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ORIGINAL PAPER

Multiple interference of the human papillomavirus-16 E7 oncoprotein with the functional role of the metastasis suppressor Nm23-H1 protein

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Abstract High-risk human papillomaviruses (HPV) are linked to human cervical and other ano-genital cancers. Integration of the viral genome in the transformed epithelial cells is restricted to the coding regions for the E6 and E7 oncoproteins. Nevertheless, E7 plays the major role in cell transformation. We report a novel interaction between HPV-16 E7 and the Nm23-H1 and Nm23-H2 proteins identified in yeast by the two-hybrid system and confirmed by co-immunoprecipitation in the human keratinocyte HaCaT cell line. Expression of the E7 oncoprotein in HaCaT cells induces modified keratinocyte proliferation and differentiation patterns, and leads to down-modulation and functional inactivation of the metastasis suppressor Nm23-H1 protein. Both transcriptional down-regulation and protein degradation contribute to reduce Nm23-H1 intracellular content. Besides metastasis suppression, Nm23-H1 displays multiple functions in cell cycle regulation and differentiation, development, DNA regulation and caspase-independent apoptosis. As a consequence of Nm23-H1 inhibition, HPV-16 E7 expressing HaCaT cells, acquire invasiveness capabilities and resistance to granzyme A-induced apoptosis. We propose that impairment of the multifunctional role of Nm23-H1 is an important feature consistent with the complex strategy carried out by HPV-16 E7 to promote cell transformation and tumor progression.

Keywords HPV-16 E7 oncoprotein · Nm23-H1 metastasis suppressor · Protein-protein interactions · HaCaT cells · Differentiation · Transformation · Invasiveness · Apoptosis

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Introduction

The human papillomavirus (HPV) family is composed of more than 100 different genotypes, characterized by a remarkable affinity for epithelial cells. "Low-risk" HPVs are responsible for benign warts, while "high-risk" HPVs, besides their infecting capabilities, are linked to the majority of cervical cancer and to more than 50% of other ano-genital cancers in humans (Munoz et al., 2003; zur Hausen, 2002). Transformed epithelial cells are characterized by the integration in their genome of the coding regions for the viral E6 and E7 oncoproteins (Munger, 2002). Indeed, these oncoproteins play a major role in virus reproduction as well in inducing immortalization and transformation by re-programming cell cycle control in the differentiating host epithelial cells, to allow DNA replication and cell division. The E7 oncoprotein is considered mandatory for "high-risk" HPVs transforming properties, while a cooperating role is played by the partner E6 oncoprotein (Munger et al., 2001; Zwerschke and Jansen-Durr, 2000).

We screened a HeLa cDNA library, by the yeast two-hybrid system (Fields and Song, 1989; Chien et al., 1991) using full-length HPV-16 E7 as bait, to identify novel HPV-16 E7 targets in the host cell. We found a novel interaction between HPV-16 E7 and the Nm23-H1 and Nm23-H2 proteins. These factors are the most characterized components of a human protein family composed of eight members

endowed with nucleoside di-phosphate kinase (NDPK) activity (Lacombe et al., 2000), and participate in multiple cellular functions, such as: 1) Suppression of metastasis and cell invasiveness operated *in vitro* and *in vivo* by Nm23-H1 and its murine cognate Nm23-M1 (Steeg et al., 1988; Steeg, 2004); 2) Embryo development (Lakso et al., 1992; Gervasi et al., 1998); 3) Cell cycle regulation and differentiation, where Nm23-H1 and Nm23–M1 play a critical role (Gervasi et al., 1996; Lombardi et al., 2001); 4) Gene regulation (Postel et al., 2000; Postel, 2003); and 5) DNA repair (Postel, 2003; Ma et al., 2004). Moreover, Nm23-H1 is the Granzyme A (GzmA)-activated DNase during cytotoxic T lymphocytes (CTL)-induced caspase-independent apoptosis of virus infected or transformed cells (Fan et al., 2003). In addition, Nm23-H1 is involved in several protein-protein interactions (Lombardi and Mileo, 2003), such as with Epstein-Barr virus EBNA-3C and EBNA-1 nuclear antigens. Suppression of cell motility dependent on *nm23*-H1 over-expression was reverted by the over-expression of the viral proteins in breast carcinoma and lymphoma cell lines (Subramanian et al., 2001; Murakami et al., 2005).

Here we show that HPV-16 E7 interacts with the Nm23-H1 and Nm23–H2 proteins. HPV-16 E7-expressing human HaCaT keratinocytes display a modified differentiation pattern, coupled with a drastic Nm23-H1 and Nm23–H2 protein down-regulation due to transcriptional repression and protein degradation. Impairment of Nm23-H1 function by HPV-16 E7 expression in HaCaT cells promotes invasion capabilities and confers resistance to GzmA-induced caspase-independent cell death.

Materials and methods

Yeast two-hybrid constructs

Full-length HPV-16 E7 cDNA was amplified by polymerase chain reaction (PCR) with an appropriate set of primers using the HA-tagged pBabe Puro-HPV-16 E7 retroviral construct (Giarre et al., 2001) (pBabe-E7) as template, and sub-cloned into the pGBT9 vector (Clontech, Mountain View, CA) (pGBT9-E7). Deletion mutants of the HPV-16 E7 cDNA, were amplified by PCR using appropriate sets of primers and sub-cloned into the pGBT9 vector. The HeLa cDNA library (Clontech) was constructed into the pGAD424 vector (Clontech). Full-length *nm23*-H1 and *nm23*-H2 cDNAs were amplified by PCR with appropriate sets of primers using as templates the pCMV-*nm23*-H1 and pCMV-*nm23*-H2 constructs (MacDonald et al., 1996) and sub-cloned into the pGAD424 vector (pGAD424-*nm23*-H1, pGAD424-*nm23*-H2).

Yeast two-hybrid screening

The procedure for the two-hybrid library screening was performed as previously described (Gervasi et al., 1998) and generally following the Matchmaker Two-Hybrid System 2 (Clontech) protocol. Briefly, pGBT9-E7 (500 μg) and the pGAD424-HeLa cDNA library (500 μg) were co-transformed into the yeast strain AH109 (Clontech). Approximately 1×10^6 transformants were selected on minimal medium and screened for $\tilde{\beta}$-galactosidase activity in a filter assay. Further, pGBT9-E7 or the deletion mutant constructs were co-transformed with either pGAD424-*nm23*-H1 or pGAD424-*nm23*-H2 into yeast to validate the interaction.

Cell cultures and treatments

HaCaT cells were routinely grown at low density, i.e. 40%-confluent in DMEM (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco Invitrogen) at 37°C and 5% CO_2. To induce differentiation, 40%-confluent HaCaT cells were cultured for further 4 days to reach post-confluent density. Otherwise, 40%-confluent HaCaT cells were transferred to the synthetic Keratinocyte-SFM basal medium (Gibco Invitrogen), containing 0.09 mM calcium and supplemented with 50 μg/ml of bovine pituitary extract and 5 ng/ml of recombinant epidermal growth factor (both from Gibco Invitrogen) (low calcium-containing medium). Four days later, HaCaT cells were shifted to Keratinocyte-SFM containing 1.80 mM calcium and supplemented as above (high calcium-containing medium). In time course experiments cells were collected and analyzed after 1, 2, 4 and 6 days in high calcium-containing medium. When required, HaCaT cells were treated with either DMSO or 10 μM MG132 in DMSO for 4 h. When needed, 5×10^5 HaCaT cells in 300 μl of HBSS (Gibco Invitrogen) containing 1 mg/ml BSA were loaded for 2 h with 0.125 or 0.25 or 0.50 μg of human recombinant GzmA (Alexis Biochemicals, Axxora, CH) using a sub-lytic concentration of purified perforin (Shi et al., 1992; Beresford et al., 1999).

Phoenix (ΦNX) cells were grown in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.

Transfections and infections

ΦNX cells were treated with 25 μM chloroquine and transfected with 15 μg of either HA-tagged pBabe Puro backbone (pBabe) or pBabe-E7. Retrovirus containing ΦNX cell supernatants were collected 48 h later and filtered through a 0.45-μm filter (Millipore, Bedford, MA). 3×10^4 HaCaT cells were plated in 35-mm dishes 24 h prior to be

infected with 1 ml of the retrovirus suspensions containing 8 μg/ml polybrene. After incubation for 18 h at 32°C and 5% CO_2, cells were shifted to selection medium containing 2 μg/ml puromycine for 3 days at 37°C and 5% CO_2.

RNA isolation, RT-PCR and Real-Time RT-PCR

Total RNA was isolated from HaCaT/pBabe or HaCaT/pBabe-E7 cells by the TRIzol Reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using the First-strand cDNA synthesis kit (Amersham Biosciences, Buckinghamshire, UK). Full-length HPV16-E7 cDNA was amplified with an appropriate set of primers by PCR.

One-step quantitative real-time RT-PCR for *nm23*-H1, *nm23*-H2 and cyclophilin A was carried out with appropriate sets of primers using the LightCycler RNA amplification kit SYBR Green I in the LightCycler instrument (Roche Applied Science, Monza, Italy) following manufacturer procedure. Real-time RT-PCR for cyclophilin A was performed on each sample as an internal control (Steele et al., 2002). Relative expression of *nm23*-H1 and *nm23*-H2 mRNAs was calculated as described (Chen et al., 2003).

Flow cytometry

Cell cycle analysis was carried out as described (Gervasi *et al*., 1996).

To evaluate DNA synthesis, HaCaT cells were exposed to 45 μM bromodeoxyuridine (BrdU) for 2 h. Cells were processed and analyzed as described (Biroccio et al., 2002).

Evaluation of apoptosis was performed using the Annexin V-Fluos Staining Kit (Roche Applied Science) following the manufacturer procedure.

Cells were analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Western blot analysis

Cell lysis, SDS-PAGE and protein transfer to nitrocellulose were performed as described (Lombardi et al., 1995). Immunodetection was done by the ECL Western blotting system following the manufacturer procedure (Amersham Biosciences). Primary antibodies were: rabbit polyclonal anti-HA sc-805, rabbit polyclonal anti-pRb sc-50, mouse monoclonal anti-HSP70 sc-24 (all from Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-Nm23 (Lombardi et al., 1995) able to recognize both Nm23-H1 and Nm23-H2 proteins, mouse monoclonal anti-Involucrin (Sigma, St. Louis, MO).

Immunoprecipitation

Immunoprecipitation was performed as described (Lombardi et al., 1995). Cell lysate aliquots containing 1 mg of total proteins were immunoprecipitated with the polyclonal anti-HA antibody and ImmunoPure Immobilized Protein A-agarose (Pierce, Rockford, IL). Immunoprecipitates were resolved by reducing SDS-PAGE and electroblotted to nitrocellulose. Immunodetection was performed using the rabbit polyclonal anti-Nm23 antibody.

Glutathione S-Transferase fusion proteins expression and purification

Full-length cDNAs of *nm23*-H1 and *nm23*-H2 were subcloned into the pGEX-3X vector (Pharmacia-Biotech, Piscataway, NJ). Full length HPV-16 E7 cDNA was subcloned into the pGEX-4T-1 vector (Pharmacia-Biotech). The GST fusion proteins were expressed in *Escherichia coli* (BL21) and purified as described (Lombardi et al., 1995).

Nucleoside-diphosphate kinase assay

NDPK activity of GST-Nm23-H1 and GST-Nm23-H2 was assayed as described (Freije et al., 1997). When required, the assay was carried out in the presence of the GST-HPV16-E7.

DNA cleavage activity

Plasmid pcDNA3 cleavage was carried out with GST-Nm23-H1 as described (Fan et al., 2003). When required GST-HPV16 E7, or mouse monoclonal anti-Nm23-H1 antibody (BD Transduction Laboratories, Franklin Lakes, NJ), or mouse monoclonal anti-HSP70 sc-24 were added to the reaction.

In vitro invasion assay

The *in vitro* invasion experiments were performed using 6-well BioCoat Matrigel Invasion Chambers (Becton Dickinson) provided with 8-μm pore PET membranes covered with basement membrane matrix. HaCaT cells maintained in high calcium-containing medium for 4 days were suspended at 1×10^5 in 2 ml of serum-free DMEM containing 0.1% BSA and added to the chambers. Wells were filled with 2.5 ml of pre-confluent HaCaT cell-conditioned medium containing 0.1% BSA and 0.5% FBS. Invasion chambers were incubated for 48 h at 37°C and 5% CO_2. The invading-cells were stained with May-Grünwald Giemsa and counted under a microscope.

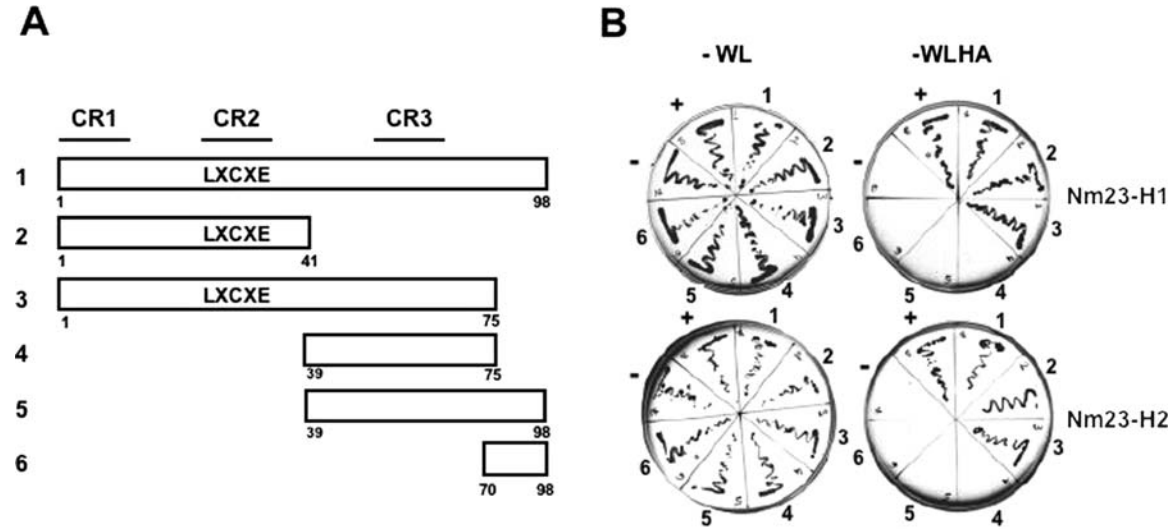


Fig. 1 HPV-16 E7 interaction with Nm23-H1 and Nm23-H2 in yeast. A) Scheme of full-length HPV-16 E7 (1) and its deletion mutants (2–6). B) Interaction of HPV-16 E7 and deletion mutants with Nm23-H1 and Nm23-H2 in yeast transformants grown on minimal medium lacking tryptophan (W), leucine (L), histidine (H) and adenine (A)

Results

HPV-16 E7 interacts with Nm23-H1 and Nm23–H2 in yeast

In order to identify novel potential targets of the E7 oncoprotein from the "high-risk" HPV-16 type (HPV-16 E7), a human HeLa cDNA library was screened by the yeast two-hybrid system using HPV-16 E7 as bait. Among the rescued plasmids, a cDNA sequence encoding for a large portion of the *nm23*-H2 gene product, from nt 241 to nt 517 (aa 57 to aa 152), was isolated. Since the Nm23-H2 protein shares 88% identity with Nm23–H1 (Lombardi et al., 2000), further yeast two-hybrid interaction studies were performed to verify the potential molecular association of HPV-16 E7 also with Nm23-H1. In addition to full-length HPV-16 E7, five deletion mutants (Fig. 1A) were also employed, in order to identify the portion(s) of the oncoprotein sufficient for the interaction with the Nm23 proteins. The results showed that HPV-16 E7 was able to interact with both full-length Nm23-H1 and Nm23-H2, and that its *N*-terminal portion, containing intact CR1 and CR2 domains, was responsible for such interaction (Fig. 1B). It is worth of note that the CR2 region embraces the LXCXE motif, responsible for the interaction with the pocket domain of all three Retinoblastoma family proteins (Munger et al., 2001).

Retrovirally-expressed HPV-16 E7 interacts with endogenous Nm23-H1 and Nm23-H2 in HaCaT keratinocytes

With the purpose of investigating the biological relevance of the interaction between HPV-16 E7 and the Nm23 proteins in a superior eukaryotic context, we chose the human HaCaT keratinocyte cell line as experimental model, being the epithelial cells the natural target of the HPVs (Fehrmann and Laimins, 2003). HaCaT cell line originated spontaneously from a long-term primary culture of human adult skin keratinocytes. HaCaT cells are immortal, not tumorigenic and retain the ability to undergo epidermal differentiation (Boukamp et al., 1988).

The HA-tagged pBabe Puro retroviral system was used to express HPV-16 E7 in HaCaT cells. The expression was confirmed by RT-PCR (Fig. 2A) and by Western blotting, using a polyclonal anti-HA antibody (Fig. 2B). The oncoprotein interaction with Nm23-H1 and Nm23-H2 was demonstrated to occur also *in vivo* in HPV-16 E7-expressing HaCaT cells (HaCaT/pBabe-E7). Proliferating, 40%-confluent, control (HaCaT/pBabe) and HaCaT/pBabe-E7 cell lysates were incubated with a polyclonal anti-HA antibody to immunoprecipitate the HA-tagged HPV-16 E7 oncoprotein. Immunoprecipitates were resolved by SDS-PAGE and the following Western blotting using a polyclonal anti-Nm23 antibody, demonstrated that the oncoprotein co-immunoprecipitated with both endogenous Nm23-H1 and Nm23-H2 proteins (Fig. 2C), thus confirming in a human cell context the protein-protein interaction identified in yeast.

HPV-16 E7 interferes with the cell cycle machinery and with Nm23-H1 and Nm23–H2 protein expression upon induction of keratinocyte differentiation

To verify whether the HPV-16 E7 oncoprotein retrovirally-expressed in HaCaT cells was functionally active, the effects on proliferation and differentiation were investigated. *In vitro* keratinocyte differentiation of HaCaT cells can be

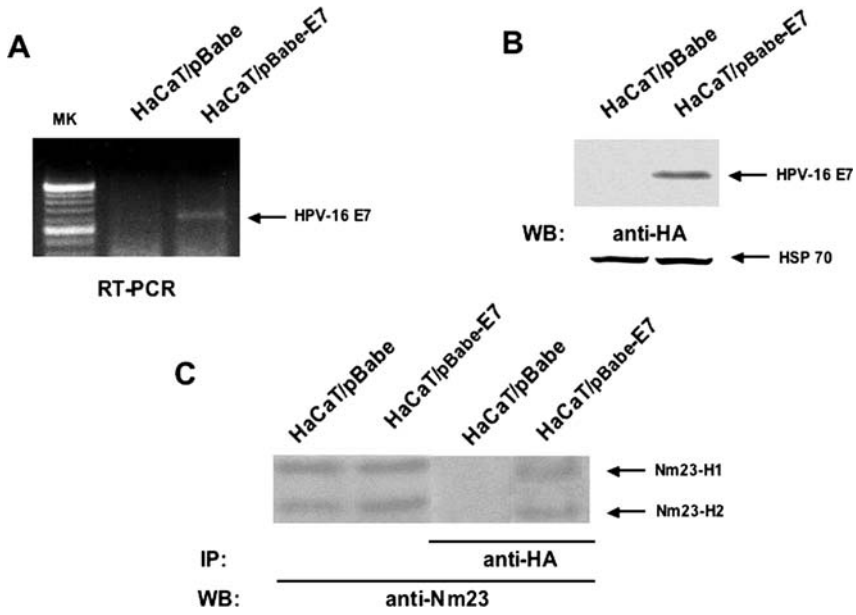


Fig. 2 HPV-16 E7 expression and interaction with Nm23-H1 and Nm23-H2 in HaCaT/pBabe-E7 cells. A) RT-PCR amplification of HPV-16 E7 cDNA from HaCaT/pBabe and HaCaT/pBabe-E7 cells analyzed by agarose gel electrophoresis. B) Western blotting for HA-tagged HPV-16 E7 in HaCaT/pBabe and HaCaT/pBabe-E7 cell lysates. HSP70 detection served as loading control. C) Western blotting for Nm23-H1 and Nm23-H2 in HaCaT/pBabe and HaCaT/pBabe-E7 cell lysates and HA-tagged HPV16-E7 immunoprecipitates. All the analyses shown are representative of three experiments, each in duplicate

easily obtained upon high cell density culture conditions in conventional medium (Ryle et al., 1989) or culturing cells in low calcium-containing medium, to allow optimal proliferation, and then shifting to high calcium-containing medium, to reproduce tissue occurring terminal differentiation (Breitkreutz et al., 1993). Cell cycle analysis was carried out on proliferating, 40%-confluent (Pro), and post-confluent (PC) control and HPV-16 E7-expressing cells. Both Pro HaCaT/pBabe and Pro HaCaT/pBabe-E7 cells displayed comparable cell cycle distribution. Indeed, PC HaCaT/pBabe-E7 cells did not undergo G0/G1 accumulation, hallmark of cell cycle arrest, as the control PC counterpart (Table 1). Moreover, bromodeoxyuridin (BrdU) incorporation analysis indicated that PC HaCaT/pBabe-E7 cells retained a DNA synthesis capability as compared to PC control cells (Table 1).

Differentiation in HaCaT cells was monitored by Western blotting for involucrin, a marker of keratinocyte terminal differentiation (Griffin and Harris, 1992; Carroll et al., 1993). Involucrin expression was remarkably increased in PC HaCaT/pBabe cells as compared to the Pro counterpart. Increased involucrin expression was also evident in control HaCaT cells differentiating in high calcium-containing medium (Fig. 3A). On the other hand, in HaCaT/pbabe-E7 cells, involucrin expression was noticeably lower than in control cells both in the PC condition and upon high calcium treatment, where a trend of slight increase could still be observed (Fig. 3B). In addition, Western blot analysis for pRb, the well-known negative regulator of cell cycle and HPV-16 E7 target (Munger et al., 2001), was done. Increased pRb expression level and dephosphorylation were evident in PC HaCaT-pBabe cells compared to the Pro counterpart, indicating cell cycle withdrawal. Moreover, as expected, pRb expression level and dephosphorylation increased throughout the whole differentiation process in high calcium-containing medium (Fig. 3A). Of note, in HaCaT/pBabe-E7 cells, pRb expression levels were always remarkably reduced in all the conditions (Fig. 3B). Overall, the above findings provide evidence that the HPV-16 E7 oncoprotein was functionally active in HPV-16 E7-expressing HaCaT cells since DNA synthesis, proliferation and pRb inhibition were induced.

Table 1 HPV-16 E7 effects on HaCaT cell proliferation

	HaCaT/pBabe		HaCaT/pBabe-E7	
	Pro	PC	Pro	PC
Cell cycle				
G0/G1	35.66% ± 1.52	52.66% ± 2.08	34.66% ± 0.57	37.00% ± 1.00
S	43.00% ± 1.00	26.66% ± 2.08	42.66% ± 1.52	36.66% ± 0.57
G2/M	21.33% ± 2.08	20.66% ± 1.15	22.66% ± 1.15	26.33% ± 1.15
BrdU incorporation				
G0/G1	25.00% ± 1.73	47.33% ± 1.52	25.66% ± 2.08	32.00% ± 1.73
S	71.33% ± 1.52	49.00% ± 2.00	68.00% ± 2.00	64.66% ± 1.15
G2/M	4.33% ± 0.57	3.66% ± 0.59	6.33% ± 0.57	6.66% ± 0.57

Note. Percentages of cell distribution in the phases of cell cycle are indicated. Values are arithmetical means ± SD for three independent experiments.

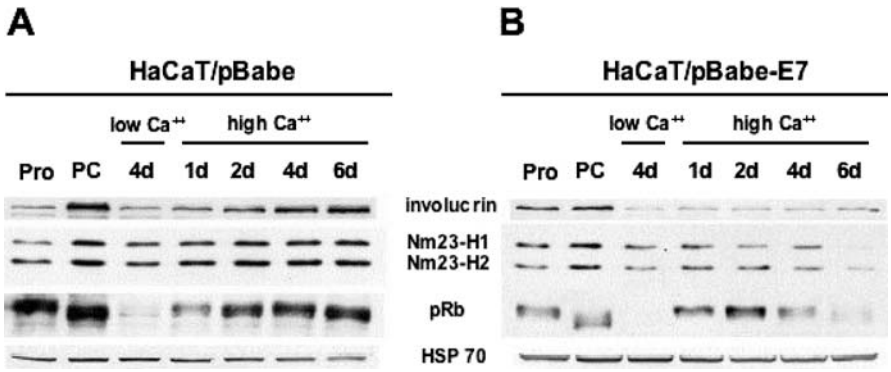


Fig. 3 HPV-16 E7 effects on HaCaT cell proteins expression. Western blotting for involucrin, Nm23-H1, Nm23-H2 and pRb in HaCaT/pBabe (A) and HaCaT/pBabe-E7 (B) cells in different culture conditions: proliferating (Pro), post-confluent (PC), cultured for 4 days in low calcium medium (low Ca^{++}) and for 1, 2, 4, 6 days in high calcium medium (high Ca^{++}). HSP70 detection served as loading control. All the analyses are representative of five experiments, each in duplicate

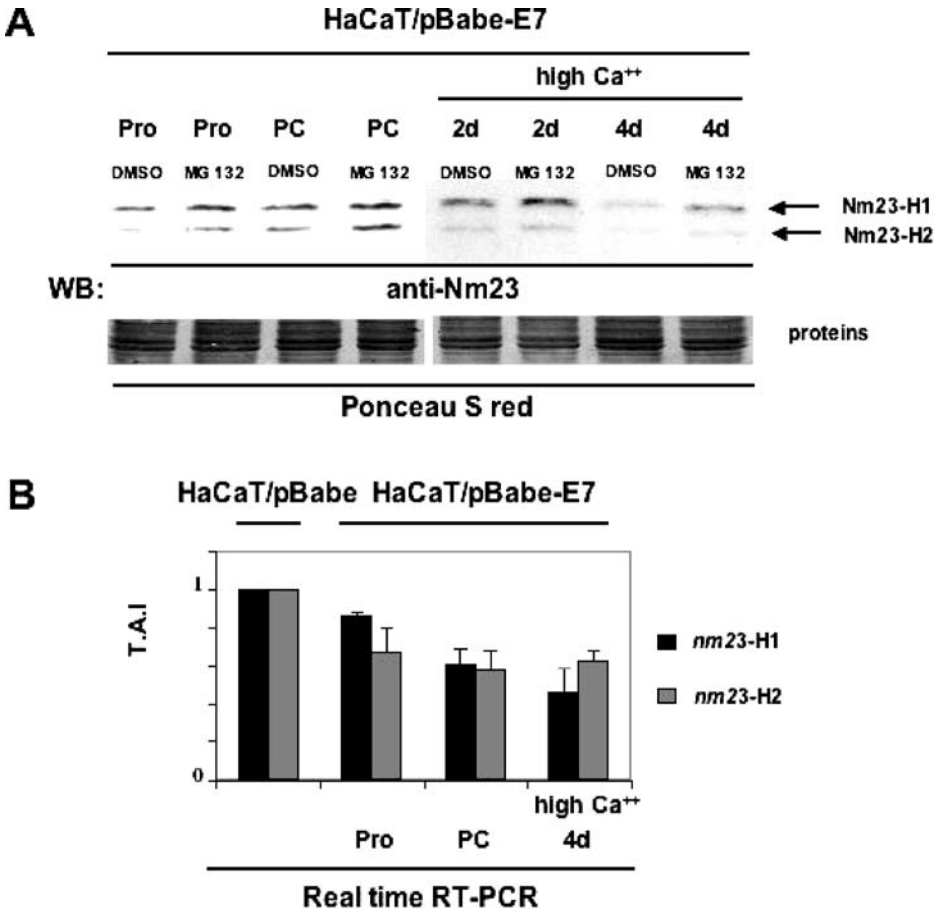


Fig. 4 HPV-16 E7 dependent Nm23 proteins down-modulation occurs via ubiquitination and transcriptional regulation. A) Western blot analysis for Nm23-H1 and Nm23-H2 in HaCaT/pBabe-E7 cells in different culture conditions: proliferating (Pro), post-confluent (PC) and cultured for 2 and 4 days in high calcium medium (high Ca^{++}) prior to and after treatment with MG 132, as indicated. Ponceau S red staining served as loading control. The analysis is representative of five experiments, each in duplicate. B) Histograms representing the transcript accumulation index (T.A.I.) of the *nm*23-H1 and *nm*23-H2 genes in HaCaT/pBabe-E7 cells under different culture conditions: proliferating (Pro), post-confluent (P.C.) and cultured for 4 days in high calcium medium (high Ca^{++}) evaluated by real time RT-PCR. T.A.I. values in HaCaT/pBabe-E7 cells are compared to T.A.I. found in HaCaT/pBabe cells, under the same culture conditions, expressed as value 1. Values are arithmetical means ± SD for three experiments, each in duplicate

In the same context, Western blotting of the Nm23 proteins was also performed. Essentially, control cells displayed a noticeable increase of expression in PC status and a progressive increment during the whole high calcium-induced differentiation process (Fig. 3A). The finding strongly suggests a link between Nm23-H1 up-modulation and growth arrest and differentiation in HaCaT cells, an association already demonstrated for other models of cell differentiation (Gervasi et al., 1996; Lombardi et al., 2001; 31); here, a similar involvement of Nm23-H2 could be also envisaged. Contrarily, the analysis of Nm23-H1 and Nm23-H2 in HaCaT/pBabe-E7 cells showed a strong decrease of expression for Nm23-H1, up to almost undetectability in the final phases of the high calcium treatment (Fig. 3B).

Down-modulation of the Nm23 proteins in HPV-16 E7 expressing HaCaT cells depends upon ubiqitination and transcriptional regulation

Because HPV-16 E7 binding to pRb functionally inactivates the target protein and induces its degradation *via* the ubiquitin-proteasome pathway (Wang et al., 2001), we investigated the role of ubiquitination in the down-modulation of the Nm23 proteins detected in HPV-16 E7-expressing cells. To this end, HaCaT/pBabe-E7 cells, in the Pro and PC status, as well as at 2 and 4 days of high calcium-induction of differentiation, were treated with 10 μM MG132, an inhibitor of the 26S proteasome, and assayed by Western blotting for Nm23-H1 and Nm23-H2 content compared to the untreated counterparts. In all the conditions, MG132-treated cells showed a partial recovery of the expression of both Nm23 proteins, more pronounced for Nm23-H1 (Fig. 4A). These results imply that HPV-16 E7-promoted degradation of the Nm23 proteins occurred through the involvement of the ubiquitin-proteasome pathway, being, at least in part, inhibited by MG132.

Quantitative real-time RT-PCR was then performed to evaluate whether HPV-16 E7-dependent down-modulation of the Nm23 proteins could be also due to transcriptional regulation of the respective genes. In HaCaT/pBabe-E7 cells, a decreased transcript accumulation for both *nm23*-H1 and *nm23*-H2 genes was observed. This transcriptional down-modulation resulted especially evident for *nm23*-H1 when cell differentiation was triggered in high calcium-containing medium (Fig. 4B).

HPV-16 E7 does not interfere with the NDPK activity, but affects the *in vitro* DNA cleavage activity of the Nm23-H1 protein

Nm23 proteins are evolutionarily conserved NDPKs (Lombardi et al., 2000). In order to evaluate whether HPV-16 E7 might interfere with the NDPK activity of the Nm23 proteins, an *in vitro* kinase assay was carried out. The presence of the recombinant GST-HPV-16 E7 protein in the reaction mixture did not affect the NDPK activity of the GST-Nm23-H1 protein (Fig. 5A). Similarly, GST-Nm23-H2 NDPK activity was not influenced by the presence of GST-HPV-16 E7 (not shown). These results indicate that Nm23 NDPK activity is not a target of the HPV-16 E7 oncoprotein. Noteworthy, the NDPK activity is not required for Nm23-H1 function in cell differentiation and metastasis suppression (Lombardi et al., 2001; MacDonald et al., 1996)

Since Nm23-H1 is endowed with DNase activity *in vitro* towards plasmid and eukaryotic DNA (Ma et al., 2004), the possibility that the HPV-16 E7 oncoprotein might interfere with such activity was determined. In an *in vitro* DNA cleavage assay, GST-Nm23-H1 was able to linearize a pcDNA3 supercoiled plasmid used as substrate, an activity that was specifically inhibited by the anti-Nm23-H1 antibody but not affected by the unspecific anti-HSP70 antibody

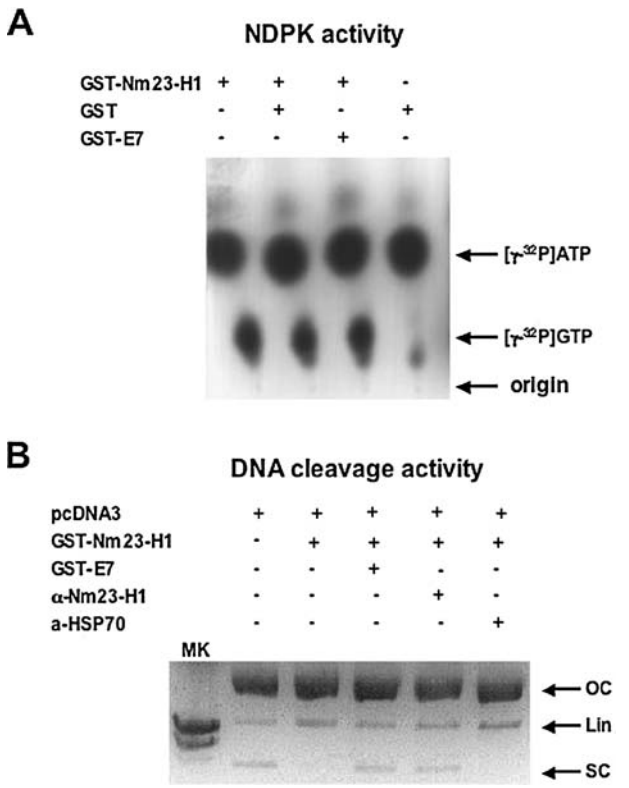
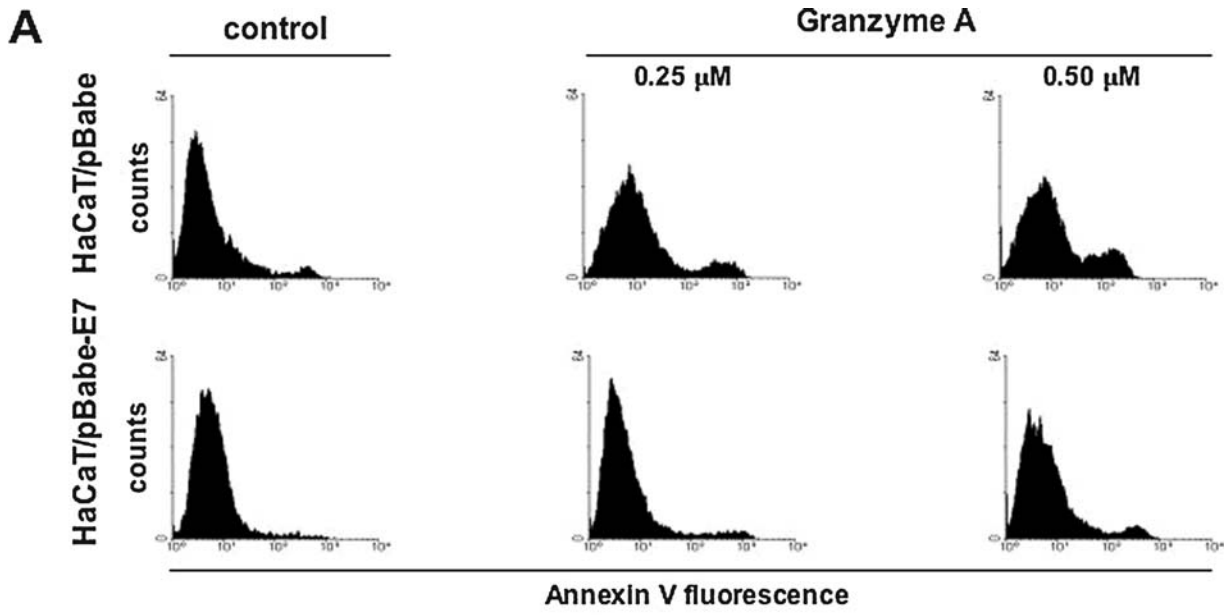


Fig. 5 HPV-16 E7 effects on Nm23-H1 NDPK activity and DNA cleavage activity. A) Purified recombinant GST-Nm23-H1 was incubated with [γ-^{32}P]ATP and cold GDP in the presence or absence of recombinant GST-HPV-16 E7 (GST-E7), and the formation of [γ-^{32}P]GTP was analyzed by thin layer chromatography and autoradiography. Positions of [γ-^{32}P]ATP (substrate) and [γ-^{32}P]GTP (product) are indicated. The analysis is representative of three experiments, each in duplicate. B) Cleavage of pcDNA3 plasmid by GST-Nm23-H1 in the presence of absence of GST-HPV-16 E7 (GST-E7) monitored by agarose gel electrophoresis. Plasmid states are indicated: SC, supercoiled; Lin, linear; and OC, open circle/nicked circular. The analysis is representative of three experiments, each in duplicate



B

	HaCaT/pBabe		HaCaT/pBabe-E7	
	live	apoptotic	live	apoptotic
control	94.4 % ± 3.2	5.6 % ± 3.2	96.6 % ± 2.4	3.4 % ± 3.5
0.125 μM Gzm A	88.0 % ± 0.9	12.0 % ± 0.9	95.1 % ± 0.7	4.9 % ± 0.7
0.25 μM Gzm A	79.4 % ± 0.1	20.6 % ± 0.1	92.1 % ± 0.3	7.9 % ± 0.3
0.50 μM Gzm A	74.2 % ± 0.2	25.8 % ± 0.3	90.1 % ± 0.1	9.9 % ± 0.1

Fig. 6 HPV-16 E7 dependent Nm23-H1 down-modulation promotes HaCaT cell survival upon Granzyme A treatment. A) Induction of apoptosis in HaCaT/pBabe and HaCaT/pBabe-E7 cells by GzmA was monitored as Annexin V binding by flow cytometry. The analysis is representative of five experiments, each in duplicate. B) Percentages of live and apoptotic HaCaT/pBabe and HaCaT/pBabe-E7 cells upon treatment with different concentrations of GzmA. Values are arithmetical means ± SD for five experiments, each in duplicate

used as control. In the presence of GST-HPV-16 E7, the DNA substrate was partially protected from cleavage, thus indicating an interference of HPV-16 E7 with the Nm23-H1 DNase activity (Fig. 5B).

HPV-16 E7 prevents Granzyme A-induced, Nm23-H1-mediated apoptosis in HaCaT cells

Recently, Nm23-H1 has been demonstrated to be the GzmA-activated DNase responsible for a caspase-independent apoptosis pathway triggered by CTL in virus-infected or tumor cells (Fan et al., 2003). Control and HPV-16 E7-expressing cells were shifted from low calcium to high calcium-containing medium and cultured for 4 days. Cells were thereafter loaded with perforin and human recombinant GzmA at three different concentrations for 2 h. Apoptosis was evaluated by flow-cytometry detection of annexin V-positive cells. Treatment with increasing concentrations of GzmA proportionally increased the percentage of annexin V-positive control cells, whereas induced almost no apoptosis in HPV-16 E7-expressing cells (Fig. 6). These results provide evidence that HPV-16 E7 interference with the Nm23-H1 protein inhibited the onset of the caspase-independent apoptosis that involves the Nm23-H1 DNase activity.

HPV-16 E7-dependent Nm23-H1 down-modulation promotes HaCaT cell invasiveness

Nm23–H1 has been extensively documented as a negative regulator of cancer cells invasiveness (Steeg, 2004). In order to investigate whether HPV-16 E7 interference on Nm23–H1 could promote HaCaT cell motility and invasiveness, a chemoinvasion assay was performed. Control and HPV-16 E7-expressing cells were shifted from low calcium to high calcium-containing medium and cultured for 4 days. Cells were thereafter transferred to the invasion chambers. Fig. 7A shows that HPV-16 E7-expressing cells, were able to migrate through an *in vitro* reconstituted basement membrane, due

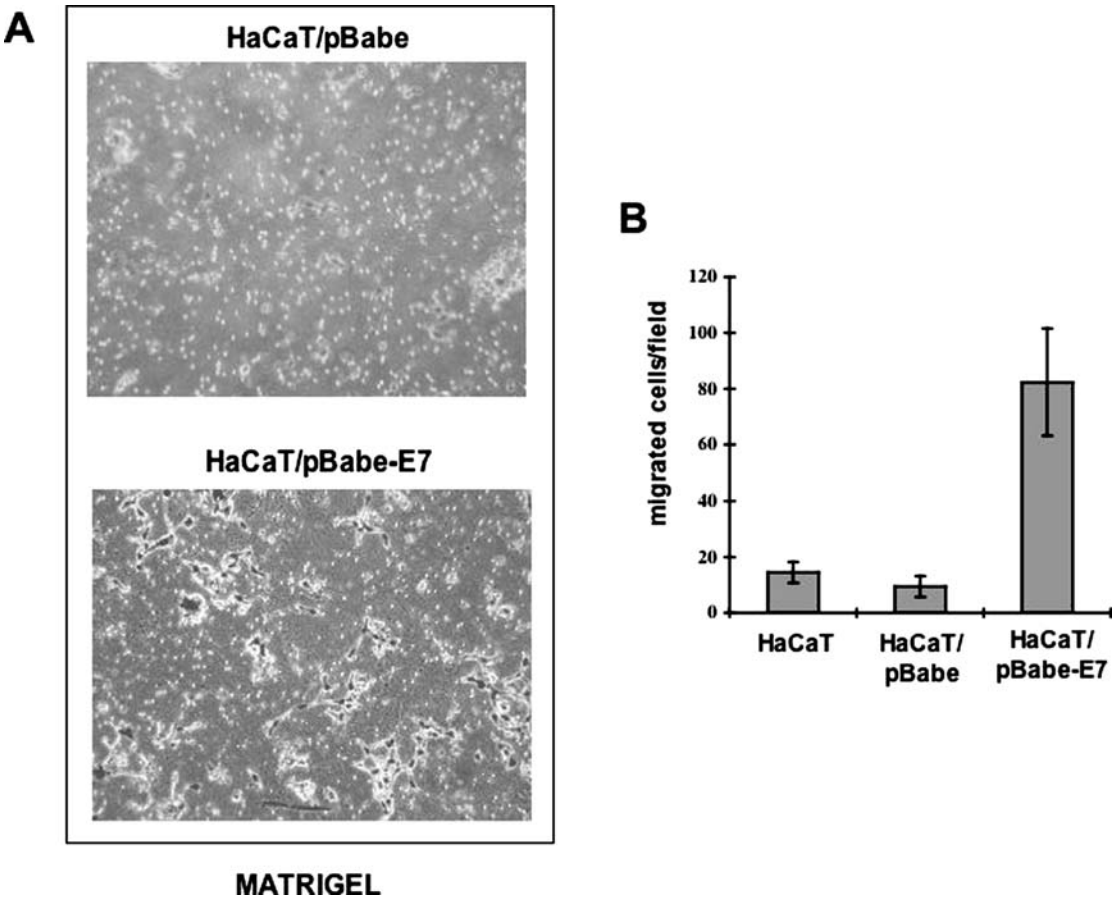


Fig. 7 HPV-16 E7 dependent Nm23-H1 down-modulation promotes HaCaT cell invasiveness. A) HaCaT/pBabe and HaCaT/pBabe-E7 cells migrated through a matrigel-coated 8-μm filter (100× magnification). B) Histograms of the migrated HaCaT, HaCaT/pBabe and HaCaT/pBabe-E7 cells per field. Values are arithmetical means ± SD of 20 fields counted for five experiments, each in duplicate

to HPV-16 E7-mediated Nm23-H1 down-modulation and/or inactivation. Histograms in Fig. 7B indicate that the number of invaded HaCaT/pBabe-E7 cells was at least 8-fold the number of invaded control cells. These data clearly indicate that Nm23-H1 inactivation induced by HPV-16 E7 results in the attainment of an invasive phenotype by the HaCaT cells.

Discussion

Here we report the identification, by the yeast two-hybrid system, of a novel interaction of the HPV-16 E7 oncoprotein with the Nm23-H1 and Nm23-H2 proteins. Moreover, we show that retrovirally-expressed HPV-16 E7 was able to physically interact with both the Nm23-H1 and Nm23–H2 proteins in a human cellular context, the HaCaT keratinocytes. HPV-16 E7-expressing HaCaT cells displayed, upon triggering of differentiation, strong down-modulation of Nm23-H1 and Nm23–H2, due to the activation of ubiquitin-proteasome protein degradation and to a reduced transcriptional activity of both the *nm23* genes. Noteworthy, HPV-16 E7-expressing HaCaT cells exhibited reduced differentiation capabilities and enhanced invasion properties. In addition, physical interaction with HPV-16 E7 reduced Nm23-H1 DNA cleavage activity *in vitro*, and HPV-16 E7 expression in HaCaT cells inhibited GzmA-activated apoptosis, a process in which Nm23-H1 plays a pivotal role by its DNase activity (Fan et al., 2003).

The expression of the E6 and E7 viral factors, associated to the integration of the respective genes in the host cell genome, is a characteristic of HPV-related human cancers, but E7 plays the main transforming role (Munger et al., 2001; Zwerschke and Jansen-Durr, 2000). The intracellular amount of the E7 oncoprotein is, however, kept at a very low level, possibly due to its intrinsic toxicity for the host cell (Smotkin and Wettstein, 1987). For this reason, the 98-aa HPV-16 E7 protein seems able to develop combined and synergistic strategies to hit key factors in the target cells, in spite of its stoichiometrically low intracellular concentration. As an example, pRb, the product of the *RB* oncosuppressor gene, is directly targeted by the LXCXE motif present in the CR2 region of HPV-16 E7, thus neutralizing several pRb-related functions mainly by releasing transcription E2F factors. In

addition, HPV-16 E7 is able to activate destabilization of pRb as well (Wang et al., 2001).

From our results, HPV-16 E7 appeared to behave with a complex strategy, in which physical interaction with Nm23-H1 compromised stability and function of the antimetastatic protein.

We showed that the expression level of the Nm23 proteins increased during *in vitro*-induced HaCaT cells differentiation, which is in agreement with several reports indicating Nm23-H1 up-modulation during ectodermal cell differentiation (Lombardi et al., 2000). The role of the Nm23-H1 and Nm23-M1 proteins in promoting cell cycle withdrawal and subsequent differentiation has been extensively studied. Their down-modulation or mutations have been associated to increased cell proliferation and prevention of neural differentiation, (Gervasi et al., 1996; Lombardi et al., 2001). Similarly, in this context, it is conceivable that Nm23-H1 contributes to HaCaT cell cycle negative control during keratinocyte differentiation. Conversely, when HPV-16 E7-expressing HaCaT cells were induced to differentiate, Nm23-H1 protein down-modulation appeared dramatically evident, which could belong to the strategy accomplished by HPV-16 E7 to maintain keratinocytes in a proliferative competent status.

Among the multiple features of the Nm23 proteins, the ability to reduce tumor invasiveness is validated by a wealth of experimental *in vitro* and *in vivo* data (Steeg, 2004). Remarkably, we also demonstrated that HPV-16 E7-dependent Nm23-H1 down-modulation enabled HaCaT cells to acquire an invasive phenotype, as revealed by the ability to degrade and move through an *in vitro* reconstituted extra-cellular matrix, a biological phenomenon that underlies metastasis dissemination.

HPV-16 E7 was able to inhibit Nm23-H1 DNA cleaving activity *in vitro*, suggested to be part of a DNA repair mechanism associated with faithful DNA replication (Ma et al., 2004), and demonstrated as responsible *in vivo* for the GzmA-activated pathway inducing caspase-independent apoptosis by CTL in virus-infected or transformed cells (Fan et al., 2003). Notably, the interference, herein shown, with these two features can concur to the tumorigenic potential of the HPV-16 E7 oncoprotein, favoring genomic instability and cell survival. Being HPV-16 a pivotal player in human epithelia tumorigenesis, additional data on Nm23-H1 expression level in specimens from HPV-16-induced tumors might be informative for patient's staging and prognosis.

In conclusion, all the findings described here, started from the evidence of the physical protein-protein interaction between HPV-16 E7 and Nm23-H1, can represent an essential component of the composite strategy employed by the E7 oncoprotein in constituting the ideal scenario for cell transformation and tumor progression.

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References

Beresford PJ, Xia Z, Greenberg AH, Lieberman J (1999) Immunity 10:585–594
Biroccio A, Amodei S, Benassi B, Scarsella M, Cianciulli A, Mottolese M, Del Bufalo D, Leonetti C, Zupi G (2002) Oncogene 21:3011–3019
Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE (1988) J Cell Biol 106:761–771
Breitkreutz D, Stark HJ, Plein P, Baur M, Fusenig NE (1993) Differentiation 54:201–217
Carroll JM, Albers KM, Garlick JA, Harrington R, Taichman LB (1993) Proc Natl Acad Sci (USA) 90:10270–10274
Chen YP, Higgins JA, Gundersen-Rindal DE (2003) J Virol Methods 114:125–133
Chien C-T, Bartel PL, Sternglanz R, Fields S (1991) Proc Natl Acad Sci (USA) 88:9578–9582
Fan Z, Beresford PJ, Oh DY, Zhang D, Lieberman J (2003) Cell 112:659–672
Fehrmann F, Laimins LA (2003) Oncogene 22:5201–5207
Fields S, Song O-K (1989) Nature 340:245–246
Freije JM, Blay P, MacDonald NJ, Manrow RE, Steeg PS (1997) J Biol Chem 272:5525–5532
Gervasi F, D'Agnano I, Vossio S, Zupi G, Sacchi A, Lombardi D (1996) Cell Growth Differ 7:1689–1695
Gervasi F, Capozza F, Bruno T, Fanciulli M, Lombardi D (1998) DNA and Cell Biol 17:1047–1055
Giarre M, Caldeira S, Malanchi I, Ciccolini F, Leao MJ, Tommasino M (2001) J Virol 75:4705–4712
Griffin EF, Harris H (1992) J Cell Sci 102:799–805
Lacombe M-L, Milon L, Munier A, Mehus JG, Lambeth DO (2000) J Bioenerg Biomembr 32:247–257
Lakso M, Steeg PS, Westphal H (1992) Cell Growth Differ 3:873–879
Lombardi D, Sacchi A, D'Agostino G, Tibursi G (1995) Exp Cell Res 217:267–271
Lombardi D, Lacombe M-L, Paggi MG (2000) J Cell Physiol 182:144–149
Lombardi D, Palescandolo E, Giordano A, Paggi MG (2001) Cell Death Differ 8:470–476
Lombardi D, Mileo AM (2003) J Bioenerg Biomembr 35:67–71
Ma D, McCorkle JR, Kaetzel DM (2004) J Biol Chem 279:18073–18084
MacDonald NJ, Freije JM, Stracke ML, Manrow RE, Steeg PS (1996) J Biol Chem 271:25107–25116
Munger K, Basile JR, Duensing S, Eichten A, Gonzalez SL, Grace M, Zacny VL (2001) Oncogene 20:7888–7898
Munger K (2002) Front Biosci 7:641–649
Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ (2003) N Engl J Med 348:518–527
Murakami M, Lan K, Subramanian C, Robertson ES (2005) J Virol 79:1559–1568
Postel EH, Berberich SJ, Rooney JW, Kaetzel DM (2000) J Bioener Biomembr 32:277–284
Postel EH (2003) J Bioener Biomembr 35:31–40
Ryle CM, Breitkreutz D, Stark HJ, Leigh IM, Steinert PM, Roop D, Fusenig NE (1989) Differentiation 40:42–54

Shi L, Kraut RP, Aebersold R, Greenberg AH (1992) J Exp Med 175:553–566
Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge JE, Liotta LA, Sobel ME (1988) J Natl Cancer Inst 80:200–204
Steeg PS (2004) J Natl Cancer Inst 96:E4
Steele BK, Meyers C, Ozbun MA (2002) Anal Biochem 307:341–347
Subramanian C, Cotter MA II, Robertson ES (2001) Nat Med 7:350–355
Smotkin D, Wettstein FO (1987) J Virol 61:1686–1689
Wang J, Sampath A, Raychaudhuri P, Bagchi S (2001) Oncogene 20:4740–4749
zur Hausen H (2002) Nat Rev Cancer 5:342–350
Zwerschke W, Jansen-Durr P (2000) Adv Cancer Res 78:1–29