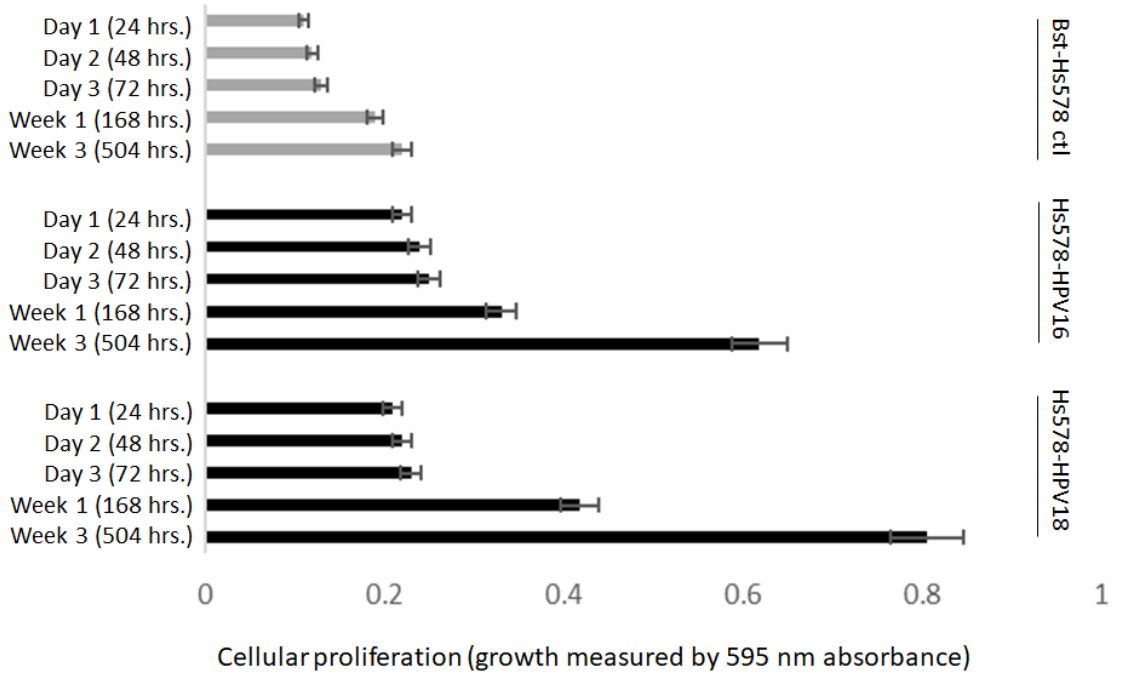

Fig. 3. Changes to cellular proliferation among Bst-Hs578 cells with HPV. The addition of high-risk HPV-16 and HPV-18 significantly increased growth over three days by approximately two-fold. These increases were sustained over Week 1 and Week 3 with a more robust increase observed with HPV-18 at the final time point

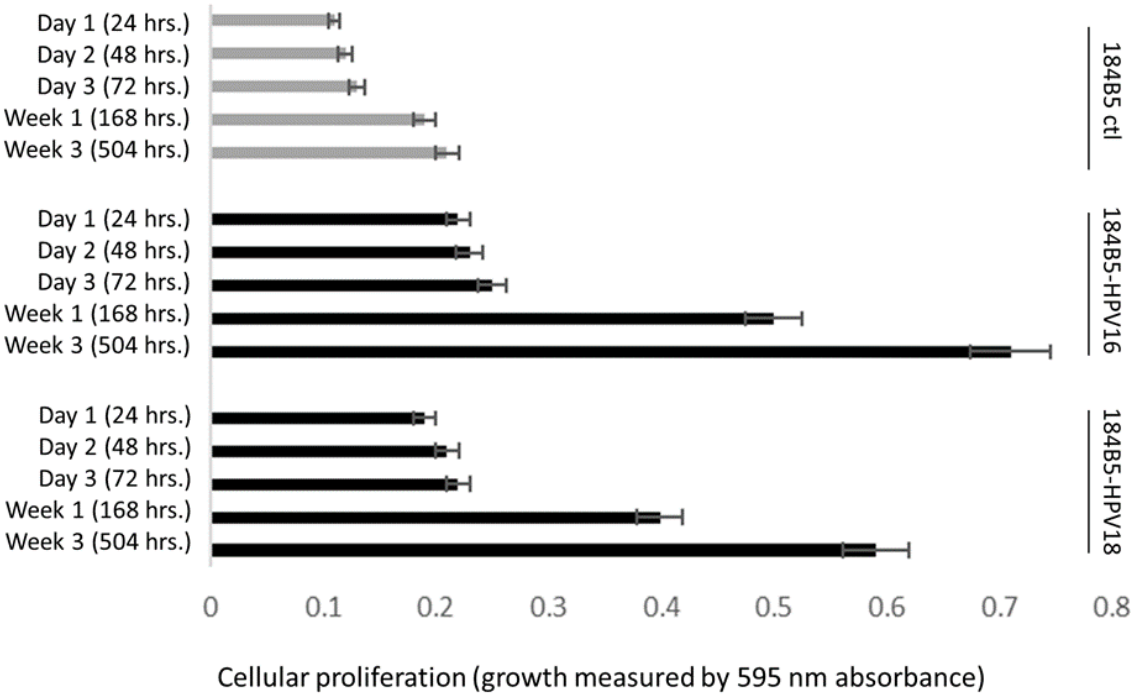

Fig. 4. Changes to cellular proliferation among 184B5 cells with HPV. The addition of high-risk HPV-16 and HPV-18 significantly increased growth over three days by approximately two-fold. These increases were sustained over Week 1 and Week 3 with a more robust increase observed with HPV-16 at the final time point

To evaluate whether the observed increases in cellular viability were correlated with other cellular phenotypes, 96-well growth assays were performed (Fig. 3) above. These data demonstrated significant changes over the first three days. For example, the addition of HPV-16 increased growth among the Bst-Hs578 cells between 1.92- and 2.01-fold at 24, 48 and 72 hrs. (Day 1, Day 2, Day 3). Similar increases between 1.77- and 1.91-fold were observed with the addition of HPV-18. These increases in growth with the addition of HPV-16 and HPV-18 were apparent at both of the additional time points of Week 1 (1.74-fold and 2.2-fold increase) and Week 3 (2.81-fold and 3.66-fold increase), which were all significantly different from the non-transfected control cells, $p<0.05$.

These proliferation and growth assays were also performed with 184B5 cells and the addition of HPV-16 and HPV18 (Fig. 4). These results also demonstrated significant changes over the entire time course of the assay. More specifically, HPV-16 increased 184B5 growth between 1.91- and 2.01-fold at 24, 48 and 72 hrs. (Day 1, Day 2, Day 3).Similar increases between 1.69- and 1.72-fold were observed with the addition of HPV-18. These increases associated with HPV-16 and HPV-18 were sustained through Week 1 (2.63-fold and 2.1-fold increase, respectively) and Week 3 (3.38-fold and 2.81-fold increase), which were all significantly different from the non-transfected control cells, $p<0.05$.

4. DISCUSSION

Because most studies to date have focused on the effects of HPV in already developing or developed breast cancers, far less is known about the effects of HPV on non-cancerous breast tissue cells [19]. Based upon this information, the objective of this study was to evaluate the effects of HPV on normal breast tissue. The results of this study demonstrated that the addition of high-risk HPV strains HPV-16 and HPV-18 were sufficient to induce significant changes to cellular phenotypes, including viability and growth.

These data support the observations from other researchers that high-risk HPV strains may be found in normal tissue from breast cancer patients, suggesting a potential role in the oncogenesis and development of these HPV-positive tumors [14,26,27]. In fact, several studies have suggested this mechanism and pathway for the development of some breast cancers – although further research into these areas will be needed to fully understand the oncogenic potential of high-risk HPV in these tissues [28,29].

In fact, new research has suggested that high-risk HPV strains, such as HPV-16 and HPV-18, may in fact be considered primary etiologic agents in the development of some breast cancers [30]. Some evidence supports these hypotheses, with findings that suggest these high-risk HPV strains may be associated with other clinical and pathological conditions that may present prior to the development of breast cancer [31,32]. In fact, as more data become available the association between these high-risk HPV strains and the development of breast cancer becomes more compelling [5,12,33].

As public health awareness regarding the potential benefits of HPV vaccination becomes more prevalent, each potential benefit and aspect of HPV prevention should be considered by both healthcare providers and parents considering these measures for the prevention of HPV-related disease [34,35]. Given that HPV may modulate or even initiate a variety of cancers, that are not just restricted to cervical and gynecological cancers, an increased awareness and understanding of HPV-related diseases and pathologies will be needed to guide public health and healthcare officials in their recommendations for patient care and disease prevention [36].

5. CONCLUSION

Although studies have demonstrated that HPV can modulate oral and breast cancer cells, no studies to date have demonstrated that HPV has the potential to mediate the growth or viability of normal, non-cancerous breast tissue. This study may be among the first to demonstrate that HPV is capable of modulating these phenotypes in normal, non-cancerous breast tissue – which will be important for dentists, oral healthcare professionals and epidemiologists who are interested in the mechanisms of HPV-induced disease and HPV-related prevention and vaccination.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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