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HPV16 E6 regulates annexin 1 (ANXA1) protein expression in cervical carcinoma cell lines

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ABSTRACT

Annexin 1 (ANXA1) is a substrate for E6AP mediated ubiquitylation. It has been hypothesized that HPV 16 E6 protein redirects E6AP away from ANXA1, increasing its stability and possibly contributing to viral pathogenesis. We analyzed ANXA1 expression in HPV-positive and negative cervical carcinoma-derived cells, in cells expressing HPV-16 oncogenes and in cells transduced with shRNA targeting E6AP. We observed that ANXA1 protein expression increased in HPV-16-positive tumor cells, in keratinocytes expressing HPV-16 E6wt (wild-type) or E6/E7 and C33 cells expressing HPV-16 E6wt. ANXA1 protein expression decreased in cells transfected with E6 Dicer-substrate RNAs (DsiRNA) and C33 cells co-transduced with HPV-16 E6wt and E6AP shRNA. Moreover, colony number and proliferation rate decreased in HPV16-positive cells transduced with ANXA1 shRNA. We observed that in cells infected with HPV16, the E6 binds to E6AP to degrade p53 and upregulate ANXA1. We suggest that ANXA1 may play a role in HPV-mediated carcinogenesis.

1. Introduction

Cervical carcinogenesis is strongly associated with infection with high-risk human papillomavirus (HPV). HPV is an epitheliotropic virus associated with several benign and malignant cutaneous and mucosal pathological conditions (Dunne and Park, 2013; Ljubojevic and Skerlev, 2014). The E6 and E7 proteins of the high-risk mucosal HPV types target several cellular factors involved in immunosurveillance, allowing viral persistence. Moreover, they alter cell cycle and apoptosis control, facilitating the accumulation of DNA damage/mutations that ultimately contribute to malignant transformation (Ghittoni et al., 2015). In the productive phase of the HPV life cycle, E6 and E7 promote the proliferation of undifferentiated and differentiated suprabasal cells, preventing apoptosis (Moody and Laimins, 2010).

The primary transforming activity of high-risk HPVs is provided by the E6 and E7 oncoproteins (McLaughlin-Drubin and Munger, 2009). During natural infection, the activity of oncogenes E6 and E7 allows the small number of infected cells to expand, increasing the number of cells that subsequently go on to produce infectious virions (Doorbar, 2006).

E6 can interact with proteins or interfere in the expression of some genes to circumvent the antiviral response of infected cells. One of the main targets of E6 from high-risk HPV types is the p53 tumor suppressor. E6 forms a complex with a 100-kDa cellular ubiquitin ligase, E6AP, which mediates p53 polyubiquitination and proteasomal degradation (Huibregtse et al., 1993). Shimoji and co-workers identified ANXA1 as a normal substrate for E6AP-mediated ubiquitylation (Shimoji et al., 2009) and suggested that viral proteins may redirect E6AP away from ANXA1, increasing the stability of this protein and thereby contributing to viral pathogenesis.

The annexin superfamily of proteins has been implicated in several cellular processes, including the regulation of differentiation, apoptosis, proliferation and inflammation. ANXA1 may have important regulatory roles in tumor development and progression. Evidence for this relies on the observation that its expression is reduced in prostate cancer, gastric and breast cancer (Kang et al., 2002; Wang et al., 2010; Yu et al., 2008), while it is increased in pancreatic cancer, melanoma and triple negative breast cancer (Belvedere et al., 2014; Boudhraa et al., 2014; Okano et al., 2015).

In the present study, we analyzed ANXA1 the role of ANXA1 in HPV16-positive cervical carcinoma derived cell lines biology.

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2. Results

2.1. ANXA1 protein expression in HPV positive cell lines and keratinocytes expressing HPV-16 E6

The analysis of ANXA1 and p53 expression was evaluated by Western blotting in HPV-16-positive cervical carcinoma-derived cell lines (SiHa and CasKi), in human foreskin keratinocytes immortalized with HPV-16 (HF698) and in the HPV-negative cervical carcinoma-derived cell line C33. We observed lower levels of ANXA1 expression in C33 compared to HPV-16 positive cells. In contrast, and as expected, higher levels of p53 were detected in C33 cells compared to HPV-16-positive cells (Fig. 1A).

In order to verify whether higher ANXA1 expression occurs exclusively in tumor cells, we analyzed protein expression levels in keratinocytes transduced with HPV-16 E6wt, E7wt and E6/E7. We observed an increase in ANXA1 expression in keratinocytes transduced with E6wt or E6/E7 HPV-16 compared to normal keratinocytes and keratinocytes expressing HPV-16 E7wt alone or transduced with pLXSN empty vector. On the other hand, downregulation of p53 expression was observed in cells expressing HPV16 E6 wt (Fig. 1B).

Next, we analyzed p53 and ANXA1 protein levels in C33 cells stably expressing HPV-16 E6wt. HPV-16 E6wt C33 cells showed higher expression of ANXA1 protein when compared to cells transduced with empty vector or not transduced (Fig. 1C). These results suggest that ANXA1 expression is influenced by HPV16 E6 oncoprotein.

2.2. ANXA1 mRNA expression is not altered but ANXA1 and p53 proteins expressions are altered in cells transfected with HPV-16 E6 DsiRNA in HPV-positive cell lines

In order to further confirm the correlation of ANXA1 upregulation in cells expressing HPV-16 E6wt, we analyzed the effect of E6 silencing using specific DsiRNAs. As expected, we observed a decrease in the levels of HPV-16 E6 mRNA compared to cells transfected with the negative control (scrambled DsiRNA), whereas we did not observe any alteration in the levels of ANXA1 mRNA in CasKi and SiHa cells transfected with HPV16 E6 DsiRNA (Fig. 2A). The results of the analysis of ANXA1 mRNA prompted us to study the effect of E6 suppression on ANXA1 protein levels. We observed that after E6 suppression, p53 protein levels were upregulated in Caski and SiHa cells. On the other hand, the expression of ANXA1 protein was decreased in cells transfected with HPV16 E6 DsiRNA compared to cells transfected with negative control DsiRNA (Fig. 2B).

2.3. Upregulation of ANXA1 protein expression requires the presence of HPV-16 E6wt and E6AP

To further investigate the role of E6AP in ANXA1 protein stability in the context of E6 expression, we infected Caski, SiHa and C33 cells with retroviral particles expressing a shRNA targeting E6AP. We observed that p53 protein levels increased in CasKi and SiHa cells upon E6AP suppression. Unexpectedly, ANXA1 protein expression decreased in these cells after E6AP downregulation (Fig. 3A).

Interestingly, C33 cells expressing HPV-16 E6wt exhibited higher ANXA1 protein levels and p53 protein downregulation compared to control C33 cells (Fig. 3B). However, in C33 cells expressing HPV16 E6 wt and shRNA targeting E6AP, the level of ANXA1 protein expression decreased and the level of p53 protein increased when compared to C33 cells expressing HPV16 E6 wt and endogenous E6AP. In this case, and as observed for SiHa and CasKi cell lines, E6AP suppression was associated with ANXA1 downregulation and p53 stabilization. Altogether, our results indicate that ANXA1 expression requires the presence of E6 and E6AP.

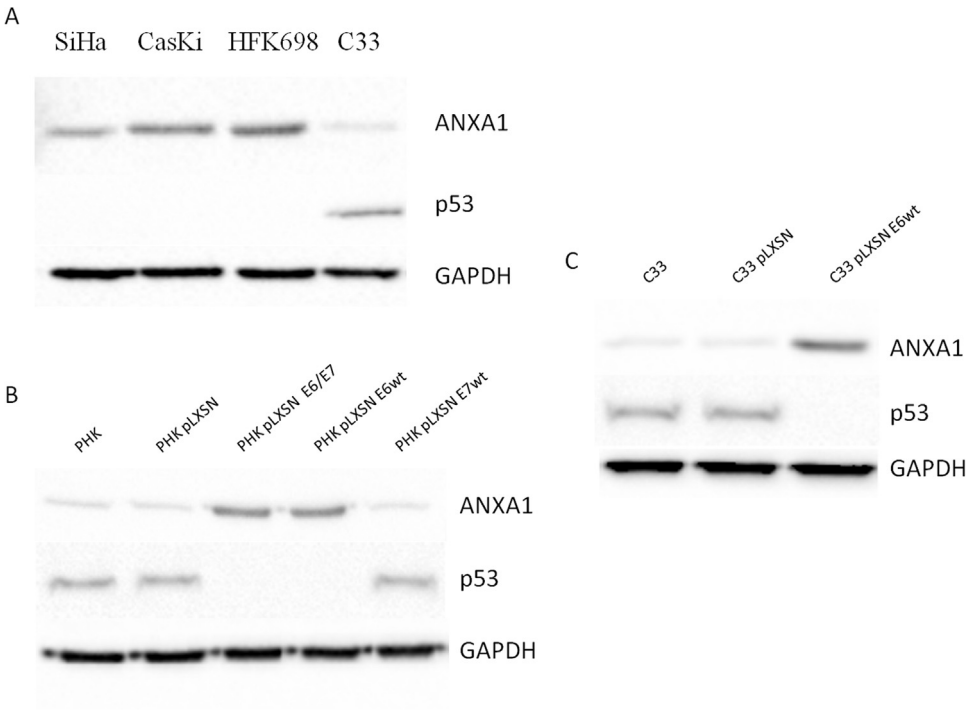

Fig. 1. Expression of HPV16-E6 induces ANXA1 expression in normal and transformed cells. (A) Western blotting analysis of ANXA1 and p53 expression in HPV-16-positive (SiHa and Caski) and HPV-negative (C33) cervical cancer cell lines and in HPV-16 immortalized keratinocytes (HFK698). (B) Primary keratinocytes (PHK) were or were not transduced with the indicated vectors (top) and analyzed by Western blotting using ANXA1 and p53 antibodies as indicated (right). (C) Cervical carcinoma cells C33 were or were not transduced with the indicated vectors (top) and subjected to Western blotting using ANXA1, p53 antibodies as indicated (right). Total proteins isolated from cells as indicated were blotted with antibodies for ANXA1 and p53. The level of GAPDH served as a loading control.

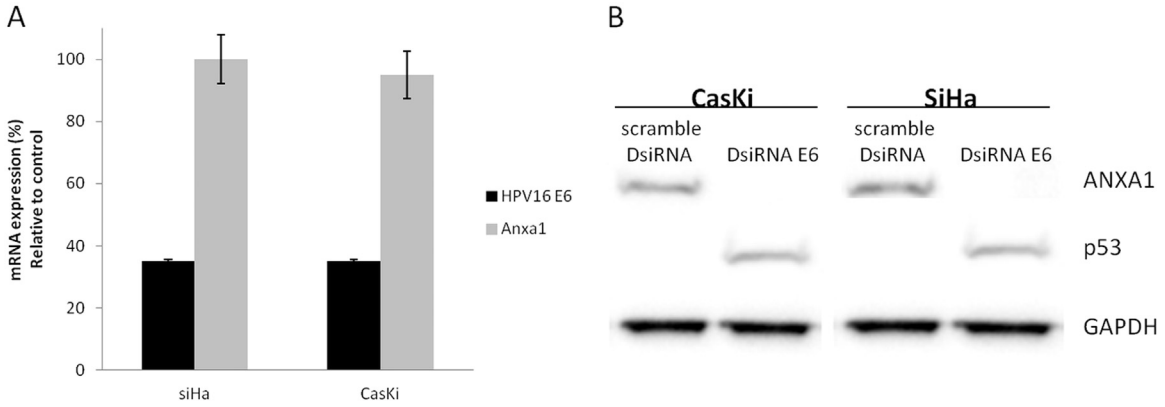

Fig. 2. ANXA1 mRNA expression were not altered but ANXA1 and p53 protein expression was altered in cells transfected with HPV-16 E6 DsiRNA in HPV-positive cell lines. (A) qPCR analysis of ANXA1 and HPV16 E6 mRNA expression in cervical carcinoma cell lines (SiHa and CasKi) transfected with DsiRNAs targeting HPV16E6 (DsiRNA E6) for 72 h. Cells transfected with a non-relevant siRNA sequence pool (scrambled DsiRNA) were used as control. GAPDH: internal standard. (B) Western blotting of ANXA1 and p53 expressions in HPV16-positive cervical cancer cell lines (SiHa and Caski) transfected with DsiRNAs targeting HPV16E6 or scrambled DsiRNA gene expression for 72 h. Total proteins isolated from cells as indicated were blotted with antibodies for ANXA1 and p53. The level of GAPDH served as a loading control.

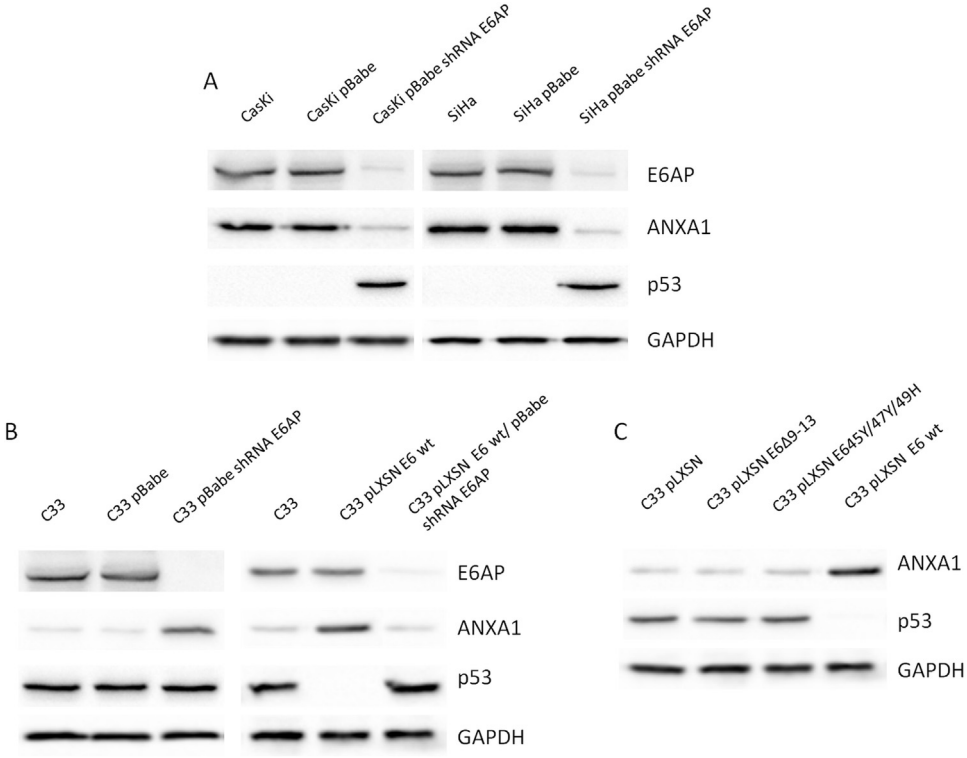

Fig. 3. Low ANXA1 protein expression occurred in E6AP knockdown cells expressing HPV16 E6wt and in cells expressing E6AP and HPV16 E6 mutants. (A) Western blotting analysis of ANXA1 and p53 expression in HPV-16-positive (SiHa and Caski) transduced with pBabe and pBabe shRNA targeting E6AP. (B) Western blotting analysis of ANXA1 and p53 expression in HPV-negative (C33) cervical cancer cell lines transduced with pBabe and pBabe shRNA targeting E6AP and C33 cells cotransduced with pLXSN E6wt and pBabe shRNA targeting E6AP. (C) Western blotting analysis of ANXA1 and p53 expression in C33 cervical carcinoma cells transduced with C33 pLXSN, pLXSN E6wt and with the E6 mutants pLXSN E6 Δ9–13 and pLXSN E6 45Y/47Y/49H. Total proteins isolated from cells as indicated were blotted with antibodies for ANXA1 and p53. The level of GAPDH served as a loading control.

2.4. ANXA1 protein induction depends on HPV-16 E6 domains required for p53 degradation

In order to determine the E6 domains required for ANXA1 upregulation, we analyzed ANXA1 and p53 protein expression in C33 cells expressing different HPV16 E6 mutants. We observed that C33 cells expressing HPV-16 E6 Δ9–13 (deletion in the PRKLP motif) and HPV-16 E6 45Y/47Y/49H exhibited ANXA1 down-regulation and increased p53 levels when compared to C33 cells expressing HPV-16 E6 wt (Fig. 3C). These observations suggest that E6-mediated ANXA1 upregulation requires the presence of E6 and is dependent on its integrity.

2.5. ANXA1 knockdown reduces cell growth and clonogenic potential in Caski and SiHa cells

Aiming to define the role of ANXA1 in HPV-positive cervical cancer derived cell line biology, the expression of this gene was suppressed using shRNA (Fig. 4A). These cells were further analyzed for proliferation and colony formation efficiencies. Our

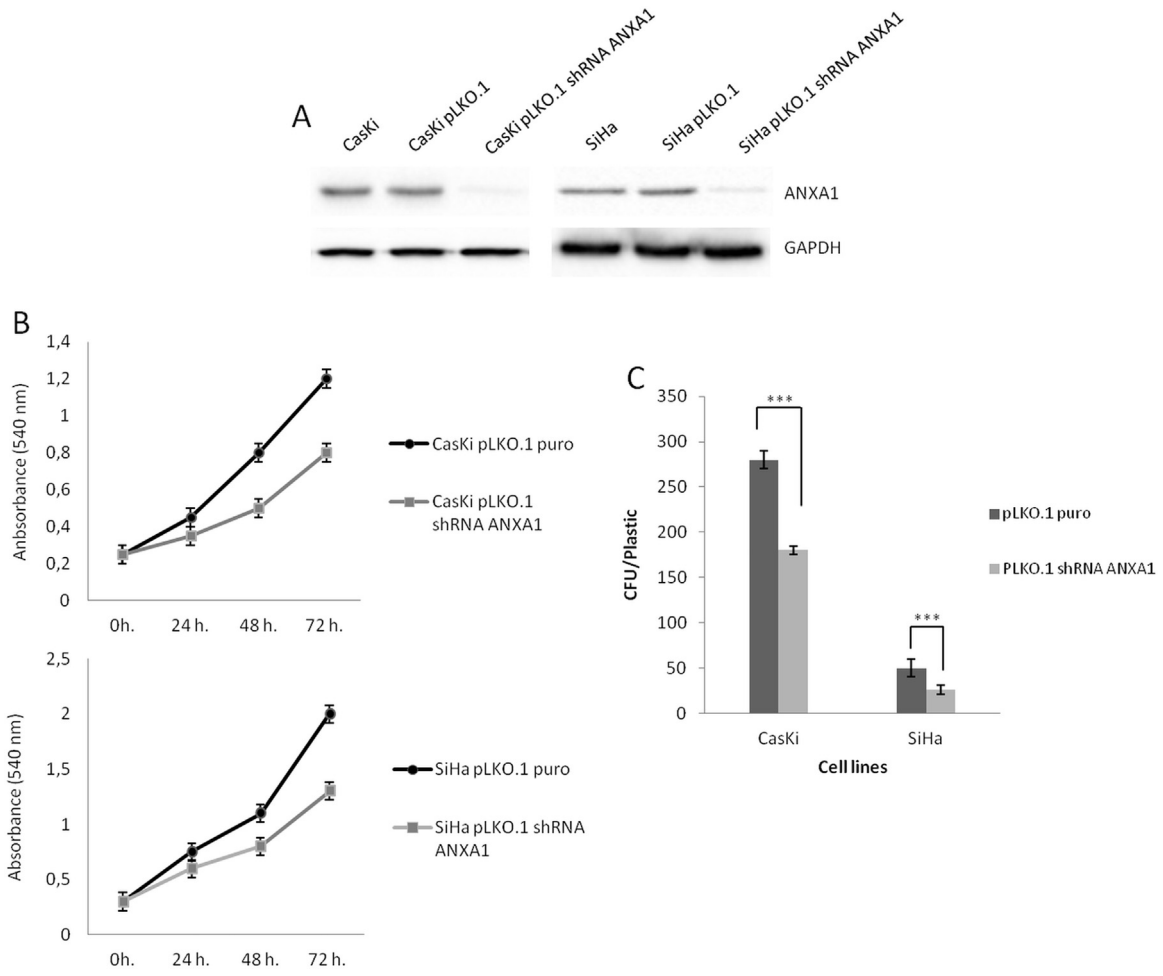

Fig. 4. Growth inhibition of Caski and SiHa cells after ANXA1 knockdown. (A) Western blots were performed to verify the knockdown efficiencies of the ANXA1 shRNA lentivirus in CasKi and SiHa cells. (B) Growth curve analysis showed that inhibition of ANXA1 using shRNA targeting ANXA1 significantly decreased cellular proliferation in SiHa and CasKi cells compared with that of the control groups (SiHa and CasKi cells transduced with pLKO.1 puro). (C) Colony formation assay showed a marked reduction in the number of colonies in the ANXA1 knockdown CasKi and SiHa cells. Data presented are the mean of 2 independent experiments $\pm$SEM. Group comparisons were carried out using Student's *t* test, $P^{***} < 0.0001$.

results demonstrate that ANXA1 suppression markedly decreased the proliferation rate of CasKi and SiHa cells (Fig. 4B) and significantly inhibited colony formation compared with the corresponding control cells (transduced with pLKO.1 puro) ($p < 0.0001$) (Fig. 4C).

3. Discussion

During the last twenty-five years, the interaction of E6 with at least 30 different cellular proteins was reported, including E6AP and p53 (Martinez-Zapien et al., 2016; Nomine et al., 2006; Vande Pol and Klingelhutz, 2013; Zanier et al., 2005; Zanier et al., 2012). It is well known that E6 oncoprotein binds to E6-associated protein (E6AP) and the E6-E6AP complex interacts with p53, resulting in the rapid ubiquitin-dependent degradation of p53 (Huibregtse et al., 1991; Scheffner et al., 1993). Previous report has shown that E6AP also binds to ANXA1 *in vivo* and *in vitro* and suggests that HPV-16 E6 redirect E6AP to other targets, such as p53 and PDZ proteins, increasing the stability of ANXA1 and contributing to viral pathogenesis (Shimoji et al., 2009).

In the present study, we show that ANXA1 expression is significantly increased in HPV-16-positive cervical carcinoma-derived cell lines and in keratinocytes expressing HPV-16 E6 compared to HPV-negative cervical carcinoma cells and control keratinocytes. We further show that ANXA1 protein upregulation depends on the presence of E6AP and intact E6 domains required to degrade p53. Besides, our results show an inverse correlation between p53 and ANXA1 levels suggesting that p53 may negatively regulate ANXA1 expression.

A direct inhibitory effect of p53 on ANXA1 expression has been previously reported (Lecona et al., 2008). In this study it was observed that down-regulation depends on the presence of a functional binding site for the tumor suppressor p53 near the proximal CCAAT box of the ANXA1 promoter. Therefore, it is expected that in cells expressing E6, where p53 are low due its degradation mediated by the viral protein, ANXA1 promoter would not be inhibited. In order to confirm this hypothesis we analyzed ANXA1 and p53 mRNA and protein levels in SiHa and CaSki cell lines after E6 suppression. Unexpectedly, ANXA1 mRNA levels remained unchanged in SiHa and CasKi cells after HPV-16 E6 silencing. On the other hand, ANXA1 protein levels decreased while p53 increased. Altogether, our data suggest that the regulation of ANXA1 expression in our experimental system occurs at a post-transcriptional level. Thus, post-transcriptional p53-dependent mechanisms may take place in cervical cells to regulate ANXA1 expression as observed in human colon adenocarcinoma cells (Lecona et al., 2008). These mechanisms may add further complexity to the regulation of ANXA1 protein expression.

To the best of our knowledge, our report is the first to describe E6-mediated ANXA1 upregulation. Moreover, we suggest that the model proposed by Shimoji and col. (Shimoji et al., 2009) that

indicates that HPV-16 E6 redirects E6AP away from ANXA1, increasing its stability may be inaccurate since we demonstrated ANXA1 downregulation upon E6 or E6AP silencing. Our results indicates that ANXA1 protein expression requires the presence of E6wt and E6AP since E6-E6AP complex degrades p53 and this protein may be regulating ANXA1 protein levels by a yet undefined mechanism.

We observed a decrease in ANXA1 levels upon E6AP suppression in CasKi, SiHa and C33 cells expressing HPV16 E6wt. This probably occurs as a consequence of the E6-E6AP complex disruption that leads to increased p53 levels and ANXA1 downregulation.

Interestingly, we observed that ANXA1 protein expression was not upregulated in cells expressing HPV-16 E6 Δ9–13, a HPV-16 E6 mutant that has a deletion in PRKLP motif that results in great decrease in E6AP-binding. In fact, the ability of this mutant in binding to E6AP is similar to that exhibited by E6 from HPV-11, a low oncogenic risk viral type (Cooper et al., 2003). Moreover, C33 cells expressing HPV-16 E6 45Y/47Y/49H, another mutant protein that does not target p53 for degradation (Foster et al., 1994), also exhibited lower ANXA1 protein expression when compared to C33 cells expressing HPV16 E6 wt. Our data suggest that in cells expressing HPV-16 E6, p53 degradation mediated by E6-E6AP leads to ANXA1 upregulation. On the other hand, the expression of ANXA1 is reduced in cells lacking HPV-16 E6 or expressing E6 mutants that do not bind to E6AP and do not degrade p53. Besides, our observations highlight the importance of E6-E6AP interaction in ANXA1 upregulation. Other studies have described similar mechanisms involving E6-mediated cellular telomerase activation. It was described that HPV16 E6 mutants E6 proteins unable to bind to E6AP were also unable to activate telomerase activity and hTERT expression (Gewin and Galloway, 2001; Liu et al., 2005).

It has been reported that ANXA1 upregulation correlates with the development of hepatocarcinoma, colorectal cancer, lung cancer, pancreatic cancer, melanoma, skin cancer, endometrial carcinoma, and penile carcinoma (Calmon et al., 2013; Clifton et al., 2006; Hummerich et al., 2006; Rondepierre et al., 2009; Voisin et al., 2011; Wong et al., 2012; Yoshida et al., 2011; Zimmermann et al., 2007). Besides, increase ANXA1 expression promotes skin carcinogenesis and favors the invasion and metastasis of melanoma (Sakaguchi et al., 2007). Moreover, it has been shown that ANXA1 is strongly expressed in the liver of transgenic animals that develop hepatocarcinoma and is overexpressed during hepatocyte proliferation in the regenerating liver after hepatectomy (de Coupade et al., 2000). In this study, we observed that ANXA1 knockdown reduces the proliferation rates and the clonogenic potential in HPV16 E6-positive cells. Our results corroborate those obtained in a study in which ANXA1 targeting with small interfering RNA (siRNA) attenuated cell proliferation induced by estradiol or epidermal growth factor in MCF-7 cells (Khau et al., 2011).

In high-risk HPVs associate cervical lesions the increased proliferation of suprabasal epithelial cells is caused by the expression of the viral oncogenes *E6* and *E7*. E6 promotes p53 degradation, altering cell cycle control arrest and apoptosis regulation. This may induce the accumulation of cellular alterations in HPV-infected cells and favor the progression towards malignancy (Ghittoni et al., 2015). Our data indicate that E6 also upregulates ANXA1 protein levels, which in turn plays a role in HPV-positive cervical carcinoma-derived cells line proliferation and clonogenic potential. We can speculate that E6-meidated ANXA1 upregulation may contribute to HPV-mediated cell transformation. Further studies are necessary to obtain insights into the molecular mechanisms underlying this phenomenon and to further determine the role of ANXA1 in cervical carcinogenesis.

4. Conclusion

HPV16 E6 expression upregulates ANXA1 protein levels. This effect requires the interaction of E6 with E6AP and resulting p53 degradation. ANXA1 silencing reduces HPV-transformed cells proliferation and clonogenic potential. We suggest that ANXA1 may play a role in HPV-mediated carcinogenesis.

5. Material and methods

5.1. Cell lines

HPV-16 immortalized keratinocytes (HFK698 cell line), kindly provided by Richard Schlegel, Georgetown University, Washington, USA to Luisa Lina Villa, was cultured in a 3:1 mixture of keratinocyte serum-free medium (LifeTech) and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Cultilab, Brazil), 100 U/mL penicillin (Romanczuk et al., 1991). The cervical cancer-derived cell lines SiHa, CasKi and C33 were cultured according to American Type Culture Collection recommendations. Pooled neonatal human foreskin keratinocytes (PHK, Lonza, USA) were cultured in keratinocyte serum-free medium (Lonza, USA). PHK cells infected with pLXSN empty retroviral vectors or transducing HPV-16 E6wt, E7wt or E6/E7 are described elsewhere (Cardeal et al., 2012). All human cell lines were maintained in a humidified cell culture chamber at 37 °C and 5% CO_2.

5.2. Retroviral vectors and transductions

Recombinant pLXSN retrovirus transducing HPV-16 E6wt (wild-type), HPV16 E6 mutant sequences (45Y/47Y/49H and Δ9–13), E7wt (wild-type) or E6/E7 were kindly provided by Dr. Denise Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA) and are described elsewhere (Helt and Galloway, 2001). C33, CasKi and SiHa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Cultilab, Brazil). Cells were acutely infected with recombinant pLXSN and pBabe retroviruses expressing the neomycin and puromycin resistance marker, respectively, as described elsewhere (Boccardo et al., 2010). C33 cells infected with pLXSN retrovirus vectors were selected with 800 μg/mL of G418 for 14 days and C33, CasKi and SiHa cells infected with pBabe vectors were selected with 1 μg/mL of puromycin for 14 days when 100% of mock infected controls were dead.

5.3. Western blot analysis

Cells were lysed in cold cell lytic MT lysis buffer (Sigma, St. Louis, USA). After 15 min on ice, centrifugation was conducted for 10 min at 12,000 × *g* and 4 °C to separate the insoluble material. Protein concentrations were determined by a commercial protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent amounts of protein (50 μg) were resolved by SDS-PAGE (8–12%) and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Little Chalfont, UK) using the Mini Trans-Blot Electrophoretic Transfer system (Bio-Rad Laboratories, Hercules, USA). Membranes were blocked for 1 h in 5% non-fat milk and probed overnight with the following primary antibodies: anti-ANXA1 (2.5 μg/mL) (Abcam, San Francisco, USA) and anti-p53 at 1:500 (Abcam, San Francisco, USA). GAPDH expression was used as a loading control using anti-GAPDH at 1:5000 (MilliPore Corporation Bedford, MA, USA). Membranes were washed and then reprobed with horseradish peroxidase (HRP)-conjugated secondary antibodies. The bands were revealed using Enhanced Chemiluminescence procedures according to the manufacturer's

recommendations (Thermo Fisher Scientific, USA).

5.4. DsiRNA transfection

HPV16 E6 DsiRNA was used to knock down endogenous HPV-16 E6 expression. The nucleotide sequences of HPV16 E6 or control DsiRNAs used in this study are as follows: for HPV16 E6 (sense: 5′ CAGAAAGUUACCACAGUUAUGCACA3′; antisense: 5′UGUGCAUAA-CUGUGGUAACUUUCUGGG3′) and its control (sense: 5′ CGUUAAUCGCGUAAAUACGCGUAT 3′; antisense: AUACGC-GUAUUAUACGCGAUUAACGAC 3′) (Integrated DNA Technologies, Coralville, USA). Transfection of SiHa and CasKi cells was carried out using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, USA) according to the manufacturer's protocol. For DsiRNA transfection, cells were incubated with 30 nM of the DsiRNA pool targeting HPV-16 E6 for 72 h before RNA and protein extraction. For control, a non-relevant siRNA sequence pool (scrambled DsiRNA) (Integrated DNA Technologies, Coralville, USA) was used.

5.5. RNA extraction and qPCR

Total RNA was isolated from cell lines using TRIzol reagent (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions. qPCR was used to assess the expression of ANXA1 and HPV-16 E6. Gene-specific primers for qPCR were designed for optimal hybridization kinetics using the Primer 3.0 program (provided by the Whitehead/MIT Center for Genome Research, MA, USA). Quantitative real-time PCR was performed using an ABI prism 7300 sequencer detector system and SybrGreen PCR Core Reagent (Applied Biosystems, Foster City, USA) following the manufacturer's protocol. In brief, the reaction mixture (20 μl total volume) was composed of 25 ng of cDNA, gene-specific forward and reverse primers for each gene, and 10 μl of 2x Quantitative Sybr Green PCR Master Mix (Applied Biosystems, Foster City, USA). Relative quantification was given by CT values, determined for triplicate reactions of cell lines and reference samples for each gene and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an endogenous control. Primer sequences are described in Supplementary Table 1. The relative expression of each gene was calculated using the formula: R=(E target)ΔCt target (control - sample)/(E endogenous) ΔCt endogenous (control - sample), as previously described (Pfaffl, 2001). The cut-off for analysis of gene expression was ≥ 2 increased or decreased expression.

5.6. ANXA1 knockdown by shRNA

The lentivirus-based shRNA expression plasmids, pLKO.1 puro and pLKO.1 expressing shRNA targeting ANXA1, were purchased from Sigma (Sigma-Aldrich, St. Louis, USA). The viral particles were produced and purified by cotransfecting pLKO.1-Axl-shRNA and the lentivirus packaging plasmids (psPAX2 and PMD2.G) into HEK293T cells. ANXA1 knockdown cells generated from CasKi and SiHa cells were selected in the culture medium containing 1.0 μg/mL puromycin.

5.7. Colony formation assay

After infection with the indicated shRNA lentiviruses for 48 h, atotal of 5000 cells were seeded in 6-wells plates in triplicate and cultured for 14days in complete medium containing 1.0 μg/mL puromycin. Surviving colonies were stained with crystal violet after formaldehyde fixation.

5.8. Proliferation assay

CasKi and SiHa transduced with PLKO.1 puro and PLKO.1 shRNA targeting ANXA1 were seeded into 96-well plates. Proliferation rates were measured at 48, 72 and 96 h after seeding using MTT. Then, 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)−2, 5-diphenyl-tetrazolium bromide (MTT) (Sigma Aldrich, St Louis, MO, USA) was added to the wells and incubated for 30 min at 37 °C. The MTT was removed, 100 μl of 100% DMSO (Sigma Aldrich, St Louis, MO, USA) was added to each well and the absorbance was measured at 562 nm. Each experiment was performed in triplicate and in two independent assays.

5.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 Software. Group comparisons in the colony formation assay and analysis of the proliferation rate were performed with Student's *t* test. In all analyses, the differences were considered statistically significant when $p < 0.05$.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2016.05.016.

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