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A Functional RNAi-Based Knockdown System: A Tool to Investigate HPV Entry?

Caroline Horvath[1], Gaelle Boulet[1], Shaira Sahebali[1], John-Paul Bogers[1,2] and Davy Vanden Broeck[1,2*]

[1]*Applied Molecular Biology Research Group (AMBIOR), University of Antwerp, Antwerp*
[2]*International Centre for Reproductive Health (ICRH), Ghent University, Ghent*
Belgium

1. Introduction

Worldwide, cervical cancer is an important health issue leading to high morbidity and mortality rates. The HPV virus plays an essential in the development of this disease. Of the more than 100 different HPV types identified, 40 are known to infect the genital tract (Woodman et al., 2007). These mucosal HPV types are classified as "low-risk" and "high-risk" types based on the prevalence ratio in cervical cancer and its precursors. Low-risk HPV types, such as 6 and 11, induce benign lesions with minimum risk of progression to malignancy. In contrast, high-risk HPVs have higher oncogenic potential (Fehrmann & Laimins, 2003). Approximately 99% of cervical cancers contain HPV DNA of high-risk types, with type HPV16 being the most prevalent, followed by types 18, 31, 33, and 45. Cervical HPV infection is one of the most common sexually transmitted infections (Walboomers et al. 1999; Woodman et al., 2007).

HPVs are obligatory intracellular parasites that must deliver their genome and accessory proteins into host cells and subsequently make use of the biosynthetic cellular machinery for viral replication. The journey of a HPV particle from the cell surface to the cytosol and nucleus consists of a series of consecutive steps that move it closer to its site of replication (Day & Schiller, 2006; Marsh & Helenius, 2006). Host cell entry of HPV is initiated by binding of the virus particle to cell surface receptors followed by internalization. Since HPV replication and assembly requires infected basal keratinocytes to undergo the stepwise differentiation program of the epithelium, HPV propagation in cell culture is a major challenge. This has prompted many researchers to study the HPV-host cell interactions by generating VLPs, PsVs and QVs. Because of many difficulties, including viral particle heterogeneity due to the maturity state of the "artificial" viral particles used in many studies, data concerning the HPV entry mechanisms are contradictory and still a subject of scientific debate (Horvath et al., 2010).

To prevent and cure HPV infections and its complications, it is important to identify the viral infection mechanisms by means of investigating the viral biology. Viral attachment and

* Corresponding author

internalization are first fundamental steps in the infection process and therefore, the ideal points to interfere with the progression of the viral life cycle. Identifying HPV uptake mechanisms may lead to a more specific pharmacological control of viral processing hereby improving therapeutic and prevention strategies.

The design of a tool to study HPV internalization is the important goal in this research project. HPVs are small, non-enveloped DNA viruses that generally enter through clathrin-coated endocytic vesicles or via the clathrin-independent caveolae system (Day et al., 2003). To date, most of the studies proved that different types of HPVs entered cells in distinct pathways, including clathrin-mediated endocytosis, caveolar endocytosis and clathrin- and caveolae-independent uptake mechanisms, although it was found that clathrin-mediated endocytosis was the major cellular entry route (Horvath et al., 2010). Therefore, it is important to design an experimental model to study the exact role of clathrin-mediated endocytosis in the uptake of HPV.

The use of molecular tools with such precise specificity to individual cellular functions allows the defined examination of endocytic pathways (Day et al., 2006). In an experimental setting, it is challenging to identify a particular endocytotic pathway that is unique for the uptake of a specific ligand. It is important that the internalization route of interest is exclusively suppressed without affecting other endocytotic pathways. Chemical inhibitors like cholesterol extracting or sequestrating drugs such as cyclodextrins, filipin and nystatin not only disrupt caveolae/lipid rafts, but also lead to the destabilization of CCPs (Rodal et al., 1999; Subtil et al., 1999). The development of molecular inhibitors in the form of dominant negative molecules has surpassed the use of these pharmacological inhibitors in terms of decreasing non-specific effects (Day et al., 2003). Different accessory proteins such as dynamin, amphiphysin and Eps15 have been subjected to dominant negative overexpression (Benmerah et al., 1998; Benmerah et al., 1999; Yao et al., 2005).

Targeting viral mRNA is one of the most active areas of research and development. Several strategies have emerged over the years such as antisense-oligonucleotides, ribozymes and RNA interference (RNAi). All these strategies share the features of conceptual simplicity, straightforward drug design and quick route to identify drug leads (Le Calvez et al., 2004). RNA interference is the inhibition of expression of specific genes by double-stranded RNAs (dsRNAs). It is becoming the method of choice to knockdown gene expression rapidly and robustly in mammalian cells. Comparing to the traditional antisense method, RNAi technology has the advantage of significantly enhanced potency, therefore, only lower concentrations may be needed to achieve the same level of gene knockdown. RNAi gained rapid acceptance by researchers after Tuschl *et al*. discovered that *in vitro* synthesized small interfering RNAs (siRNAs) of 21 to 23 nucleotides in length can effectively silence targeted genes in mammalian cells without triggering interferon production (Le Calvez et al., 2004; Tuschl et al., 1999).

The transient nature of siRNA, due to the short lifespan of synthetic RNA molecules and the absence of a RNA-dependent RNA polymerase in mammalian cells, limits its applications (Dykxhoorn et al., 2008; Kim & Rossi, 2007; Rao et al., 2009). To overcome these limitations, a vector-based production of short hairpin RNA (shRNA) is used, hereby mediating a long-term and stable knockdown of the target transcripts for as long as transcription of the shRNAs takes place (Brummelkamp et al., 2002, Kim & Rossi, 2007; Paddison et al., 2002).

As described above, epsin is an essential accessory protein involved in clathrin-mediated endocytosis. Therefore, epsin is the target protein to generate a shRNA-based knockdown construct to investigate the role of clathrin-mediated endocytosis in the uptake of HPV.

Another approach to investigate the role of clathrin-mediated endocytosis in the uptake of HPV, is the use of dominant-negative inhibitors of Eps15. Eps15 (epidermal growth factor pathway substrate 15) is a protein that is associated with clathrin-mediated endocytosis and consists of three different domains. The N-terminal region contains three copies of the conserved Eps15 homology domain (EH-domain). The central region of the protein is important in the homodimerization/oligodimerization of Eps15 and the C-terminal region contains binding sites for AP2. Overexpression of the C-terminal region (GFP-Eps15 DIII mutant) has a strong inhibitory effect on the uptake of the clathrin markers transferrin and epidermal growth factor receptor (EGFR) (figure 1) (Benmerah et al., 1998). Another Eps15 mutant (GFP-Eps15 EΔ95/295), missing the second and third EH-domain in its N-terminal region has a negative influence on clathrin-mediated internalization (figure 1) (Benmerah et al., 1999). Both vectors, in combination with a control plasmid (GFP-Eps15 D3Δ2), can be used in order to study the involvement of clathrin-mediated endocytosis in the uptake of HPV.

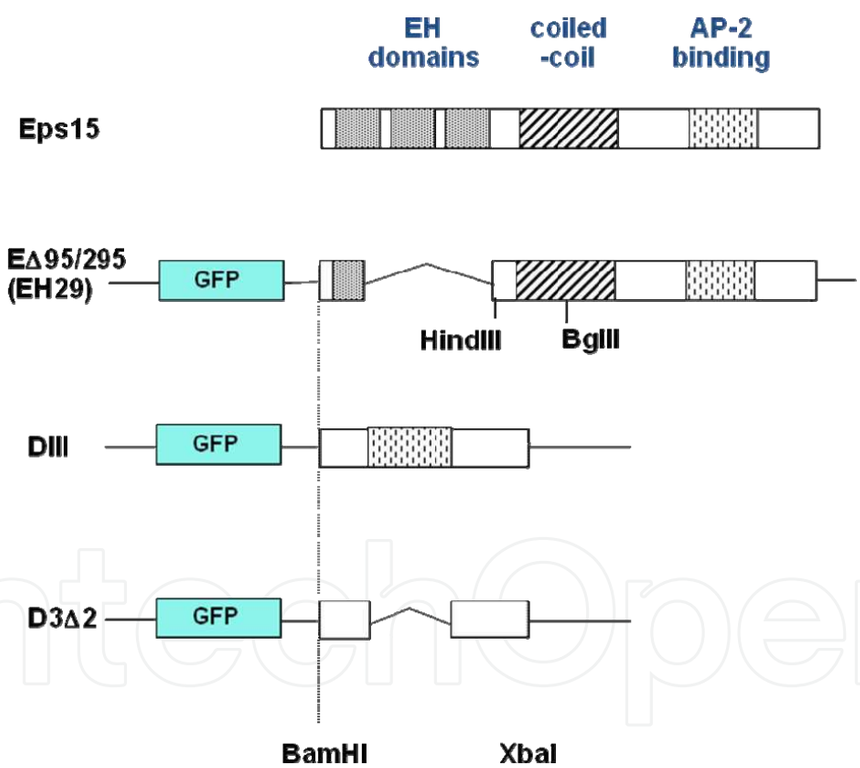


Fig. 1. Eps15 mutants
The EH29 mutant was generated by deleting 537 nucleotides (+288/+825) resulting in a construct length of 2163 nucleotides versus 2700 for wild type Eps15. This construct has been cloned between the BamHI/XbaI sites of the EGFP-C2 vector.
The DIII construct corresponds to the C-terminal domain of Eps15 (+1587/+2700) resulting in a construct length of 1200 bp. The D3D2 construct corresponds to DIII from which AP-2 binding sites has been removed (+1863/+2217) resulting in a 760 nucleotides long insert (Benmerah et al,, 1998; Benmerah et al., 1999).

2. Materials & methods

2.1 Cell culture

The human cervical cell line HeLa (ATCC CCL-2), which contains 10-50 copies of integrated HPV-18 and the human keratinocyte cell line HaCaT (Prof. Boukamp, Deutsches Krebsforschungszentrum (DKFZ), German Cancer Research Centre, Heidelberg, Germany), were grown under a humidified carbon dioxide atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 200mM L-glutamin, 50mg/L penicillin/streptomycin, 50mg/L gentamicin, non-essential amino acids (Gibco, Carlsbad, CA, USA) and 50mg/L amphotericin (Sigma-Aldrich, Milan, Italy).

Human intestinal colon carcinoma cells, Caco-2 cells (ATCC HTB-37) were cultured in Modified Eagle's Medium (MEM) supplemented with 10% fetal bovine serum, non essential amino acids, 50mg/L penicillin/streptomycin, 50mg/L gentamicin, 55mg/L sodium pyruvate (Gibco, Carlsbad, CA, USA) and 1mg/L amphotericin (Sigma-Aldrich, Milan, Italy).

2.2 RNAi selection and plasmid preparation

Based on the primary sequences of the different epsin isoforms a multiple alignment and homology search was performed using ClustalW (searchlauncher.bcm.tmc.edu/multi-align/.html). A stretch of 21 nucleotides was selected from the conserved ENTH region located <0.2 kb after the start codon, according to the guidelines and parameters (Tuschl et al., 1999). **(**table 1**)**.

pRNATin-H1.2/Neo is a siRNA inducible expression vector (GenScript). The H1.2 promotor is an engineered inducible H1 promotor (RNA polIII) containing a tetracycline operator (TetO1). The tetracycline operator itself has no effect on expression. When the tetracycline repressor (TetR) is present, it effectively binds the TetO1 and blocks the transcription. In the presence of tetracycline or doxycycline, the inducer binds TetR and causes the TetR protein to release the TetO1 site, and derepressing the transcription from the H1 promotor. The pRNATin-H1.2/Neo vector is designed for mammalian transfection and carries a neomycin resistance gene which can be used for establishing stable cell lines and a cGFP (coral GFP) marker under CMV promotor control to track the transfection efficiency. The insert is cloned after the H1 promotor and transcribed into short double stranded RNA (dsRNA) with a hairpin structure.

The parental plasmid was digested with *Bam*H1 and *Hin*dIII, separated on a 1% agarose gel and purified using the Qiagen Extraction kit. The shRNA insert was designed by using siRNA construct builder (GenScript; http://www.genscript.com/rnai.html). The shRNA sequences (table 1) were allowed to anneal and ligated into the digested vector using Ready-to-Go T4 ligase. In parallel, a functional non-targeting shRNA, a sequence with no matching mRNA (scrambled sequence) (table 1), was ligated into the same vector.

Correct insertion of the construct was confirmed by sequencing (data not shown). After transformation of *E.coli*, ampicillin recombinant strains were selected by using Fast-Media® Amp XGal Agar. Bacterial cells were grown in medium supplemented with 5µl/ml ampicillin and plasmids were purified using the PureYield Plasmid Midiprep System (Promega, Madison, WI, USA). Transformation and purification for the Eps15 mutant

vectors was performed in a similar way, hereby selecting recombinant strains by using kanamycin selection.

Name	Sequence (5′→3′)
siRNA epsin	Forward: GAAGAACATCGTCCACAACTA Reverse: CTTCTTGTAGCAGGTGTTGAT
siRNA scrambled	Forward: GCATATGTGCGTACCTAGCAT Reverse: CGTATACACGCATGGATCGTA
shRNA epsin	GGATCCCGAAGAACATCGTGCACAACTATTCAAGAGATAGTTGTGC ACGATGTTCTTCTTTTTTCCAAAAGCTT
shRNA scrambled	GGATCCCGCATATGTGCGTACCTAGCATTTCAAGAGAATGCTAGGTA CGCACATATGCTTTTTTCCAAAAGCTT

Table 1. siRNA and shRNA sequences

2.3 Transfection and selection

Liposomal transfection using Lipofectamine2000 (Invitrogen, San Diego, CA, USA) was performed according to the manufacture's protocol. In brief, 4 µg plasmid DNA was added to 250µl OptiMEM and gently mixed, 5µl Lipofectamine2000 was suspended in 250µl OptiMEM, both mixtures were combined after a 5 minute incubation at room temperature and added to HeLa and HaCaT cells, with a 90-95% cell density, in a 6-well plate.

HeLa and HaCaT cells were trypsinized 24h after transfection and seeded into a selection medium containing 100 µg/ml G418 as a starting point. A kill curve was created by doubling the G418 concentration with each medium change eventually culturing the HeLa and HaCaT cells in selection medium containing 1500 µg/ml G418.

To estimate the transfection efficiency through GFP expression, cells were grown in MatTek glass bottom dishes (MatTek Corp., Ashland, MA, USA), fixed with 4% paraformaldehyde for 30 min at RT and mounted in Citifluor. Confocal fluorescence microscopy (Zeiss CLSM 510) was used to discriminate between transfected and non-transfected cells. GFP-positive cells were counted by fluorescence microscopy and expressed as the percentage of the total amount of cells visualized by light microscopy.

2.4 Real-time RT-PCR

The effect of shRNA against epsin was evaluated by monitoring the epsin mRNA expression level.

Total RNA was isolated using the reagent-based method TRIzol® (Invitrogen, Carlsbad, CA, USA) and RNA concentration and purity were monitored by UV-spectrophotometry. Prior to cDNA synthesis, DNA was removed from the total RNA extracts. An amount of 12µl DNase-treated total RNA was reversed transcribed in a 20µl reaction volume using the

Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions.

Real-time RT-PCR analyses were performed using the LightCycler system based on the TaqMan methodology (Roche Applied Science, Indianapolis, IN, USA). PCR-efficiencies (E) were tested for hypoxanthine phosphoribosyltransferase (HPRT) and epsin (EPN), ranging from 92%-132%. Specific primers and probes for these genes were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) (table 2).

For the LightCycler reaction a Mastermix of the following reaction components was prepared to the indicated end-volume (20µl): 7µl PCR-H_2O, 1µl forward primer (0.5µM), 1µl reverse primer (0.5µM), 2µl probe (0.2µM), 4µl LightCycler Mastermix (LightCycler TaqMan Master kit; Roche Applied Science, IN, USA) was filled in the LightCycler glass capillaries and 5µl cDNA was added as PCR template. The following protocol was used: denaturation program (95°C for 10min), amplification and quantification program repeated 50 cycles (95°C for 10s, 58°C for 10s, 72°C for 10s with a single fluorescence measurement), melting curve program (55-95°C, with a heating step of 0.1°C per second and a continuous fluorescence measurement) and finally a cooling step to 40°C.

Name	Sequence 5′→3′
EPN	Forward: ACTTCCAGTACGTGGACCGC Reverse: GCTGCTTAGCTTTCTCACGCA TaqMan probe: FAM-ACGGCAAGGACCAGGGCGTGA-Dabcyl
HPRT	Forward: GGCAGTATAATCCAAAGATGGTCA A Reverse: GTCTGGCTTATATCCAACACTTCGT TaqMan probe: FAM-CAAGCTTGCTGGTGAAAAGGACCCC-Dabcyl

Table 2: Epsin and HPRT primer and probe sequences

2.5 Western blotting

Cells were lysed 60 min on ice in standard lysis buffer (50 mM NaCl, 10 mM HEPES, 3 mM $MgCl_2$, 300 mM sucrose and 0.5% Triton X-100) supplemented with Complete Mini EDTA-free protease inhibitor (Roche). Lysates were centrifuged at 15,000xg for 20 min at 4°C and supernatans was collected.

Protein A/G agarose beads were coated with goat-anti-epsin polyclonal antibodies (Santa Cruz Biotechnology, 1:1000) and incubated overnight with the cell lysate on ice. After centrifugation at 15,000xg for 5 min at 4°C, the sediment was suspended in NuPAGE LDS Sample Buffer (Invitrogen), heat denaturated for 10 min at 95°C and loaded on a NuPAGE 12% bis-tris polyacrylamide gel (Invitrogen). After electrophoresis in NuPAGE MOPS SDS running buffer using the NOVEX XCell II unit (40 min, 200V, 120mA), proteins were transferred to Immun-Blot® I PVDF membranes (Bio-Rad) (1h, 30V, 170mA). Membranes were blocked in Tris buffered saline containing 0.05% Tween-20 (TBST) and 5% non-fat dry milk for 1h. After blocking, membranes were probed overnight at 4°C with polyclonal goat-anti-epsin in TBST containing 5% dry non-fat milk. Membranes were washed five times with TBST during 5 minutes and incubated with TBST containing 5% dry non-fat milk and mouse anti-goat peroxidase conjugated antibody (Calbiochem, 1:2500) for 2h at room temperature.

After several washing steps, 5 times for 5 minutes, immunoblots were detected using the ECL® Plus Western blot detection system by the ChemiGenius2.

3. Results

3.1 Transfection efficiency

Efficiency of transfection with pRNATin-H1.2/Neo was measured by confocal fluorescence microscopy and the amount of GFP-positive cells were counted by fluorescence microscopy and expressed as the percentage of the total amount of cells visualized by light microscopy. Figure 2 shows the efficiency of transfection with pRNATin-H1.2/Neo in HeLa cells which amounted to 81%.

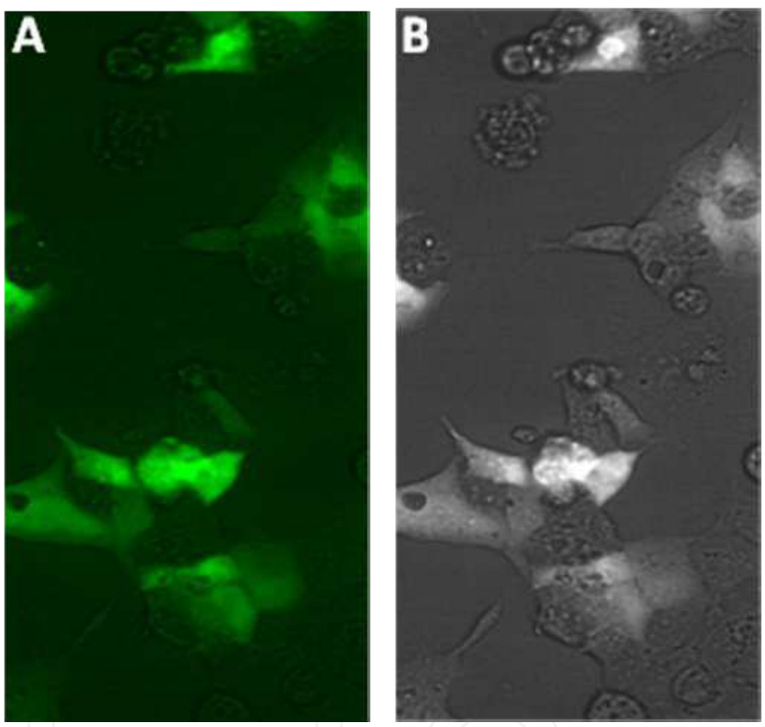


Fig. 2. Efficiency of transfection with pRNATin-H1.2/Neo in HeLa cells. The amount of transfected cells was determined by counting GFP-positive cells versus the total amount of cells, determined by fluorescence **(A)** and light microscopy **(B)** respectively

3.2 Real-time RT-PCR and western blotting

3.2.1 Synthetic siRNA

The synthetic siRNA (table 3) reduced the epsin expression with a factor $>10^3$ (figure 3 A) without affecting expression profiles of the housekeeping gene. In negative controls (HeLa scrambled) the expression of epsin was barely affected, an indication that the transfection procedure did not elicit non-specific effects (figure 3 A).

Western blot analysis shows a reduction of the epsin protein in HeLa epsin deficient cells (HeLa eps-; figure 3 B) compared to HeLa control and HeLa scrambled.

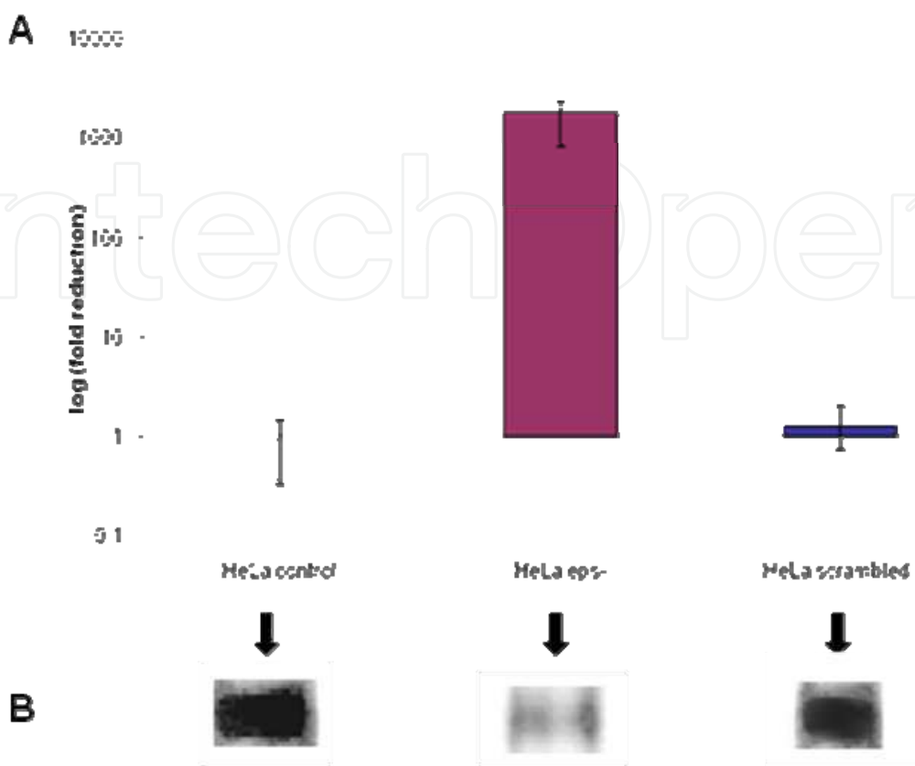
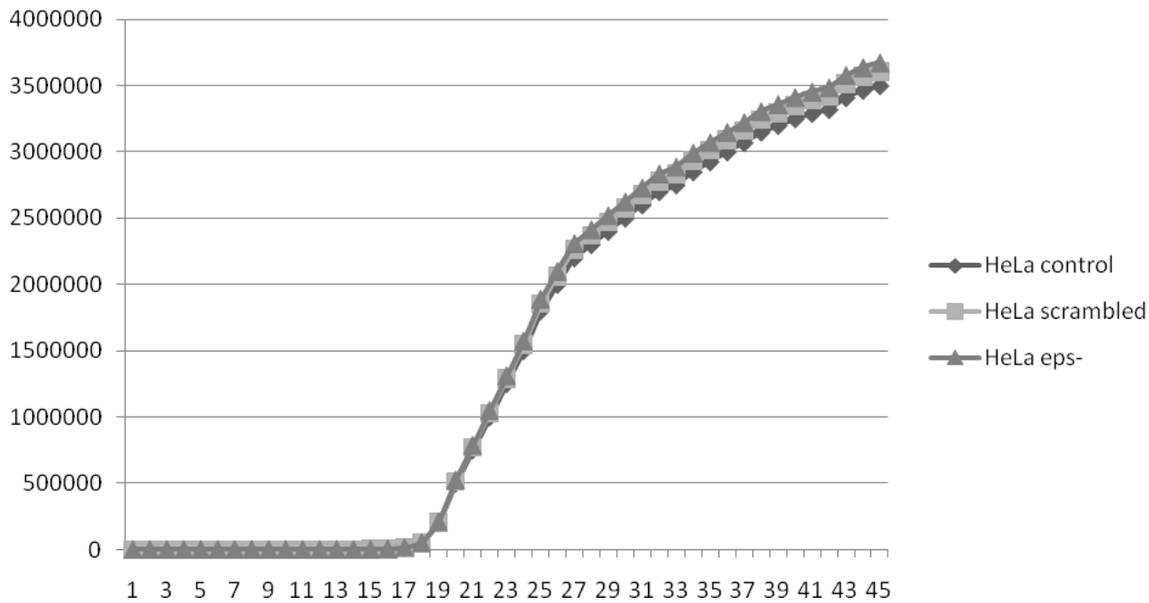


Fig. 3. **A.** The synthetic siRNA reduced the epsin expression with a factor >10^3. **B.** Western blot analysis shows a reduction of the epsin protein in HeLa epsin deficient cells (HeLa eps-).

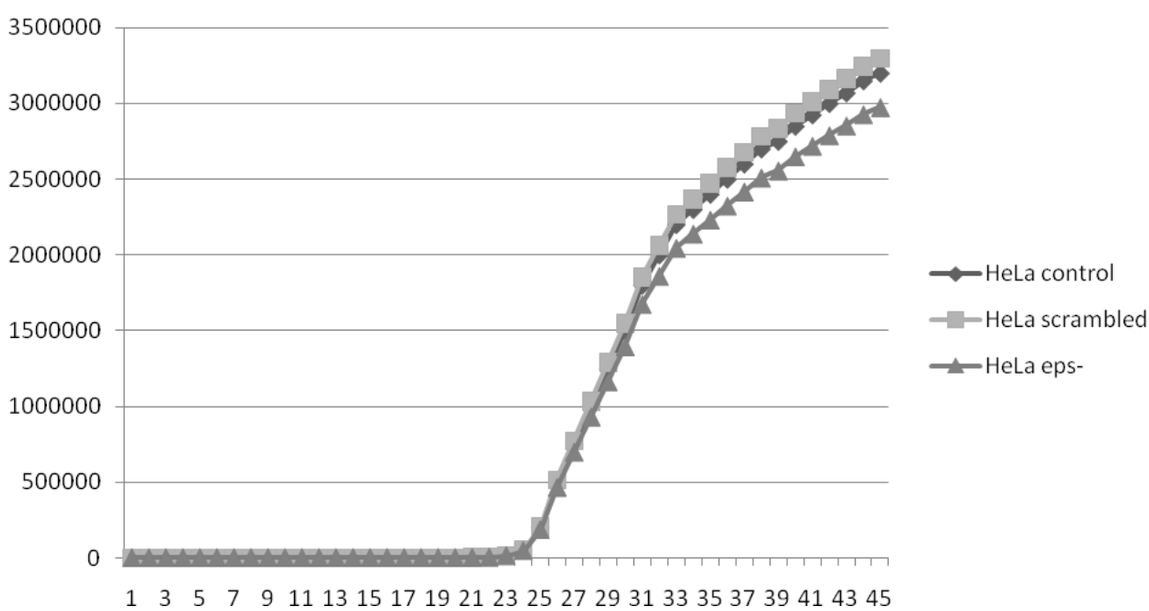
3.2.2 Plasmid transfection shRNA

The extent of siRNA-mediated silencing of epsin expression in HeLa cells (both control and transfected cells) by using a shRNA-based knockdown construct, was controlled by means of real-time RT-PCR and Western blotting (figure 4-5).

Real-time RT-PCR for epsin and HPRT expression was performed on HeLa control cells and on HeLa cells transfected with a vector expressing siRNA against epsin (HeLa eps-) and a scrambled sequence (HeLa scrambled). In order to exclude secondary transfection effects, the expression profile of a housekeeping gene HPRT was compared in both transfected cells and control cells. The real time RT-PCR curves of HeLa control, HeLa scrambled and HeLa eps- nearly coincided, resulting in almost identical C_P values. These data reveal that neither the carbohydrate nor nucleic acid metabolism were compromised by the transfection procedure, an essential requisite for application of siRNA-induced silencing of specific target genes (figure 4 A). However, the same results were obtained for epsin, indicating that there is no suppression of epsin in cells transfected with the vector expressing siRNA against epsin (figure 4 B).



A



B

Fig. 4. The extent of siRNA-mediated silencing of epsin expression in HeLa control and transfected cells (HeLa scrambled/HeLa eps-) by using a shRNA-based knockdown construct, control by means of real-time RT-PCR. Real-time RT-PCR was performed on HeLa control cells and cells transfected with a vector expressing siRNA against epsin and a scrambled sequence. Total RNA was isolated and used for real-time RT-PCR with primers against the housekeeping gene HPRT (**A**) and against epsin (**B**).

In order to evaluate expression of epsin at the protein level, Western blotting was performed. These results revealed that there was no reduction of cellular epsin in transfected HeLa cells versus controls (figure 5).

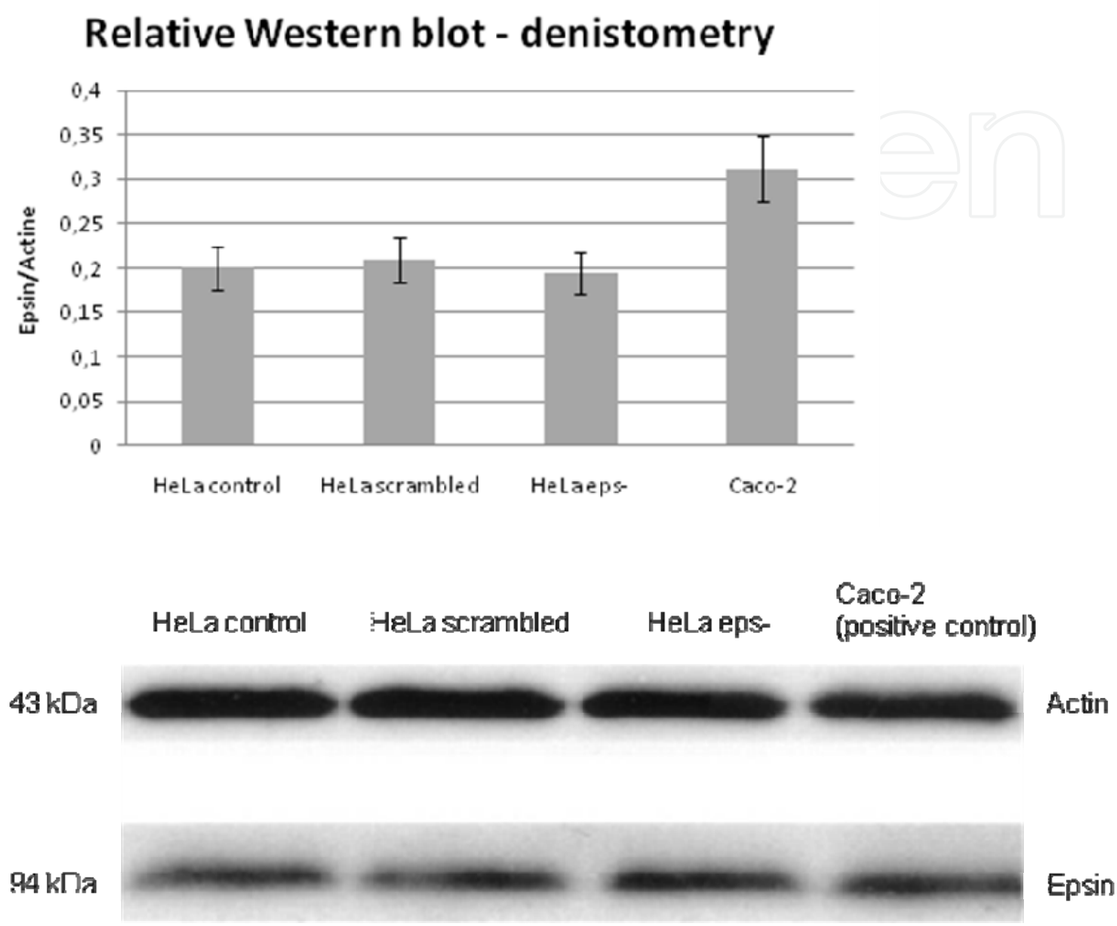


Fig. 5. Western blotting was performed on HeLa control and transfected cells. Caco-2 cells were used as a positive control. Densitometric scans of the protein bands were performed and normalized. There was no reduction of epsin at the protein level measured.

4. Discussion

Despite the significant advances and the emergence of a general picture of the infectious HPV entry pathway, many details remain to be clarified. This study intended to design an experimental model to study the exact role of clathrin-mediated endocytosis in the uptake of HPV, hereby exploiting the technique of RNAi. Because proteins as clathrin heavy chain (CHC) and AP2 suffer from cross reaction phenomena with other endocytotic pathways (Hinrichsen et al., 2003), the essential accessory protein of clathrin-mediated endocytosis, epsin was used as the target protein to generate a shRNA-based knockdown construct.

CHC is inherently specific for clathrin-mediated endocytosis, although it is not unique for internalization at the plasma membrane, because of its involvement in vesicular transport at

the trans-Golgi network (TGN) (Alberts et al., 2002). AP2 has an important function in the recognition of certain cargo motifs, although it is not recognized by all cargo molecules that are internalized via coated pits. Moreover, coated pits can be formed independently of AP2 interaction with cargo (Nesterov et al., 1999; Rappoport et al., 2004). To date, for epsin there are no specific restrictions in specificity for clathrin-mediated endocytosis at the plasma membrane reported.

It is important to know that complete inhibition of clathrin-mediated uptake would undoubtedly lead to induction of apoptosis, hence partial blocking of clathrin-mediated endocytosis has the advantage that cell viability is not significantly reduced. Hinrichsen *et al.*, have shown that a reduction of clathrin to 20% is the absolute limit to ensure cell viability (Hinrichsen et al., 2003). In addition, it has been reported that the uptake of the transferrin receptor, which is an important marker for clathrin-mediated endocytosis, can never be abolished completely in living cells (Iversen et al., 2003).

In this study, a inducible shRNA-based epsin knockdown construct was used. Transient or constitutive expression of either siRNA or shRNA results in temporal or persistent inhibition of gene expression, respectively. A tightly regulated and reversibly inducible RNAi-mediated gene silencing approach could conditionally control gene expression in a temporal or spatial manner that provides an extremely useful tool for studying gene function. It provides an ideal genetic switcher allowing the inducible and reversible control of specific gene activity in mammalian cells (Wu et al., 2007). Moreover, it is less likely that the use of such a system would compromise cell viability.

In our experiments, there was no reduction of the epsin expression in HeLa cells on both mRNA and protein level. However, Vanden Broeck *et al.*, showed a highly specific reduction of epsin in Caco-2 cells using a constitutive shRNA-based knockdown construct. Epsin expression at the protein level was reduced by ~75%, resulting in an equal inhibition of clathrin-mediated endocytosis (Vanden Broeck & De Wolf, 2006).

There are different reasons to explain to obtained results in this study. First of all there might be some cell type specific differences that could clarify these data. Different cells express different genes and feature different cellular pathways. It is likely that different cell types will reveal different results. Moreover, certain cell types could have more profound off-target effect compared to other cell types. Although HeLa cells are probably the most frequently used cell type in published RNA studies, Koller *et al.*, showed that in general, higher concentration of siRNA were needed to see effects in HeLa cells compared to other cell types (Koller et al., 2006).

Furthermore, the copy number of the plasmid could be too low in transfected HeLa cells or the reduction of epsin is detrimental to the cells, leading to the selection against those with reduced levels of expression (counter-selection of knockdown cells). Although we have used high concentration of antibiotics in our experiments, even much higher concentrations of antibiotics may lead to survival of those cells with higher integration numbers and therefore probably more knockdown efficiency.

5. Future perspectives

To prevent and cure HPV infections and its complications, it is important to identify the viral infection mechanisms by means of investigating the viral biology. Viral attachment and

internalization are first fundamental steps in the infection process and therefore, the ideal points to interfere with the progression of the viral life cycle.

The use of molecular tools with such precise specificity to individual cellular functions allows the defined examination of endocytic pathways. The use of an inducible vector system, expressing shRNA against pathway-specific markers is an important step in achieving this goal. This study used such an inducible system in order to knockdown epsin in HeLa, herby interfering with clathrin-mediated endocytosis. Although no reduction of epsin has been achieved, it is important to adjust and optimize the experimental setting, hereby using different vector systems and multiple cell types.

The use of live cell imaging and single virus tracking are important means to dissect multiple infection pathways and multiple infection steps.

Finally, it is important to note that cultured cells are simplified model system. A complete understanding of viral infection would greatly benefit from *in vivo* experiments. It is therefore particularly exciting to track virus particles in live tissues and animals to see how viruses break host defence barriers to reach target cells for infection.

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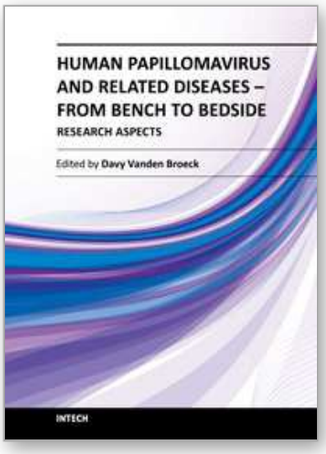

Human Papillomavirus and Related Diseases - From Bench to Bedside - Research aspects

Edited by Dr. Davy Vanden Broeck

ISBN 978-953-307-855-7
Hard cover, 406 pages
Publisher InTech
Published online 25, January, 2012
Published in print edition January, 2012

Cervical cancer is the second most prevalent cancer among women worldwide, and infection with Human Papilloma Virus (HPV) has been identified as the causal agent for this condition. The natural history of cervical cancer is characterized by slow disease progression, rendering the condition, in essence, preventable and even treatable when diagnosed in early stages. Pap smear and the recently introduced prophylactic vaccines are the most prominent prevention options, but despite the availability of these primary and secondary screening tools, the global burden of disease is unfortunately still very high. This book will focus on epidemiological and fundamental research aspects in the area of HPV, and it will update those working in this fast-progressing field with the latest information.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Caroline Horvath, Gaelle Boulet, Shaira Sahebali, John-Paul Bogers and Davy Vanden Broeck (2012). A Functional RNAi-Based Knockdown System: A Tool to Investigate HPV Entry?, Human Papillomavirus and Related Diseases - From Bench to Bedside - Research aspects, Dr. Davy Vanden Broeck (Ed.), ISBN: 978-953-307-855-7, InTech, Available from: http://www.intechopen.com/books/human-papillomavirus-and-related-diseases-from-bench-to-bedside-research-aspects/a-functional-rnai-based-knockdown-system-a-tool-to-investigate-hpv-entry-

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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821