

Title: Therapeutic HPV16 vaccines

The invention relates to the field of medicine and more in particular to nucleic acid constructs and polypeptides that can be used in therapeutic vaccines against human
5 papillomavirus type 16.

Background of the invention

The family of human papillomaviruses (HPVs) consist of more than 100 types
10 (also referred to as subtypes) that are capable of infecting keratinocytes of the skin or mucosal membranes. Over 40 types of HPV are typically transmitted through sexual contact and HPV infections of the anogenital region are very common in both men and women. Some sexually transmitted HPV types may cause genital warts. Persistent infections with "high-risk" HPV types (e.g. types 16, 18, 31, 45) — different from the
15 ones that cause skin warts — may progress to precancerous lesions and invasive cancer, e.g. of the cervix, vulva, vagina, penis, oropharynx and anus. The majority of HPV infections are spontaneously cleared within one to two years after infection. In healthy individuals circulating Th1- and Th2-type CD4+ T-cells specific for the viral early proteins E2, E6 and E7 of HPV-16 as well as E6- specific CD8+ T-cells,
20 migrate into the skin upon antigenic challenge, indicating that successful defense against HPV-16 infection is commonly associated with a systemic effector T-cell response against these viral early antigens. In a minority (~1%) of infected individuals, HPV infection persists, ultimately resulting in genital neoplastic lesions. Among the high-risk HPVs, HPV16 and HPV18 are the main cause of cervical
25 cancer, together causing about 70% of the cases, and these two types also play a major role in other HPV-induced cancers such as anal and oropharyngeal cancer. Worldwide, HPV is one of the most important infectious agents causing cancer.

Vaccination against HPV is deemed a feasible strategy to reduce the incidence or effects of infection by HPV (van der Burg and Melief, 2011, *Curr Opinion*
30 *Immunol* 23: 252–257).

Prophylactic HPV vaccines based on virus like particles (VLPs) formed by the (envelope) protein L1 of the HPV types 16 and 18, are very efficient in the prevention of persistent infection and the associated disease by HPV16 and HPV18. These vaccines are believed to provide sterile immunity via the induction of neutralizing

antibodies against the L1 proteins. Addition of L1-based VLPs from additional high-risk HPV types may further increase the breadth of protection conferred by such vaccines.

5 However, while such vaccines can *prevent* initial infection (i.e., they result in prophylaxis), there is no evidence of a beneficial effect on established genital lesions caused by HPV16 and HPV18, so they are not considered *therapeutic* vaccines against HPV (Hildesheim *et al.*, 2007, *JAMA* 298: 743-53).

10 Despite the introduction of these prophylactic vaccines, large numbers of people have already obtained or are still at risk of obtaining persistent high-risk HPV infections and, therefore, are at risk of getting cancer. Therapeutic vaccines for the eradication of established HPV infections and associated diseases are an urgent unmet medical need.

Some attempts to address this need have been described. For example, clinical trials have been carried out with a variety of different vaccination strategies, such as a
15 fusion protein consisting of a heat shock protein (Hsp) from *Mycobacterium bovis* and HPV-16 E7 or consisting of a fusion protein of E6, E7 and L2 from HPV-16 and HPV-18, chimeric L1-E7 VLPs, recombinant vaccinia viruses expressing either E6 and E7 of HPV-16 and HPV-18 or bovine papilloma virus E2, DNA vaccines expressing CTL epitopes of E6 and E7 of HPV-16 and HPV-18, a live-attenuated
20 *Listeria monocytogenes* (Lm) that secretes the HPV-16 E7 antigen, and synthetic long-peptides (SLPs) comprising HPV-16 E6 and E7 peptides. While some of these approaches show some, but limited, clinical efficacy, most have failed, demonstrating that improvement of the current strategies is needed.

Integration of the early HPV proteins E6 and E7 is a necessary step in the
25 process from infection to cancer and continuous expression of E6 and E7 is required for the maintenance of the neoplastic phenotype of cervical cancer cells. E6 and E7 are therefore considered good targets for therapeutic vaccination. As mentioned some studies have shown that therapeutic vaccination of women infected with high-risk HPV can induce regression of existing lesions. Kenter *et al* showed a durable and
30 complete regression in 47% of patients having Vulvar Intraepithelial Neoplasia (VIN) using SLPs derived from the HPV16 E6 and E7 proteins and an adjuvant as a therapeutic vaccine (Kenter *et al.*, 2009, *N Engl J Med* 361: 1838-47). Similarly, a study in which a protein-based vaccine (TA-CIN, consisting of a fusion protein of HPV16 E6, E7 and L2) was combined with local immune modulation in VIN 2/3

patients, showed complete regression in 63% of patients (Daayana *et al.*, 2010, *Br J Cancer* 102: 1129-36). Possible drawbacks of the synthetic long peptides as a vaccine include manufacturability at large scale and costs associated therewith, the need for potentially reactogenic adjuvant and the associated adverse effects associated with immunization (especially pain and swelling). Due to the high level of discomfort it is not likely that SLPs will be used in early stage disease when the spontaneous clearance rate is still high. Similarly, due to the need for local imiquimod treatment in the case of TA-CIN treatment, tolerability is a significant issue as the majority of women experience local and systemic side effects lasting for the duration of imiquimod treatment, which may affect daily activities.

A possible alternative is to use nucleic acid based vaccination such as DNA vaccines or viral vaccines encoding the HPV E6 and/or E7 protein for vaccination.

However, the HPV E6 and E7 proteins have oncogenic potential and thus vaccination with vaccines that comprise nucleic acids encoding these proteins poses a risk of inducing cellular transformation due to the possibility of prolonged expression of the antigens.

Therefore, in case of genetic vaccination, non-oncogenic/detoxified versions of E6 and/or E7 can be used in order to exclude any risk of cellular transformation due to the vaccination. Loss of oncogenic potential of wild-type E6 and E7 is commonly achieved by deletion and/or substitution of residues known to be important for the function of these proteins (e.g., Smahel *et al.*, 2001, *Virology* 281:231-38; Yan *et al.*, 2009, *Vaccine* 27: 431-40; Wieking *et al.*, 2012, *Cancer Gene Ther* 19: 667-74; WO 2009/106362). However, a disadvantage of these approaches is that they carry the risk of removing important T-cell epitopes from and/or introducing new undesired T-cell epitopes into the proteins, and may thus not lead to the desired immune response.

In an alternative strategy to remove the oncogenic potential of HPV16 E6 and E7, shuffled versions (i.e. polypeptides wherein fragments of the wild-type protein are re-ordered) of the E6 and E7 proteins have been constructed (e.g. Öhlschläger *et al.*, 2006, *Vaccine* 24: 2880-93; Oosterhuis *et al.*, 2011, *Int J Cancer* 129: 397-406; Oosterhuis *et al.*, 2012, *Hum Gen Ther* 23: 1301-12). However, these approaches would still require manufacturing, formulation and administration of multiple molecules to ensure inclusion of all possible epitopes of both the E6 and E7 proteins, resulting in sub-optimal logistics and relatively high costs, and moreover the

strategies described introduce potentially strong non-natural epitopes that are not present in E6 and E7 and since immune responses could be diverted from relevant E6/E7 epitopes toward such non-natural epitopes, the described constructs may not have the optimal immunological characteristics.

5 Thus, there remains a need in the art for therapeutic vaccines against HPV, preferably having less of the drawbacks of the approaches described before.

Summary of the invention

10 The present invention provides nucleic acid molecules that encode polypeptides that comprise essentially all possible T-cell epitopes of HPV16 oncoproteins E6 and E7, but nevertheless have a strongly reduced (as compared to wt E6 and E7), up to non-detectable, transforming activity, by comprising fragments of the E6 and E7 proteins that have been re-ordered, while at the same time containing a
15 minimized number of undesired neo-epitopes. This is in contrast to molecules previously presented by others.

 The invention provides a nucleic acid molecule encoding a polypeptide comprising a sequence as set forth in SEQ ID NO: 1.

 The encoded polypeptide may further comprise a leader sequence.

20 In certain embodiments, the encoded polypeptide further comprises at least one epitope of a human papillomavirus (HPV) E2 protein, for example an HPV16 E2 protein. The E2 protein may be mutated to decrease DNA binding, e.g. by a deletion or mutation(s) in its DNA binding domain. In certain embodiments, the encoded polypeptide comprises a sequence as set forth in SEQ ID NO: 3 or SEQ ID NO: 5.

25 In certain embodiments, the nucleic acid sequence is codon-optimized, e.g. for expression in human cells.

 In certain embodiments, the nucleic acid sequence comprises a sequence as set forth in SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NO: 6.

 The invention also provides a vector comprising a nucleic acid
30 molecule according to the invention, wherein the sequence encoding the polypeptide is operably linked to a promoter.

 In certain embodiments, the vector is a DNA vector such as a plasmid. In other embodiments the vector is a viral vector, such as an MVA vector or a recombinant

adenoviral vector. In certain preferred embodiments, the vector is a recombinant adenovirus.

In certain embodiments, the promoter in the vector is operably coupled to a repressor operator sequence, to which a repressor protein can bind in order to repress
5 expression of the promoter in the presence of said repressor protein. In certain embodiments, the repressor operator sequence is a TetO sequence or a CuO sequence.

The invention also provides a vaccine composition comprising a vector according to the invention, and a pharmaceutically acceptable excipient.

The invention also provides a method of inducing an immune response against
10 HPV, in particular HPV16, in a subject, the method comprising administering to the subject a vaccine composition according to the invention. The invention also provides a vaccine according to the invention for use in inducing an immune response against HPV, in particular HPV16.

In certain embodiments, the vaccine is administered to the subject more than
15 once.

The invention also provides a method for treating any of: persistent HPV infection (in particular persistent HPV16 infection), vulvar intraepithelial neoplasia (VIN), cervical intraepithelial neoplasia (CIN), vaginal intraepithelial neoplasia (VaIN), anal intraepithelial neoplasia (AIN), cervical cancer (such as cervical
20 squamous cell carcinoma (SCC), oropharyngeal cancer, penile cancer, vaginal cancer or anal cancer in a subject, the method comprising administering to the subject a vaccine according to the invention. The invention also provides a vaccine according to the invention for use in treatment of any of: persistent HPV infection (in particular persistent HPV16 infection), vulvar intraepithelial neoplasia (VIN), cervical
25 intraepithelial neoplasia (CIN), vaginal intraepithelial neoplasia (VaIN), anal intraepithelial neoplasia (AIN), cervical cancer (such as cervical squamous cell carcinoma (SCC), oropharyngeal cancer, penile cancer, vaginal cancer or anal cancer in a subject.

The invention also provides a polypeptide comprising a sequence as set forth
30 in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5.

Brief description of the Figures

Fig. 1. Expression of fusion proteins of HPV16 E6 and E7. HEK-293T cells were transiently transfected with DNA vectors expressing the transgenes indicated above the figure. 24 hr after transfection the cells were harvested and cell extracts were
5 analyzed by SDS-PAGE and western blotting with an antibody against HPV16 E7 (upper panel). A loading control showing NF- κ B (lower panel) confirms similar loading of cell lysates in all lanes. A molecular weight marker is indicated on the left. Expected sizes of the fusion proteins: E6E7SH approx. 38kDa; E2E6E7SH and E6E7E2SH approx. 75kDa, LSE2E6E7SH approx. 78kDa.

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Fig. 2. Colony formation in soft agar. A) Schematic representation of the setup of the soft-agar assay. B) Representative microscopic images at 40x magnification of the cells in agar six weeks post seeding. The white arrows highlight colonies observed in the E7wt transfected cells. C) Colony quantification six weeks post seeding in agar
15 using the GelcountTM and associated software. *: $p < 0.05$ (Poisson regression model); **: non-inferior (generalized linear model with non-inferiority margin of 5%).

Fig. 3. E6E7SH has lost E6 and E7 activities. A) Representative western blot demonstrating absence of p53 degradation by E6E7SH. Human p53 null NCI-H1299
20 cells were co-transfected with a plasmid expressing p53 in combination with a plasmid expressing HPV16 E6 wild-type, E6E7SH or the empty vector. Non-TF indicates non-transfected cells. 24 hours after transfection cell lysates were prepared and 30 μ g of total protein was loaded on gel. Upper panel - p53 staining, middle panel - E6 staining, lower panel - NF- κ B staining (loading control). (B) Quantification of
25 p53 levels in four independent assays. The p53 signal was normalized to the NF- κ B signal. C) Western blot demonstrating lack of pRb degradation by E6E7SH. pRb null Saos-2 cells were transfected with a plasmid expressing pRb in combination with a plasmid expressing HPV16 E7 wild-type, E6E7SH or the empty vector. Non-TF indicates non-transfected cells. 24 hours after transfection cell lysates were prepared
30 and 10 μ g of total protein was loaded on gel. Upper panel - pRb staining, middle panel - E7 staining, lower panel - NF- κ B staining (loading control). D) Quantification of pRb levels in four independent assays. The pRb signal was normalized to the NF-

κB signal. *: $p < 0.05$ (ANOVA models); **: non-inferior (testing was based on 95% CI's derived from ANOVA models. Non-inferiority margin was set at 75%).

Fig. 4. E6E7SH does not immortalize primary human epidermal keratinocytes.

- 5 Primary human epidermal keratinocytes were transduced with lentiviruses encoding either the wild-type E6- and E7-encoding open reading frame of HPV16 (E6E7wt), the E6E7SH sequence or eGFP. Non-transduced donor cells were used as a control. Only expression of E6E7wt induces immortalization of primary keratinocytes as indicated by the extended lifespan and hTERT activation around day 200 (not shown).
- 10 The cross symbol indicates that the cells died in senescence and could not be further cultured. For details see example 2. Similar results were obtained in two additional donors (not shown).

Fig. 5. Immune response induced by E6E7SH after DNA immunization – IFN γ

- 15 **ELISPOT analysis.** A. Immunization scheme. CB6F1 mice were immunized with DNA plasmids expressing E6E7SH or a plasmid expressing no transgene (control). Two weeks after immunization the mice were sacrificed and isolated splenocytes were stimulated overnight with 15mer peptide pools corresponding to E7. B. E7-specific immune responses in individual mice as measured by IFN γ ELISPOT assays are given
- 20 as spot forming units (SFU) per 10^6 splenocytes.

Fig. 6. Immunogenicity of E6E7SH – IFN γ ELISPOT analysis. (A). Immunization

- scheme. Mice were immunized with adenovectors with inserts as indicated. E7-specific responses at two weeks (B) and at eight weeks (C) were analyzed by IFN γ
- 25 ELISPOT (represented as spot-forming units (SFU) per 10^6 splenocytes). The closed circles represent mice immunized with a dosage of 1×10^{10} vp, and open circles represent mice immunized with 5×10^9 vp. The black bar represents the geometric mean of the responses. The dotted line indicates the lower detection limit in the ELISPOT assay. ANOVA Post-hoc Bonferroni statistical analysis was performed on
- 30 log transformed data. *: $p < 0.05$. For details see example 3.

Fig. 7. Immunogenicity of E2E6E7SH – E7-tetramer staining. (A). Immunization

scheme. CB6F1 mice were immunized with 1×10^{10} vp of adenovectors expressing the

transgenes as indicated. Two weeks after immunization the mice were sacrificed and isolated splenocytes analyzed for the presence of CD8⁺ cells capable of interacting with E7₄₉₋₅₇-H2-Db tetramers (B). The percentage of E7-tetramer positive CD8⁺ T-cells is indicated on the y-axis. ANOVA Post-hoc Bonferroni statistical analysis was performed on log transformed data, the differences between the different E6E7SH variants were not statistically significant.

Fig. 8. Immunogenicity of E2E6E7SH – IFN γ ELISPOT analysis. (A).

Immunization scheme. CB6F1 mice were immunized with adenovectors expressing the transgenes indicated below panels B and C. Two weeks after immunization the mice were sacrificed and isolated splenocytes were stimulated overnight with 15mer peptide pools corresponding to E2 (B), E6 (not shown) or E7 (C). Responses are given as SFU per 10⁶ splenocytes. ANOVA Post-hoc Bonferroni statistical analysis was performed on log transformed data. The E2 response induced by Adenovectors encoding E2 alone is higher than the response induced by the polypeptides of the invention that include the E6 and E7 fragments. The difference is significant for E2 vs E2E6E7SH and E2 vs E6E7E2SH (*: p<0.05). ANOVA Post-hoc Bonferroni statistical analysis was performed on log transformed data.

Fig. 9. Sustained responses in immunized mice. (A) Immunization scheme. CB6F1 mice were immunized with 1*10¹⁰ vp of Ad35 vectors expressing variants LSE2E6E7SH, E2E6E7SH, E6E7SH, or with an adenovector not expressing a transgene (Empty). Blood samples were taken every two weeks to determine the percentage E7-specific CD8⁺ T-cells by tetramer staining. (B) Immune responses two weeks after immunization. The vector including a leader sequence induced a higher response than vectors without the leader sequence; LSE2E6E7SH vs E2E6E7SH (*: p<0.05). (C) Kinetics of the responses. ANOVA Post-hoc Bonferroni statistical analysis was performed on log transformed data of the week 2 data set. The E7 response induced by molecules including E2 tend to be higher compared to the molecule without E2, though the results were not statistically significant.

Fig. 10. Use of different Adenoviral vectors to boost immune responses. (A).

Immunization scheme. CB6F1 mice were immunized with an Ad26 vector expressing

HPV16 E2E6E7SH (HPV16-Tx) or with an Ad26 vector expressing no transgene (empty). Two weeks later the immunizations were repeated with Ad35-based vectors as indicated below the figure. Four weeks after the second immunization the mice were sacrificed and blood samples were used to determine the percentage of E7-specific CD8⁺ T-cells by tetramer staining (B). * indicates the comparison of Ad26.HPV16-Tx/Ad35.HPV16-Tx versus Ad26.HPV16-Tx/Ad35.Empty, $p < 0.05$ (student t-test on log transformed data, with $\alpha = 0.01$ for multiple comparisons).

Fig. 11. Cellular immunogenicity of E2E6E7SH in Rhesus macaques.. (A)

10 Immunization scheme. Rhesus macaques were immunized at day 0: Eight animals received Ad26.HPV16-E2E6E7SH and two control animals received Ad26.Empty by intramuscular immunization (i.m). A boost immunization was given (Ad26.HPV16-E2E6E7SH or Ad26.Empty) at 8 weeks. At 16 weeks, animals received a second boost immunization with Ad35 vectors expressing the same E2E6E7SH, while control animals received Ad35.Empty. The dose of adenovectors was 1×10^{11} vp per
15 immunization. Blood drawings were performed at several time points. (B) Cellular immune responses in PBMCs were measured by IFN γ ELISPOT. PBMCs were stimulated with peptide pools corresponding to HPV16 E2, E6 or E7 and the number of spot-forming units (SFU) in 1×10^6 PBMCs are depicted. The empty control animal
20 (n=2) showed no detectable response. For details see example 4.

Fig. 12. Therapeutic effect of Adenovectors expressing HPV16-E2E6E7SH. (A)

TC-1 injection and immunization scheme. CB6F1 mice were injected sub-cutaneously with 1×10^5 TC-1 cells at day 0. After six days, when tumors were palpable, mice were
25 immunized with two SLPs covering HPV16 E6 and E7 immunodominant epitopes (i.e., HPV16 E6, aa41-65 (KQQLLRREVYDFAFRDLCIVYRDGN; SEQ ID NO: 18) and HPV16 E7 aa 43-77 (GQAEPDRAHYNIVTFCKCDSTLRLCVQSTHVDIR; SEQ ID NO: 19)) at 150 μ g in a final volume of 200 μ l 0.9% saline supplemented with 5 nmol ODN1826-CpG (B) or Ad26.HPV16-E2E6E7SH (C). Control mice
30 received either CpG alone (D) or Ad26.Empty (E). All mice received a boost immunization at day 20. Mice that received Ad26 vectors in the prime immunization were subsequently immunized with the corresponding Ad35 vectors. The other mice received, SLP adjuvanted with CpG or CpG alone as in the prime immunizations. (B-

E) Tumor measurement in TC-1 injected mice. Tumor volume was calculated as $(\text{width}^2 * \text{length})/2$. Mice were sacrificed when tumor volumes surpassed 1000 mm³. Two mice had to be sacrificed due to weight loss of more than 20% (indicated with asterisks). (F-G) Close up of panels B and C for first 35 days. (H) Survival after TC-1
5 injection. The survival of mice treated with Ad.HPV16-E2E6E7SH was significantly increased compared with mice immunized with SLP and CpG (Log-rank test $p < 0.05$). Three mice immunized with the Ad.HPV16-E2E6E7SH were tumor free at the end of the experiment (at day 92).

10 **Fig. 13. Adenoviral vectors carrying transgenes encoding either HPV Ag or LSE2E6E7SH show increased viral yields on cells capable of repressing transgene expression.** A) Viral yield assay for Ad35 vectors. PER.C6, PER.C6/CymR, and PER.C6/TetR cells were infected by Ad35 vectors carrying GFP-Luc- or HPVAg-encoding transgenes. These transgenes were driven by either CuO- or
15 TetO-containing CMV promoters. Viral yields were determined four days after infection by an Ad35 hexon-specific qPCR-based method. B) Viral yield assay for Ad26 vectors. PER.C6 and PER.C6/TetR cells were infected by Ad26 vectors carrying GFP-Luc, HPVAg, or LSE2E6E7SH-encoding transgenes, which were all driven by a TetO-containing CMV promoter. Viral yields were determined three days
20 after infection by an Ad26 hexon-specific qPCR-based method. For details see Example 6.

**Fig. 14. Employment of a repressor system to repress transgene expression during vector production prevents transgene cassette instability in an adenoviral
25 vector carrying an HPVAg-encoding transgene.** An Ad35 vector expressing HPVAg under the control of CMVCuO was rescued by DNA transfection on either PER.C6 or PER.C6/CymR cell lines. Resultant viral plaques were picked – five per cell line – and used for consecutive infection rounds on the respective cell lines. A) Analysis of the integrity of the vector transgene cassette region by PCR after 10 viral passages.
30 PCR products obtained from viral isolates passaged on PER.C6 and PER.C6/CymR are shown in the middle and right panels, respectively. The full-length-appearing PCR products obtained for PER.C6-passaged viral isolates 1, 2, 4, and 5, and those seen for PER.C6/CymR-passaged isolates 1 to 5 were analyzed by Sanger DNA sequencing. Analysis of the chromatogram traces (not shown) revealed that all isolates grown on

PER.C6, but not those grown on PER.C6/CymR, contained either frameshifting small deletions or premature stop mutations within the coding sequence for HPVAg. B) Analysis of the ability of the vectors to express HPVAg after seven viral passages. A549 cells were transduced by the PER.C6- and PER.C6/CymR-grown viral isolates and HPVAg expression was analyzed by Western Blot using an HPV16 E7-specific antibody. The predicted size for HPVAg is 83 kDa. For details see Example 6.

Detailed description of the invention

The invention provides a nucleic acid molecule encoding a polypeptide comprising SEQ ID NO: 1. The polypeptide is a fusion polypeptide, and is sometimes referred to herein as the polypeptide of the invention, or the fusion polypeptide of the invention. This polypeptide is useful to generate an immune response against the E6 and E7 proteins of HPV16, and thus the nucleic acid molecule can be used as a therapeutic vaccine to prevent persistent HPV16 infection, and diseases associated therewith.

The polypeptide of the invention is a carefully designed molecule that contains virtually the complete E6 and E7 amino acid sequences of HPV16 (it lacks only one amino acid from the C-terminus of the native HPV16 E6 protein) in the form of fragments that are re-ordered and partly overlapping such that (essentially) all T-cell epitopes of the HPV16 E6 and E7 protein are present. Earlier molecules with some potential as HPV vaccines have been described by others (e.g. Kenter *et al.*, 2009, *N Engl J Med* 361: 1838-47; Daayana *et al.*, 2010, *Br J Cancer* 102: 1129-36; Smahel *et al.*, 2001, *Virology* 281: 231-38; Yan *et al.*, 2009, *Vaccine* 27: 431-40; Öhlschläger *et al.*, 2006, *Vaccine* 24: 2880-93; Oosterhuis *et al.*, 2011, *Int J Cancer* 129: 397-406; EP1183368, WO 2013/083287), but each of these molecules has one or more drawbacks. The designer polypeptide molecules of the invention are advantageous in at least one and typically several aspects with respect to the approaches described earlier. In particular, advantages of the molecules and/or vectors of the present invention include: (i) they have a desired safety profile, as the nucleic acid has a strongly reduced (as compared to native E6 and E7 proteins), up to non-detectable, transforming activity; (ii) they are single nucleic acid molecules, which are easy to manufacture at industrial scale in an economically feasible manner, and do not pose logistic challenges unlike multiple molecule approaches; (iii) the encoded polypeptides comprise essentially all T-cell epitopes of the native HPV16 E6 and E7

proteins; (iv) the design of the encoded polypeptides has minimized the introduction of undesired potential strong neo-epitopes (i.e. epitopes not present in the native E6 and E7 proteins); and (v) in certain embodiments, they are not dependent on highly reactogenic adjuvants to raise a desired immune response. Thus, the molecules of the invention represent a major step forward by combining various advantageous characteristics in a single design, and are excellent candidates primarily for therapeutic vaccination against HPV16. These molecules could also possibly work as prophylactic vaccines against HPV16, meaning that they are likely to prevent persistent infection with HPV16 of vaccinated subjects.

In certain embodiments, by careful design the number of neo-epitopes with a length of nine amino acids with a predicted binding affinity <50 nM for the 20 most common HLA-A, 20 most common HLA-B and 20 most common HLA-C alleles was minimized to only 1. This is a significant improvement over constructs described by others, which for a single shuffled E6 protein already contained more than 30 of such neo-epitopes, and which constructs will highly likely comprise even several more neo-epitopes in sequences that were appended to these constructs to prevent loss of epitopes (Öhlschläger *et al.*, 2006, *Vaccine* 24: 2880–93). Hence the constructs of the invention have a significantly improved immunologic profile since chances of an altered immune response as compared to native E6 and E7 have been minimized in the molecules of the invention, as compared to approaches described by others.

Skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the polypeptide sequence encoded by the polynucleotides described to reflect the codon usage of any particular host organism in which the polypeptides are to be expressed. Therefore, unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

In a preferred embodiment, the nucleic acid encoding the polypeptide according to the invention is codon optimized for expression in mammalian cells, preferably human cells. Methods of codon-optimization are known and have been described previously (e.g. WO 96/09378). A sequence is considered codon optimized if at least one non-preferred codon as compared to a wild type sequence is replaced by a codon that is more preferred. Herein, a non-preferred codon is a codon that is used

less frequently in an organism than another codon coding for the same amino acid, and a codon that is more preferred is a codon that is used more frequently in an organism than a non-preferred codon. The frequency of codon usage for a specific organism can be found in codon frequency tables, such as in

5 <http://www.kazusa.or.jp/codon>. Preferably more than one non-preferred codon, e.g. more than 10%, 40%, 60%, 80% of non-preferred codons, preferably most (e.g. at least 90%) or all non-preferred codons, are replaced by codons that are more preferred. Preferably the most frequently used codons in an organism are used in a codon-optimized sequence. Replacement by preferred codons generally leads to
10 higher expression.

Nucleic acid sequences can be cloned using routine molecular biology techniques, or generated de novo by DNA synthesis, which can be performed using routine procedures by service companies having business in the field of DNA synthesis and/or molecular cloning (e.g. GeneArt, GenScripts, Invitrogen, Eurofins).

15 It will be appreciated by a skilled person that changes can be made to a protein, e.g. by amino acid substitutions, deletions, additions, etc, e.g. using routine molecular biology procedures. Generally, conservative amino acid substitutions may be applied without loss of function or immunogenicity of a polypeptide. This can be checked according to routine procedures well known to the skilled person.

20 In certain embodiments, the encoded polypeptide according to the invention further comprises a leader sequence, also referred to as signal sequence or signal peptide. This is a short (typically 5-30 amino acids long) peptide present at the N-terminus of the majority of newly synthesized proteins that are destined towards the secretory pathway. The presence of such a sequence may lead to increased expression
25 and immunogenicity. Non-limiting examples that can be used are an IgE leader peptide (see e.g. US 6,733,994; e.g. having sequence MDWTWILFLVAAATRVHS (SEQ ID NO: 7)) or a HAVT20 leader peptide (e.g. having sequence MACPGFLWALVISTCLEFSMA (SEQ ID NO: 9)). One of these can optionally be added to the N-terminus of a polypeptide of the invention. In other embodiments, a
30 polypeptide according to the invention does not comprise a leader sequence.

Diverse types of HPV exist (over 120 types have been identified and are referred to by number), and generally for each type that needs to be covered by a vaccine, type-specific antigens may need to be incorporated in the vaccine, although for certain antigens some cross-reactivity might exist. Types 16, 18, 31, 33, 35, 39,

45, 51, 52, 56, 58, 59, 68, 73, and 82 are carcinogenic "high-risk" sexually transmitted HPVs and may lead to the development of cervical intraepithelial neoplasia (CIN), vulvar intraepithelial neoplasia (VIN), vaginal intraepithelial neoplasia (VaIN), penile intraepithelial neoplasia (PIN), and/or anal intraepithelial neoplasia (AIN). The HPV according to the invention (i.e. the HPV from which the E6 and E7 fragments in the encoded polypeptide are derived) is HPV16. It can be used for subjects that are infected with HPV16. It may in certain embodiments also suitably be combined with vaccines against other HPV types. In certain embodiments, this combination is with a vaccine against HPV of a high risk type as identified above, e.g. with a vaccine against HPV18. In other embodiments, the vaccine of the invention is combined with a vaccine against one or more of HPV-18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, or -82. Such combinations could for instance be used if the exact type of HPV infection is not yet certain, or if an immune response with a prophylactic effect is desired against more than one HPV type. Also combinations of the vaccines of the invention with vaccines against HPV types that cause genital warts, such as HPV6 and/or HPV11, are envisaged. Sequences of these HPV types and the proteins encoded thereby (e.g. E6, E7, E2) are available to the skilled person in public databases, such as the GenBank sequence database provided by the National Center for of technology Information (NCBI).

The polypeptide according to the invention comprises SEQ ID NO: 1, and in one embodiment the nucleic acid molecule according to the invention comprises SEQ ID NO: 2.

Sequences herein are provided from 5' to 3' direction or from N- to C-terminus, as custom in the art.

The polypeptide according to the invention comprises the epitopes of HPV16 E6 and E7 proteins. In certain embodiments, the polypeptide according to the invention further comprises (and hence the nucleic acid encoding the polypeptide further encodes) at least one further antigen or epitope(s) of such further antigen. Such a further antigen preferably is an HPV antigen, preferably of the same HPV type as the E6 and E7 proteins in the polypeptide, i.e. HPV16. Such a further antigen can thus be an HPV protein or an immunogenic fragment thereof, and in certain embodiments comprises an E2 protein or a fragment thereof comprising at least one epitope of E2 of HPV, preferably from HPV16. Such further antigens or epitopes could be placed internally between two fragments of E6 and/or E7 in the polypeptide comprising SEQ

ID NO: 1, but preferably are fused N-terminally or C-terminally to the E6/E7 polypeptide comprising SEQ ID NO: 1. Alternatively or in addition, amino acid sequences can be present that stimulate the immune response. Thus, in certain embodiments the invention provides nucleic acid molecules according to the

5 invention, encoding a polypeptide comprising SEQ ID NO: 1, and wherein the polypeptide further comprises at least one other antigen, e.g. HPV E2 protein or at least one epitope, but preferably more epitopes, thereof. One advantage of the addition of E2 antigen for the instant invention is that E2 is known to be expressed early during infection/in low grade lesions where E6 and E7 expression is still very low.

10 During the development towards cervical cancer E2 expression is lost and as a result E6 and E7 levels are increased (Yugawa and Kiyono, 2009, *Rev Med Virol* 19: 97–113). Combining epitopes from E2, E6 and E7 in one vaccine allows for treatment in a broad target group of patients, ranging from having persistent infection to invasive cervical cancer (or other HPV16-caused cancers). In certain embodiments, the E2

15 protein is a wild-type E2 protein. In certain other embodiments, the E2 protein has a deletion or one or more mutations in its DNA binding domain (as compared to a wild type E2 protein). The sequence of the HPV16 E2 protein (NP_041328.1) can be found in the NCBI protein database (www.ncbi.nlm.nih.gov/protein) under number NP_041328.1. Several single amino acid changes in E2 such as G293V, K299M, or

20 C300R in the C-terminal part of this protein are known to abrogate DNA binding. An advantage of using a variant or fragment of E2 that lacks DNA binding capacity is that it could prevent unpredictable transcriptional changes via direct binding to host cell DNA in the cells where it is expressed. The E2 protein or part or variant thereof can be added internally, but preferably to the N-terminus or to the C-terminus of the

25 polypeptide of the invention having SEQ ID NO: 1. In one embodiment, the nucleic acid molecule of the invention encodes a polypeptide comprising SEQ ID NO: 3. In one embodiment thereof, the nucleic acid molecule of the invention comprises SEQ ID NO: 4. In another embodiment, the nucleic acid molecule of the invention encodes a polypeptide comprising SEQ ID NO: 5. In one embodiment thereof, the nucleic acid

30 molecule of the invention comprises SEQ ID NO: 6.

It is also possible to make further fusions of the designer polypeptides of the invention with further proteins, e.g. so called carrier proteins, such as Calreticulin, Mycobacterium Tuberculosis heat shock protein-70, IP10, or Tetanus toxin fragment C (see Oosterhuis et al., *Human Gene Ther*, 2012, *supra*, for more examples), which

could further enhance the immune response to the HPV E6 and E7 (and optionally E2) epitopes. The invention thus also provides such further fusion proteins, and nucleic acids encoding such.

In certain embodiments, a nucleic acid molecule according to the invention is
5 incorporated into a vector. A "vector" as used herein, is typically a vehicle to artificially carry foreign genetic material into another cell, where it can be replicated and/or expressed, and according to the invention can be any nucleic acid molecule that incorporates a nucleic acid molecule according to the invention. These can be prepared according to routine molecular biology techniques such as cloning. Typically
10 such vectors can be propagated in at least one type of suitable hosts such as bacteria, yeast, insect cells, mammalian cells, and the like. Four major types of vectors are plasmids, viral vectors, cosmids, and artificial chromosomes. The vector itself is generally a DNA sequence that consists of an insert (transgene; in the present invention the nucleic acid encoding the fusion polypeptide of the invention) and a
15 sequence that serves as the "backbone" of the vector. The purpose of a vector which transfers genetic information to another cell is typically to isolate, multiply, or express the insert in the target cell. Preferably, the sequence encoding the polypeptide is operably linked to a promoter in the vector. The term "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the promoter in a manner
20 that allows for expression of the nucleotide sequence (e.g., in a host cell when the vector is introduced into the host cell). Expression regulatory sequences can be operably linked to a transgene. In certain embodiments, vectors are designed for the expression of the transgene in the target cell, and generally have a promoter sequence that drives expression of the transgene. In certain embodiments, one or more of
25 routinely used vector elements such as transcription terminator sequences, polyadenylation tail sequences, Kozak sequences, UTRs, origin of replication, multiple cloning sites, genetic markers, antibiotic resistance, and further sequences may be present, and the skilled person can design a vector such that it has the desired properties, e.g. for replication in certain cells for propagation and multiplication of the
30 vector, and for expression of the transgene of the vector in target cells into which the vector is introduced. Vectors comprising the nucleic acid encoding the fusion polypeptide according to the invention, preferably designed for expression in mammalian cells, are suitable as vaccines according to the invention. In certain embodiments, a vector according to the invention is a plasmid, a cosmid, a yeast

artificial chromosome, a bacterial artificial chromosome, a viral vector, or the like.

The person skilled in the art is aware that various promoters can be used to obtain expression of a gene in host cells. Some well-known and much used promoters for expression in eukaryotic cells comprise promoters derived from viruses, such as

5 adenovirus, e.g. the E1A promoter, promoters derived from cytomegalovirus (CMV), such as the CMV immediate early (IE) promoter (referred to herein as the CMV promoter) (obtainable for instance from pcDNA, Invitrogen), promoters derived from Simian Virus 40 (SV40) (e.g. obtainable from pIRES, cat.no. 631605, BD Sciences), and the like. Suitable promoters can also be derived from eukaryotic cells, such as
10 methallothionein (MT) promoters, elongation factor 1 α (EF-1 α) promoter, ubiquitin C or UB6 promoter, actin promoter, an immunoglobulin promoter, heat shock promoters, and the like (see e.g. WO 2006/048459). A non-limiting example of a suitable promoter for obtaining expression in eukaryotic cells is a CMV-promoter (US 5,385,839), e.g. the CMV immediate early promoter, for instance comprising nt. -735
15 to +95 from the CMV immediate early gene enhancer/promoter, e.g. a CMV promoter as provided herein with a sequence as set forth in SEQ ID NO: 13. A polyadenylation signal, for example the bovine growth hormone polyA signal (US 5,122,458), may be present behind the transgene(s).

Further regulatory sequences may also be added. The term “regulatory
20 sequence” is used interchangeably with “regulatory element” herein and refers to a segment of nucleic acid, typically but not limited to DNA, that modulate the transcription of the nucleic acid sequence to which it is operatively linked, and thus acts as a transcriptional modulator. A regulatory sequence often comprises nucleic acid sequences that are transcription binding domains that are recognized by the
25 nucleic acid-binding domains of transcriptional proteins and/or transcription factors, enhancers or repressors etc. For example, it is possible to operably couple a repressor sequence to the promoter, which repressor sequence can be bound by a repressor protein that can decrease or prevent the expression of the transgene in a production cell line that expresses this repressor protein. This may improve genetic stability
30 and/or expression levels of the nucleic acid molecule upon passaging and/or when this is produced at high quantities in the production cell line. Such systems have been described in the art. For example, a regulatory sequence could include one or more tetracycline operon operator sequences (tetO), such that expression is inhibited in the

presence of the tetracycline operon repressor protein (tetR). In the absence of tetracycline, the tetR protein is able to bind to the tetO sites and repress transcription of a gene operably linked to the tetO sites. In the presence of tetracycline, however, a conformational change in the tetR protein prevents it from binding to the operator sequences, allowing transcription of operably linked genes to occur. In certain embodiments, a nucleic acid molecule, e.g. when present in a recombinant adenovirus vector, of the present invention can optionally include tetO operatively linked to a promoter, such that expression of one or more transgenes is inhibited in recombinant adenoviruses that are produced in the producer cell line in which tetR protein is expressed. Subsequently, expression would not be inhibited if the recombinant adenovirus is introduced into a subject or into cells that do not express the tetR protein (e.g., international patent application WO 07/ 073513). In certain other embodiments, a nucleic acid molecule of the present invention, e.g. when present in a recombinant adenovirus, can optionally include a cumate gene-switch system, in which regulation of expression is mediated by the binding of the repressor (CymR) to the operator site (CuO), placed downstream of the promoter (e.g., Mullick et al. *BMC Biotechnol.* 2006 6:43). As used herein, the term "repressor," refers to entities (e.g., proteins or other molecules) having the capacity to inhibit, interfere, retard and/or repress the production of heterologous protein product of a recombinant expression vector. For example, by interfering with a binding site at an appropriate location along the expression vector, such as in an expression cassette. Examples of repressors include tetR, CymR, the lac repressor, the trp repressor, the gal repressor, the lambda repressor, and other appropriate repressors known in the art. Examples of the use of the tetO/tetR operator/repressor system and of the CuO/CymR operator/repressor system are provided herein. Repression of vector transgene expression during vector propagation can prevent transgene instability, and may increase yields of vectors having a transgene of the invention during production. Hence, in some embodiments, the vectors of the invention have a promoter that can be repressed by binding of a repressor protein, e.g. by having a promoter that is operably coupled to a repressor operator sequence (e.g. in non-limiting embodiments, a TetO-containing sequence, e.g. the one set forth in SEQ ID NO: 11, or a CuO-containing sequence, e.g. the one set forth in SEQ ID NO: 12), to which a repressor protein (e.g. the TetR protein, e.g. having an amino acid sequence as set forth in SEQ ID NO: 15, or the CymR protein, e.g. having an amino acid sequence as set forth in SEQ ID NO: 17) can bind.

In certain embodiments, the vector is a plasmid DNA molecule, or a fragment thereof. These can be used for DNA vaccination. Other platforms are also possible for use as vectors, for instance live-attenuated double-deleted *Listeria monocytogenes* strains.

5 In other embodiments, the vector is a recombinant viral vector, which may be replication competent or replication deficient. In certain embodiments, a viral vector comprises a recombinant DNA genome. In certain embodiments, a vector according to the invention is for instance a recombinant adenovirus, a recombinant retrovirus, a recombinant pox virus such as a vaccinia virus (e.g. Modified Vaccinia Ankara
10 (MVA)), a recombinant alphavirus such as semliki forest virus, a recombinant paramyxovirus, such as a recombinant measles virus, or another recombinant virus. In certain embodiments, a vector according to the invention is an MVA vector.

In preferred embodiments, a vector according to the invention is a recombinant adenovirus. Advantages of adenoviruses for use as vaccines include ease
15 of manipulation, good manufacturability at large scale, and an excellent safety record based on many years of experience in research, development, manufacturing and clinical trials with numerous adenoviral vectors that have been reported. Adenoviral vectors that are used as vaccines generally provide a good immune response to the transgene-encoded protein, including a cellular immune response. An adenoviral
20 vector according to the invention can be based on any type of adenovirus, and in certain embodiments is a human adenovirus, which can be of any serotype. In other embodiments, it is a simian adenovirus, such as chimpanzee or gorilla adenovirus, which can be of any serotype. In certain embodiments, a vector according to the invention is of a human adenovirus serotype 5, 26 or 35. The preparation of
25 recombinant adenoviral vectors is well known in the art. In certain embodiments, an adenoviral vector according to the invention is deficient in at least one essential gene function of the E1 region, e.g. the E1a region and/or the E1b region, of the adenoviral genome that is required for viral replication. In certain embodiments, an adenoviral vector according to the invention is deficient in at least part of the non-essential E3
30 region. In certain embodiments, the vector is deficient in at least one essential gene function of the E1 region and at least part of the non-essential E3 region.

Adenoviral vectors, methods for construction thereof and methods for propagating thereof, are well known in the art and are described in, for example, U.S. Pat. Nos. 5,559,099, 5,837,511, 5,846,782, 5,851,806, 5,994,106, 5,994,128,

5,965,541, 5,981,225, 6,040,174, 6,020,191, and 6,113,913, and Thomas Shenk, "Adenoviridae and their Replication", M. S. Horwitz, "Adenoviruses", Chapters 67 and 68, respectively, in *Virology*, B. N. Fields *et al.*, eds., 3d ed., Raven Press, Ltd., New York (1996), and other references mentioned herein. Typically, construction of
5 adenoviral vectors involves the use of standard molecular biological techniques, such as those described in, for example, Sambrook *et al.*, *Molecular Cloning, a Laboratory Manual*, 2d ed., Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989), Watson *et al.*, *Recombinant DNA*, 2d ed., Scientific American Books (1992), and Ausubel *et al.*, *Current Protocols in Molecular Biology*, Wiley Interscience Publishers, NY
10 (1995), and other references mentioned herein.

Particularly preferred serotypes for the recombinant adenovirus are human serotype 35 or human serotype 26. Preparation of rAd26 vectors is described, for example, in WO 2007/104792 and in Abbink *et al.*, 2007 *Virology* 81: 4654-63. Exemplary genome sequences of Ad26 are found in GenBank Accession EF 153474
15 and in SEQ ID NO:1 of WO 2007/104792. Preparation of rAd35 vectors is described, for example, in US Patent No. 7,270,811, in WO 00/70071, and in Vogels *et al.*, 2003, *J Virol* 77: 8263-71. Exemplary genome sequences of Ad35 are found in GenBank Accession AC_000019 and in Fig. 6 of WO 00/70071.

In certain embodiments, the adenovirus is replication deficient, e.g. because it
20 contains a deletion in the E1 region of the genome. As known to the skilled person, in case of deletions of essential regions from the adenovirus genome, the functions encoded by these regions have to be provided in trans, preferably by the producer cell, i.e. when parts or whole of E1, E2 and/or E4 regions are deleted from the adenovirus, these have to be present in the producer cell, for instance integrated in the genome
25 thereof, or in the form of so-called helper adenovirus or helper plasmids. The adenovirus may also have a deletion in the E3 region, which is dispensable for replication, and hence such a deletion does not have to be complemented.

A producer cell (sometimes also referred to in the art and herein as 'packaging cell' or 'complementing cell') that can be used can be any producer cell wherein a
30 desired adenovirus can be propagated. For example, the propagation of recombinant adenovirus vectors is done in producer cells that complement deficiencies in the adenovirus. Such producer cells preferably have in their genome at least an adenovirus E1 sequence, and thereby are capable of complementing recombinant adenoviruses with a deletion in the E1 region. Any E1-complementing producer cell

can be used, such as human retina cells immortalized by E1, e.g. 911 or PER.C6 cells (see US patent 5,994,128), E1-transformed amniocytes (See EP patent 1230354), E1-transformed A549 cells (see e.g. WO 98/39411, US patent 5,891,690), GH329:HeLa (Gao *et al.*, 2000, *Hum Gene Ther* 11: 213-19), 293, and the like. In certain
5 embodiments, the producer cells are for instance HEK293 cells, or PER.C6 cells, or 911 cells, or IT293SF cells, and the like. Production of adenoviral vectors in producer cells is reviewed in (Kovesdi *et al.*, 2010, *Viruses* 2: 1681-703).

 In certain embodiments, an E1-deficient adenovirus comprises the E4-orf6 coding sequence of an adenovirus of subgroup C such as Ad5. This allows
10 propagation of such adenoviruses in well known complementing cell lines that express the E1 genes of Ad5, such as for example 293 cells or PER.C6 cells (see, e.g. Havenga *et al.*, 2006, *J Gen Virol* 87: 2135-43; WO 03/104467, incorporated in its entirety by reference herein).

 “Heterologous nucleic acid” (also referred to herein as ‘transgene’) in vectors
15 of the invention is nucleic acid that is not naturally present in the vector, and according to the present invention the nucleic acid encoding the fusion polypeptide of the invention is considered heterologous nucleic acid when present in a vector. It is introduced into the vector for instance by standard molecular biology techniques. It can for instance be cloned into a deleted E1 or E3 region of an adenoviral vector, or in
20 the region between the E4 region and the rITR. A transgene is generally operably linked to expression control sequences. In preferred embodiments, the transgene is cloned into the E1-region of an adenoviral vector.

 Production of vectors such as DNA vectors, or recombinant adenovirus vectors, can be performed according to various methods well known to the person
25 skilled in the art. Generally, the production entails propagation in cultured cells to generate a substantial amount of vector material, followed by harvest of the vector from the cell culture, and typically followed by further purification of the vector to remove other substances and obtain purified vectors that can be formulated into pharmaceutical compositions (e.g., Hoganson *et al.*, 2002, *BioProcessing J* 1: 43-8; Evans *et al.*, 2004, *J Pharm Sci* 93:2458-75). For example, methods for harvesting
30 adenovirus from cultures of producer cells have for instance been extensively described in WO 2005/080556. For example WO 2010/060719, and WO 2011/098592, both incorporated by reference herein, describe suitable methods for obtaining and purifying large amounts of recombinant adenoviruses.

In certain aspects, the invention also provides a polypeptide that is encoded by a nucleic acid molecule according to the invention. Such a polypeptide comprises SEQ ID NO: 1. In certain embodiments, such a polypeptide may comprise SEQ ID NO: 3 or SEQ ID NO: 5. The characteristics of such a polypeptide are as described above. Such a polypeptide can for instance be used directly as a vaccine against HPV.

The invention further provides vaccines comprising nucleic acid molecules, vectors or polypeptides according to the invention, wherein embodiments for each of these aspects can include those as described above. In preferred embodiments, a vaccine according to the invention comprises a nucleic acid molecule according to the invention. In further preferred embodiments, the vaccine comprises a vector according to the invention, preferably a DNA vector, an MVA vector, or a recombinant adenovirus vector.

In certain embodiments, a vaccine according to the invention comprises further active ingredients, e.g. nucleic acid encoding at least one epitope of E6 and/or E7 protein of at least one HPV type different from HPV16, e.g. a high risk HPV type such as HPV18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, or -82.

The term "vaccine" refers to an agent or composition containing an active component effective to induce a prophylactic and/or therapeutic degree of immunity in a subject against a certain pathogen or disease, in this case therapeutically against HPV. The vaccine typically comprises the nucleic acid molecule, or vector, according to the invention, and a pharmaceutically acceptable excipient. Upon administration to a subject, the polypeptide encoded by the nucleic acid molecule according to the invention will be expressed in the subject, which will lead to an immune response towards E6 and/or E7 antigenic fragments that are present in the polypeptide. The advantage of the instant molecules is that essentially all T-cell epitopes of HPV16 E6 and E7 are present and thus a T-cell response to any epitope present in wild-type E6 or E7 can be mounted in the vaccinee. Further, the vaccine has all the safety and efficacy advantages as outlined above for the nucleic acid molecules according to the invention.

For administering to humans, the invention may employ pharmaceutical compositions comprising the vector and a pharmaceutically acceptable carrier or excipient. In the present context, the term "Pharmaceutically acceptable" means that

the carrier or excipient, at the dosages and concentrations employed, will not cause any unwanted or harmful effects in the subjects to which they are administered. Such pharmaceutically acceptable excipients are well known in the art (see Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing

5 Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

An excipient is generally a pharmacologically inactive substance formulated with the active ingredient of a medication. Excipients are commonly used to bulk up

10 formulations that contain potent active ingredients (thus often referred to as "bulking agents," "fillers," or "diluent"), to allow convenient and accurate dispensation of a drug substance when producing a dosage form. They also can serve various therapeutic-enhancing purposes, such as facilitating drug absorption or solubility, or other pharmacokinetic considerations. Excipients can also be useful in the

15 manufacturing process, to aid in the handling of the active substance concerned such as by facilitating powder flowability or non-stick properties, in addition to aiding in vitro stability such as prevention of denaturation over the expected shelf life. The selection of appropriate excipients also depends upon the route of administration and the dosage form, as well as the active ingredient and other factors.

20 The purified nucleic acid molecule, vector or polypeptide preferably is formulated and administered as a sterile solution although it is also possible to utilize lyophilized preparations. Sterile solutions are prepared by sterile filtration or by other methods known per se in the art. The solutions are then lyophilized or filled into pharmaceutical dosage containers. The pH of the solution generally is in the range of

25 pH 3.0 to 9.5, e.g pH 5.0 to 7.5. The nucleic acid molecule or vector or polypeptide typically is in a solution having a suitable buffer, and the solution of vector may also contain a salt. Optionally stabilizing agent may be present, such as albumin. In certain embodiments, detergent is added. In certain embodiments, vaccine may be formulated into an injectable preparation. These formulations contain effective amounts of

30 nucleic acid molecule, vector or polypeptide are either sterile liquid solutions, liquid suspensions or lyophilized versions and optionally contain stabilizers or excipients.

For instance recombinant adenovirus vector may be stored in the buffer that is also used for the Adenovirus World Standard (Hoganson *et al.*, 2002, *Bioprocessing J* 1: 43-8): 20 mM Tris pH 8, 25 mM NaCl, 2.5% glycerol. Another useful

formulation buffer suitable for administration to humans is 20 mM Tris, 2 mM MgCl₂, 25 mM NaCl, sucrose 10% w/v, polysorbate-80 0.02% w/v. Another formulation buffer that is suitable for recombinant adenovirus comprises 10-25 mM citrate buffer pH 5.9-6.2, 4-6% (w/w) hydroxypropyl-beta-cyclodextrin (HBCD), 70-
5 100 mM NaCl, 0.018-0.035% (w/w) polysorbate-80, and optionally 0.3-0.45% (w/w) ethanol. Obviously, many other buffers can be used, and several examples of suitable formulations for the storage and for pharmaceutical administration of purified vectors are known.

In certain embodiments a composition comprising the vector further comprises
10 one or more adjuvants. Adjuvants are known in the art to further increase the immune response to an applied antigenic determinant. The terms "adjuvant" and "immune stimulant" are used interchangeably herein, and are defined as one or more substances that cause stimulation of the immune system. In this context, an adjuvant is used to enhance an immune response to the polypeptides encoded by the nucleic acid
15 molecules in the vectors of the invention. Examples of suitable adjuvants include aluminium salts such as aluminium hydroxide and/or aluminium phosphate and/or aluminium potassium phosphate; oil-emulsion compositions (or oil-in-water compositions), including squalene-water emulsions, such as MF59 (see e.g. WO 90/14837); saponin formulations, such as for example QS21 and Immunostimulating
20 Complexes (ISCOMS) (see e.g. US 5,057,540; WO 90/03184, WO 96/11711, WO 2004/004762, WO 2005/002620); bacterial or microbial derivatives, examples of which are monophosphoryl lipid A (MPL), 3-O-deacylated MPL (3dMPL), CpG-motif containing oligonucleotides, ADP-ribosylating bacterial toxins or mutants thereof, such as *E. coli* heat labile enterotoxin LT, cholera toxin CT, and the like. It is
25 also possible to use vector-encoded adjuvant, e.g. by using heterologous nucleic acid that encodes a fusion of the oligomerization domain of C4-binding protein (C4bp) to the antigen of interest (e.g. Solabomi *et al.*, 2008, *Infect Immun* 76: 3817-23), or by using a vector encoding both the transgene of interest and a TLR-3 agonist such as heterologous dsRNA (e.g. WO 2007/100908), or the like.

30 In other embodiments, the compositions of the invention do not comprise adjuvants.

Pharmaceutical compositions may be administered to a subject, e.g. a human subject. The total dose of the vaccine active component provided to a subject during one administration can be varied as is known to the skilled practitioner, and for

adenovirus is generally between 1×10^7 viral particles (vp) and 1×10^{12} vp, preferably between 1×10^8 vp and 1×10^{11} vp, for instance between 3×10^8 and 5×10^{10} vp, for instance between 10^9 and 3×10^{10} vp. For a DNA vaccine, total amounts of DNA per administration may for instance be between 1 μ g and 10 mg. If a gene gun is used for administration, typically low amounts are used, e.g. 10 μ g. For intramuscular injection, typically higher amounts are used, e.g. up to 5 mg.

Administration of pharmaceutical compositions can be performed using standard routes of administration. Non-limiting embodiments include parenteral administration, such as by injection, e.g. intradermal, intramuscular, etc, or subcutaneous or transcutaneous, or mucosal administration, e.g. intranasal, oral, intravaginal, rectal, and the like. In one embodiment a composition is administered by intramuscular injection, e.g. into the deltoid muscle of the arm, or vastus lateralis muscle of the thigh. In certain embodiments the vaccine is a DNA vaccine, and this can for instance be administered intradermally, e.g. by DNA tattooing (see, e.g. Oosterhuis *et al.*, 2012, *Curr Top Microbiol Immunol* 351: 221-50). This route is also feasible for adenoviral vectors. In certain embodiments a composition according to the invention comprises an adenoviral vector and is administered by intramuscular injection. The skilled person knows the various possibilities to administer a composition, such as a vaccine in order to induce an immune response to the antigen(s) in the vaccine.

A subject as used herein preferably is a mammal, for instance a rodent, e.g. a mouse, or a non-human-primate, or a human. Preferably, the subject is a human subject.

The vaccines of the invention can be used to treat patients having one of various stages of diseases caused by HPV (in particular type 16), from incident and persistent HPV infection as such (e.g. as detected by HPV DNA testing), thus before (pre-)cancerous lesions are formed, as well as cervical intraepithelial neoplasia (CIN; also known as cervical dysplasia and cervical interstitial neoplasia, which is the potentially premalignant transformation and abnormal growth (dysplasia) of squamous cells on the surface of the cervix) up to and including cervical cancer (such as cervical squamous cell carcinoma (SCC)). In addition, other HPV-induced neoplasias, such as vulvar intraepithelial neoplasia (VIN), vaginal intraepithelial neoplasia (VaIN), penile intraepithelial neoplasia (PIN), anal intraepithelial neoplasia (AIN) can be targeted as well as more advanced stages of oropharyngeal cancer (also known as head- and neck cancer), penile cancer, vaginal cancer, vulvar cancer and

anal cancer. The vaccines of the invention thus can target a wide range of HPV induced lesions, and are likely most effective at the precancerous stages of HPV-induced disease, e.g. at the (persistent) infection and/or the neoplasia stages, where expression of E2, E6 and/or E7 is highest. It is also possible to combine the treatment using a vaccine of the invention with compounds that counteract or can overcome immune escape mechanisms in advanced cancer cells e.g. anti-PD1/PD-L1 antibodies, anti CTLA-4 antibodies such as Ipilimumab, anti-LAG-3 antibodies, anti-CD25 antibodies, IDO-inhibitors, CD40 agonistic antibodies, CD137 agonistic antibodies, etc (see, e.g. Hamid and Carvajal, 2013, *Expert Opinion Biol Ther* 13: 847-861; Mellman *et al.*, 2011, *Nature Rev* 480: 480-89). The therapeutic vaccination method could in principle also be used for treating external genital warts or precursors thereof in case the vaccine comprises further (sequences encoding) E6 and/or E7 of an HPV type causing external genital warts and is administered to a subject infected by such an HPV type.

As used herein, 'treating' means administration of the vaccine to induce a therapeutic immune response against cells that express (epitopes of) HPV16 E6 and/or E7 in the patient, which leads to at least reduction of the level of and preferably complete removal of HPV16 infection, which results in at least slowing and preferably stopping the progress of HPV16-caused disease such as neoplasias and/or symptoms thereof. Preferably treatment with the vaccine results also in remission of more advanced stages of HPV-induced cancers. It is preferred to administer the vaccine to patients that have an established HPV infection that has been typed, so that the vaccine that encodes the polypeptide of the corresponding HPV type can be administered. In the absence of screening the vaccine can also be administered in the part of the population that is likely to be HPV infected, i.e. sexually active people. It is also possible to administer a vaccine of the invention to subjects that have not been infected by HPV16, e.g. for prophylactic use, possibly in combination with a vaccine against another HPV type by which the patient has been infected, or alternatively in non-infected subjects. A vaccine of the invention can also be administered to a subject that is subject to further treatment by other means, e.g. surgery (removal of a lesion caused by HPV16 infection), or treatment with imiquimod (comprising a TLR-7/8 agonist, see e.g. Dayaana *et al.*, 2010, *Br J Cancer* 102: 1129 – 36). The effect of the treatment can be measured either by cytology or by HPV testing.

The vaccination comprises administering the vaccine of the invention to a subject or patient at least once. It is also possible to provide one or more booster administrations of one or more further vaccines. If a boosting vaccination is performed, typically, such a boosting vaccination will be administered to the same subject at a moment
5 between one week and one year, preferably between two weeks and four months, after administering an immunogenic composition with the same antigen to the subject for the first time (which is in such cases referred to as 'priming vaccination'). In alternative boosting regimens, it is also possible to administer different vectors, e.g. one or more adenoviruses of different serotype, or other vectors such as MVA, or
10 DNA, or protein, to the subject as a priming or boosting vaccination. In certain embodiments, the same form of a vaccine of the invention is administered at least twice to the same patient in a prime-boost regimen, e.g. with the same recombinant adenovirus (such as Ad26) according to the invention. In certain embodiments, a vaccine of the invention is administered at least twice in a prime-boost regimen, but
15 the vector of the vaccine is different, e.g. two different serotypes of adenoviral vectors are used, e.g. priming with recombinant Ad26 and boosting with recombinant Ad35, or vice versa; or priming with DNA and boosting with an adenoviral vector, or vice versa; or priming with an adenoviral vector and boosting with an MVA vector, or vice versa. In certain embodiments, a vaccine according to the invention is administered at
20 least three times, in a prime-boost-boost regimen. Further booster administrations might be added to the regimen.

It is also an aspect of the invention to induce a CTL response against HPV16 in a subject, comprising administering a vaccine according to the invention to the subject.

25 The invention provides also the following non-limiting embodiments:

- 1) a nucleic acid encoding a polypeptide comprising SEQ ID NO: 1;
- 2) a nucleic acid according to embodiment 1, wherein the polypeptide further comprises at least part of HPV E2 protein;
- 3) a nucleic acid according to embodiment 2, wherein the at least part of the HPV E2
30 protein is from the E2 protein of HPV16;
- 4) a nucleic acid according to embodiment 2, wherein the polypeptide comprises at least part of the E2 protein fused to the N-terminal side of the polypeptide with SEQ ID NO: 1;
- 5) a nucleic acid according to embodiment 2, wherein the polypeptide comprises at

least part of the E2 protein fused to the C-terminal side of the polypeptide with SEQ ID NO: 1;

6) a nucleic acid according to embodiment 3, wherein the polypeptide comprises at least part of the E2 protein fused to the N-terminal side of the polypeptide with SEQ

5 ID NO: 1;

7) a nucleic acid according to embodiment 3, wherein the polypeptide comprises at least part of the E2 protein fused to the C-terminal side of the polypeptide with SEQ ID NO: 1;

8) a nucleic acid according to embodiment 2, wherein the at least part of the E2
10 protein comprises a variant of the E2 protein with a mutation that abrogates DNA binding of E2;

9) a nucleic acid according to embodiment 3, wherein the at least part of the E2 protein comprises a variant of the E2 protein with a mutation that abrogates DNA binding of E2;

10) a nucleic acid according to embodiment 4, wherein the at least part of the E2
15 protein comprises a variant of the E2 protein with a mutation that abrogates DNA binding of E2;

11) a nucleic acid according to embodiment 5, wherein the at least part of the E2
20 protein comprises a variant of the E2 protein with a mutation that abrogates DNA binding of E2;

12) a nucleic acid according to embodiment 6, wherein the at least part of the E2 protein comprises a variant of the E2 protein with a mutation that abrogates DNA binding of E2;

13) a nucleic acid according to embodiment 7, wherein the at least part of the E2
25 protein comprises a variant of the E2 protein with a mutation that abrogates DNA binding of E2;

14) a vector comprising a nucleic acid according to embodiment 1, wherein a sequence encoding the polypeptide is operably linked to a promoter;

15) a vector comprising a nucleic acid according to embodiment 2, wherein a
30 sequence encoding the polypeptide is operably linked to a promoter;

16) a vector comprising a nucleic acid according to embodiment 3, wherein a sequence encoding the polypeptide is operably linked to a promoter;

17) a vector comprising a nucleic acid according to embodiment 4, wherein a sequence encoding the polypeptide is operably linked to a promoter;

- 18) a vector comprising a nucleic acid according to embodiment 5, wherein a sequence encoding the polypeptide is operably linked to a promoter;
- 19) a vector comprising a nucleic acid according to embodiment 6, wherein a sequence encoding the polypeptide is operably linked to a promoter;
- 5 20) a vector comprising a nucleic acid according to embodiment 7, wherein a sequence encoding the polypeptide is operably linked to a promoter;
- 21) a vector comprising a nucleic acid according to embodiment 8, wherein a sequence encoding the polypeptide is operably linked to a promoter;
- 22) a vector comprising a nucleic acid according to embodiment 9, wherein a
10 sequence encoding the polypeptide is operably linked to a promoter;
- 23) a vector comprising a nucleic acid according to embodiment 10, wherein a sequence encoding the polypeptide is operably linked to a promoter;
- 24) a vector comprising a nucleic acid according to embodiment 11, wherein a sequence encoding the polypeptide is operably linked to a promoter;
- 15 25) a vector comprising a nucleic acid according to embodiment 12, wherein a sequence encoding the polypeptide is operably linked to a promoter;
- 26) a vector comprising a nucleic acid according to embodiment 13, wherein a sequence encoding the polypeptide is operably linked to a promoter;
- 27) a vector according to embodiment 14, wherein the vector is an adenovirus;
- 20 28) a vector according to embodiment 15, wherein the vector is an adenovirus;
- 29) a vector according to embodiment 16, wherein the vector is an adenovirus;
- 30) a vector according to embodiment 17, wherein the vector is an adenovirus;
- 31) a vector according to embodiment 18, wherein the vector is an adenovirus;
- 32) a vector according to embodiment 19, wherein the vector is an adenovirus;
- 25 33) a vector according to embodiment 20, wherein the vector is an adenovirus;
- 34) a vector according to embodiment 21, wherein the vector is an adenovirus;
- 35) a vector according to embodiment 22, wherein the vector is an adenovirus;
- 36) a vector according to embodiment 23, wherein the vector is an adenovirus;
- 37) a vector according to embodiment 24, wherein the vector is an adenovirus;
- 30 38) a vector according to embodiment 25, wherein the vector is an adenovirus;
- 39) a vector according to embodiment 26, wherein the vector is an adenovirus;
- 40) a vector according to embodiment 27, wherein the adenovirus is a human adenovirus of serotype 26;
- 41) a vector according to embodiment 28, wherein the adenovirus is a human

adenovirus of serotype 26;

42) a vector according to embodiment 29, wherein the adenovirus is a human adenovirus of serotype 26;

43) a vector according to embodiment 30, wherein the adenovirus is a human
5 adenovirus of serotype 26;

44) a vector according to embodiment 31, wherein the adenovirus is a human adenovirus of serotype 26;

45) a vector according to embodiment 32, wherein the adenovirus is a human adenovirus of serotype 26;

10 46) a vector according to embodiment 33, wherein the adenovirus is a human adenovirus of serotype 26;

47) a vector according to embodiment 34, wherein the adenovirus is a human adenovirus of serotype 26;

48) a vector according to embodiment 35, wherein the adenovirus is a human
15 adenovirus of serotype 26;

49) a vector according to embodiment 36, wherein the adenovirus is a human adenovirus of serotype 26;

50) a vector according to embodiment 37, wherein the adenovirus is a human adenovirus of serotype 26;

20 51) a vector according to embodiment 38, wherein the adenovirus is a human adenovirus of serotype 26;

52) a vector according to embodiment 39, wherein the adenovirus is a human adenovirus of serotype 26;

53) a vector according to embodiment 28, wherein the adenovirus is a human
25 adenovirus of serotype 35;

54) a vector according to embodiment 29, wherein the adenovirus is a human adenovirus of serotype 35;

55) a vector according to embodiment 30, wherein the adenovirus is a human adenovirus of serotype 35;

30 56) a vector according to embodiment 31, wherein the adenovirus is a human adenovirus of serotype 35;

57) a vector according to embodiment 32, wherein the adenovirus is a human adenovirus of serotype 35;

58) a vector according to embodiment 33, wherein the adenovirus is a human

- adenovirus of serotype 35;
- 59) a vector according to embodiment 34, wherein the adenovirus is a human adenovirus of serotype 35;
- 60) a vector according to embodiment 35, wherein the adenovirus is a human
- 5 adenovirus of serotype 35;
- 61) a vector according to embodiment 36, wherein the adenovirus is a human adenovirus of serotype 35;
- 62) a vector according to embodiment 37, wherein the adenovirus is a human adenovirus of serotype 35;
- 10 63) a vector according to embodiment 38, wherein the adenovirus is a human adenovirus of serotype 35;
- 64) a vector according to embodiment 39, wherein the adenovirus is a human adenovirus of serotype 35;
- 65) a vaccine composition comprising a vector according to embodiment 14, and a
- 15 pharmaceutically acceptable excipient;
- 66) a vaccine composition comprising a vector according to embodiment 15, and a pharmaceutically acceptable excipient;
- 67) a vaccine composition comprising a vector according to embodiment 16, and a pharmaceutically acceptable excipient;
- 20 68) a vaccine composition comprising a vector according to embodiment 17, and a pharmaceutically acceptable excipient;
- 69) a vaccine composition comprising a vector according to embodiment 18, and a pharmaceutically acceptable excipient;
- 70) a vaccine composition comprising a vector according to embodiment 19, and a
- 25 pharmaceutically acceptable excipient;
- 71) a vaccine composition comprising a vector according to embodiment 20, and a pharmaceutically acceptable excipient;
- 72) a vaccine composition comprising a vector according to embodiment 21, and a pharmaceutically acceptable excipient;
- 30 73) a vaccine composition comprising a vector according to embodiment 22, and a pharmaceutically acceptable excipient;
- 74) a vaccine composition comprising a vector according to embodiment 23, and a pharmaceutically acceptable excipient;

- 75) a vaccine composition comprising a vector according to embodiment 24, and a pharmaceutically acceptable excipient;
- 76) a vaccine composition comprising a vector according to embodiment 25, and a pharmaceutically acceptable excipient;
- 5 77) a vaccine composition comprising a vector according to embodiment 26, and a pharmaceutically acceptable excipient;
- 78) a vaccine composition comprising a vector according to embodiment 27, and a pharmaceutically acceptable excipient;
- 79) a vaccine composition comprising a vector according to embodiment 28, and a pharmaceutically acceptable excipient;
- 10 80) a vaccine composition comprising a vector according to embodiment 29, and a pharmaceutically acceptable excipient;
- 81) a vaccine composition comprising a vector according to embodiment 30, and a pharmaceutically acceptable excipient;
- 15 82) a vaccine composition comprising a vector according to embodiment 31, and a pharmaceutically acceptable excipient;
- 83) a vaccine composition comprising a vector according to embodiment 32, and a pharmaceutically acceptable excipient;
- 84) a vaccine composition comprising a vector according to embodiment 33, and a pharmaceutically acceptable excipient;
- 20 85) a vaccine composition comprising a vector according to embodiment 34, and a pharmaceutically acceptable excipient;
- 86) a vaccine composition comprising a vector according to embodiment 35, and a pharmaceutically acceptable excipient;
- 25 87) a vaccine composition comprising a vector according to embodiment 36, and a pharmaceutically acceptable excipient;
- 88) a vaccine composition comprising a vector according to embodiment 37, and a pharmaceutically acceptable excipient;
- 89) a vaccine composition comprising a vector according to embodiment 38, and a pharmaceutically acceptable excipient;
- 30 90) a vaccine composition comprising a vector according to embodiment 39, and a pharmaceutically acceptable excipient;
- 91) a vaccine composition comprising a vector according to embodiment 40, and a pharmaceutically acceptable excipient;

- 92) a vaccine composition comprising a vector according to embodiment 41, and a pharmaceutically acceptable excipient;
- 93) a vaccine composition comprising a vector according to embodiment 42, and a pharmaceutically acceptable excipient;
- 5 94) a vaccine composition comprising a vector according to embodiment 43, and a pharmaceutically acceptable excipient;
- 95) a vaccine composition comprising a vector according to embodiment 44, and a pharmaceutically acceptable excipient;
- 96) a vaccine composition comprising a vector according to embodiment 45, and a pharmaceutically acceptable excipient;
- 10 97) a vaccine composition comprising a vector according to embodiment 46, and a pharmaceutically acceptable excipient;
- 98) a vaccine composition comprising a vector according to embodiment 47, and a pharmaceutically acceptable excipient;
- 15 99) a vaccine composition comprising a vector according to embodiment 48, and a pharmaceutically acceptable excipient;
- 100) a vaccine composition comprising a vector according to embodiment 49, and a pharmaceutically acceptable excipient;
- 101) a vaccine composition comprising a vector according to embodiment 50, and a pharmaceutically acceptable excipient;
- 20 102) a vaccine composition comprising a vector according to embodiment 51, and a pharmaceutically acceptable excipient;
- 103) a vaccine composition comprising a vector according to embodiment 52, and a pharmaceutically acceptable excipient;
- 25 104) a vaccine composition comprising a vector according to embodiment 53, and a pharmaceutically acceptable excipient;
- 105) a vaccine composition comprising a vector according to embodiment 54, and a pharmaceutically acceptable excipient;
- 106) a vaccine composition comprising a vector according to embodiment 55, and a pharmaceutically acceptable excipient;
- 30 107) a vaccine composition comprising a vector according to embodiment 56, and a pharmaceutically acceptable excipient;
- 108) a vaccine composition comprising a vector according to embodiment 57, and a pharmaceutically acceptable excipient;

- 109) a vaccine composition comprising a vector according to embodiment 58, and a pharmaceutically acceptable excipient;
- 110) a vaccine composition comprising a vector according to embodiment 59, and a pharmaceutically acceptable excipient;
- 5 111) a vaccine composition comprising a vector according to embodiment 60, and a pharmaceutically acceptable excipient;
- 112) a vaccine composition comprising a vector according to embodiment 61, and a pharmaceutically acceptable excipient;
- 113) a vaccine composition comprising a vector according to embodiment 62, and a
10 pharmaceutically acceptable excipient;
- 114) a vaccine composition comprising a vector according to embodiment 63, and a pharmaceutically acceptable excipient;
- 115) a vaccine composition comprising a vector according to embodiment 64, and a pharmaceutically acceptable excipient;
- 15 116) a method for inducing an immune response against HPV in a subject, comprising administering to the subject a vaccine composition according to any one of embodiments 65-115;
- 117) a method for treating persistent HPV (type 16) infection, comprising administering a vaccine according to any one of embodiments 65-115 to a subject that
20 suffers from persistent HPV infection;
- 118) a method for treating vulvar intraepithelial neoplasia (VIN) (with underlying HPV type 16 infection), comprising administering a vaccine according to any one of embodiments 65-115 to a subject that suffers from VIN;
- 119) a method for treating vulvar cancer (with underlying HPV type 16 infection),
25 comprising administering a vaccine according to any one of embodiments 65-115 to a subject that suffers from vulvar cancer;
- 120) a method for treating cervical intraepithelial neoplasia (CIN) (with underlying HPV type 16 infection), comprising administering a vaccine according to any one of embodiments 65-115 to a subject that suffers from CIN;
- 30 121) a method for treating cervical cancer (with underlying HPV type 16 infection), comprising administering a vaccine according to any one of embodiments 65-115 to a subject that suffers from cervical cancer;

- 122) a method for treating oropharyngeal cancer (with underlying HPV type 16 infection), comprising administering a vaccine according to any one of embodiments 65-115 to a subject that suffers from oropharyngeal cancer;
- 123) a method for treating penile intraepithelial neoplasia (PIN) (with underlying HPV type 16 infection), comprising administering a vaccine according to any one of
5 embodiments 65-115 to a subject that suffers from PIN;
- 124) a method for treating penile cancer (with underlying HPV type 16 infection), comprising administering a vaccine according to any one of embodiments 65-115 to a subject that suffers from penile cancer;
- 10 125) a method for treating vaginal intraepithelial neoplasia (VaIN) (with underlying HPV type 16 infection), comprising administering a vaccine according to any one of embodiments 65-115 to a subject that suffers from VaIN;
- 126) a method for treating vaginal cancer (with underlying HPV type 16 infection), comprising administering a vaccine according to any one of embodiments 65-115 to a
15 subject that suffers from vaginal cancer;
- 127) a method for treating anal intraepithelial neoplasia (AIN) (with underlying HPV type 16 infection), comprising administering a vaccine according to any one of embodiments 65-115 to a subject that suffers from AIN;
- 128) a method for treating anal cancer (with underlying HPV type 16 infection),
20 comprising administering a vaccine according to any one of embodiments 65-115 to a subject that suffers from anal cancer;
- 129) a polypeptide comprising SEQ ID NO: 1;
- 130) a polypeptide according to embodiment 129, wherein the polypeptide further comprises at least part of HPV E2 protein;
- 25 131) a polypeptide according to embodiment 130, wherein the at least part of the HPV E2 protein is from the E2 protein of HPV16;
- 132) a polypeptide according to embodiment 130, wherein at least part of the E2 protein is fused to the N-terminal side of the polypeptide with SEQ ID NO: 1;
- 133) a polypeptide according to embodiment 130, wherein at least part of the E2
30 protein is fused to the C-terminal side of the polypeptide with SEQ ID NO: 1;
- 134) a polypeptide according to embodiment 131, wherein at least part of the E2 protein is fused to the N-terminal side of the polypeptide with SEQ ID NO: 1;
- 135) a polypeptide according to embodiment 131, wherein at least part of the E2 protein is fused to the C-terminal side of the polypeptide with SEQ ID NO: 1;

- 136) a polypeptide according to embodiment 130, wherein the at least part of the E2 protein comprises a variant of the E2 protein with a mutation that abrogates DNA binding of E2;
- 137) a polypeptide according to embodiment 131, wherein the at least part of the E2 protein comprises a variant of the E2 protein with a mutation that abrogates DNA binding of E2;
- 138) a polypeptide according to embodiment 132, wherein the at least part of the E2 protein comprises a variant of the E2 protein with a mutation that abrogates DNA binding of E2;
- 139) a polypeptide according to embodiment 133, wherein the at least part of the E2 protein comprises a variant of the E2 protein with a mutation that abrogates DNA binding of E2;
- 140) a polypeptide according to embodiment 134, wherein the at least part of the E2 protein comprises a variant of the E2 protein with a mutation that abrogates DNA binding of E2;
- 141) a polypeptide according to embodiment 135, wherein the at least part of the E2 protein comprises a variant of the E2 protein with a mutation that abrogates DNA binding of E2;
- 142) a nucleic acid according to embodiment 3, encoding a polypeptide according to SEQ ID NO: 3;
- 143) a nucleic acid according to embodiment 3, encoding a polypeptide according to SEQ ID NO: 5;
- 144) a vector encoding a nucleic acid according to embodiment 142, wherein a sequence encoding the polypeptide is operably linked to a promoter;
- 145) a vector encoding a nucleic acid according to embodiment 143, wherein a sequence encoding the polypeptide is operably linked to a promoter;
- 146) a vector according to embodiment 144, wherein the vector is an adenovirus;
- 147) a vector according to embodiment 145, wherein the vector is an adenovirus;
- 148) a vector according to embodiment 146, wherein the adenovirus is a human adenovirus of serotype 26;
- 149) a vector according to embodiment 147, wherein the adenovirus is a human adenovirus of serotype 26;
- 150) a vector according to embodiment 146, wherein the adenovirus is a human adenovirus of serotype 35;

- 151) a vector according to embodiment 147, wherein the adenovirus is a human adenovirus of serotype 35;
- 152) a vaccine composition comprising a vector according to embodiment 144, and a pharmaceutically acceptable excipient;
- 5 153) a vaccine composition comprising a vector according to embodiment 145, and a pharmaceutically acceptable excipient;
- 154) a vaccine composition comprising a vector according to embodiment 146, and a pharmaceutically acceptable excipient;
- 155) a vaccine composition comprising a vector according to embodiment 147, and a pharmaceutically acceptable excipient;
- 10 156) a vaccine composition comprising a vector according to embodiment 148, and a pharmaceutically acceptable excipient;
- 157) a vaccine composition comprising a vector according to embodiment 149, and a pharmaceutically acceptable excipient;
- 15 158) a vaccine composition comprising a vector according to embodiment 150, and a pharmaceutically acceptable excipient;
- 159) a vaccine composition comprising a vector according to embodiment 151, and a pharmaceutically acceptable excipient;
- 160) a method for inducing an immune response against HPV in a subject, comprising administering to the subject a vaccine composition according to any one of embodiments 152-159;
- 20 161) a method for treating vulvar intraepithelial neoplasia (VIN), comprising administering a vaccine according to any one of embodiments 152-159 to a subject that suffers from VIN;
- 25 162) a method for treating vulvar cancer, comprising administering a vaccine according to any one of embodiments 152-159 to a subject that suffers from vulvar cancer;
- 163) a method for treating cervical intraepithelial neoplasia (CIN), comprising administering a vaccine according to any one of embodiments 152-159 to a subject that suffers from CIN;
- 30 164) a method for treating cervical cancer, comprising administering a vaccine according to any one of embodiments 152-159 to a subject that suffers from cervical cancer;

165) a method for treating oropharyngeal cancer, comprising administering a vaccine according to any one of embodiments 152-159 to a subject that suffers from oropharyngeal cancer;

166) a method for treating penile intraepithelial neoplasia (PIN), comprising
5 administering a vaccine according to any one of embodiments 152-159 to a subject that suffers from PIN;

167) a method for treating penile cancer, comprising administering a vaccine according to any one of embodiments 152-159 to a subject that suffers from penile cancer;

10 168) a method for treating vaginal intraepithelial neoplasia (VaIN), comprising administering a vaccine according to any one of embodiments 152-159 to a subject that suffers from VaIN;

169) a method for treating vaginal cancer, comprising administering a vaccine according to any one of embodiments 152-159 to a subject that suffers from vaginal
15 cancer;

170) a method for treating anal intraepithelial neoplasia (AIN), comprising administering a vaccine according to any one of embodiments 152-159 to a subject that suffers from AIN;

171) a method for treating anal cancer, comprising administering a vaccine according
20 to any one of embodiments 152-159 to a subject that suffers from anal cancer.

The practice of this invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, cell biology, and recombinant DNA, which are within the skill of the art. See e.g.
25 Sambrook, Fritsch and Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd edition, 1989; *Current Protocols in Molecular Biology*, Ausubel FM, et al., eds, 1987; the series *Methods in Enzymology* (Academic Press, Inc.); *PCR2: A Practical Approach*, MacPherson MJ, Hams BD, Taylor GR, eds, 1995; *Antibodies: A Laboratory Manual*, Harlow and Lane, eds, 1988.

30

The invention is further explained in the following examples. The examples do not limit the invention in any way. They merely serve to clarify the invention.

EXAMPLES

Example 1: construction of a designer polypeptide comprising essentially all HPV16 E6 and E7 CTL epitopes

5 We designed a novel, non-tumorigenic polypeptide (and nucleic acid encoding such) that contains essentially all CTL epitopes of HPV16 E6 and E7 proteins, and has a minimum number of anticipated/predicted strong neo-epitopes (neo-epitopes meaning epitopes not present in the wild type HPV16 E6 and E7 proteins). The polypeptide of the invention (also sometimes referred to as 'E6E7SH' herein)
10 comprises a sequence as provided in SEQ ID NO: 1. A codon-optimized nucleic acid encoding this polypeptide is provided in SEQ ID NO: 2.

 The molecules of the invention are single molecules, which provides manufacturing advantages over strategies where multiple molecules are used. In
15 addition, the polypeptide of the invention comprises essentially all putative CTL epitopes that are present in wild-type E6 and E7 of HPV16, and at the same time have a minimum number of anticipated/predicted strong neo-epitopes that could potentially be immunodominant and thus divert the immune response from relevant wild-type CTL epitopes. Thus the constructs of the present invention are immunologically more
20 favourable than molecules described by others that either lack possible CTL epitopes and/or that contain more or stronger neo-epitopes.

 For instance, the construct of SEQ ID NO: 1 contains only one neo-epitope with a length of nine amino acids with a predicted binding affinity <50 nM for the 20
25 most common HLA-A, 20 most common HLA-B and 20 most common HLA-C alleles (HLA-A*01:01, HLA-A*02:01, HLA-A*02:03, HLA-A*02:06, HLA-A*02:07, HLA-A*03:01, HLA-A*11:01, HLA-A*23:01, HLA-A*24:02, HLA-A*26:01, HLA-A*29:02, HLA-A*30:01, HLA-A*30:02, HLA-A*31:01, HLA-A*32:01, HLA-A*33:01, HLA-A*33:03, HLA-A*34:01, HLA-A*68:01, HLA-A*68:02, HLA-B*07:02, HLA-B*07:04, HLA-B*08:01, HLA-B*13:01, HLA-B*15:01, HLA-B*18:01, HLA-B*35:01, HLA-B*37:01, HLA-B*39:01, HLA-B*40:01, HLA-B*40:02, HLA-B*40:06, HLA-B*44:02, HLA-B*44:03, HLA-B*46:01, HLA-B*48:01, HLA-B*51:01, HLA-B*52:01, HLA-B*53:01, HLA-B*58:01, HLA-C*07:02, HLA-C*04:01, HLA-C*03:04, HLA-C*01:02, HLA-

C*07:01, HLA-C*06:02, HLA-C*03:03, HLA-C*08:01, HLA-C*15:02, HLA-C*12:02, HLA-C*02:02, HLA-C*05:01, HLA-C*14:02, HLA-C*03:02, HLA-C*16:01, HLA-C*08:02, HLA-C*12:03, HLA-C*04:03, HLA-C*17:01, HLA-C*14:03), as determined using the ANN (Lundegaard *et al.*, 2008, *Nucl Acids Res* 36: W509-12) and SMM method (Peters *et al.*, 2003, *Bioinformatics* 19: 1765-72) for HLA-A and HLA-B and the NetMHCpan method (Hoof *et al.*, 2009, *Immunogenetics* 61: 1-13) for HLA-C of the prediction tool for 'Peptide binding to MHC class I molecules' at the IEDB website (http://tools.immuneepitope.org/analyze/html/mhc_binding.html, version 2009-09-01B).

As a non-limiting example, using the SMM prediction tool at the IEDB website, the shuffled E6 and E7 sequences as described by Oosterhuis *et al.*, 2011, *Int J Cancer* 129: 397-406 and Öhlschläger *et al.*, 2006, *Vaccine* 24: 2880-93 contain each nine potential strong unique neo-epitopes (ANN or SMM IC₅₀<50 nM) for the 20 most HLA-A and -B, in the core part. This even excludes the appendices used in that approach (in which appendices will further contribute to additional neo-epitopes, and may miss out on more native MHC II epitopes due to the limited length of the 'overlap'). Indeed, a reportedly improved molecule containing a variant with shuffled E6 and E7 proteins that was described in WO 2013/083287, contains 22 unique neo-epitopes with a length of nine amino acids with a predicted IC₅₀ <50 nM (ANN, SMM or NetMHCpan) for the 20 most common HLA-A, 20 most common HLA-B and 20 most common HLA-C alleles.

Hence, the designer molecules of the invention clearly are favourable in having much lower number of predicted neo-epitopes compared to other published approaches where E6 and E7 were shuffled to remove functionality.

Nucleic acid encoding our thus designed HPV16 E6E7SH molecule (i.e. a polypeptide having amino acid sequence as provided in SEQ ID NO:1) was synthesized, the nucleic acid sequence comprising SEQ ID NO: 2, and flanked by a HindIII site and a Kozak sequence on the 5' end and an XbaI site on the 3' site (custom synthesis and standard molecular cloning at Invitrogen Life technologies, Germany).

The synthesized fragments were cloned using HindIII and XbaI into a standard expression vector, pCDNA2004.Neo, harbouring both a bacterial resistance marker (Ampiciline) and a mammalian resistance marker (Neomycine), to obtain plasmid

vectors encoding a molecule of the invention, e.g. for (transient) transfection based experiments.

These molecules could be used as such, but also as the basis for further
5 molecules that contain additional features. As non-limiting examples, some further variants were prepared as described below.

The HPV16 E6E7SH fusion protein sequence can be combined with
sequences of other HPV16 early proteins to target individuals with persistent infection
10 and to broaden the immune repertoire in an immunized individual. Immune responses against E2 have been suggested to play an important role in the clearance of HPV16 infections (de Jong *et al.*, 2002, *Cancer Res* 62: 472-479). Fusion of E2 to E6E7SH will give a vaccine component that harbours antigens against the stages of HPV-related cancer from persistent infection to invasive cancer or recurrent/refractory
15 disease after LEEP surgery. Therefore, as a non-limiting example of such embodiments, we prepared a sequence coding for a fusion protein of E6E7SH with E2 at its N-terminus. In the E2 sequence modifications can be made to abrogate DNA binding activity that might affect gene expression in cells expressing the fusion protein. We mutated Glycine at position 293, Lysine at position 299 and Cysteine at
20 position 300 of the wt HPV16 E2 protein into respectively Valine, Methionine and Arginine. Each of these mutations on its own already completely abrogates the binding of E2 to DNA sequences that harbour E2 binding domains (Prakash *et al.*, 1992, *Genes Dev* 6: 105-16).

The resulting polypeptide is referred to as HPV16 E2E6E7SH and comprises
25 SEQ ID NO: 3. A codon-optimized sequence encoding this polypeptide was prepared and is provided in SEQ ID NO: 4.

We also constructed a variant wherein the same E2 mutant protein was fused to the C-terminus of the HPV16 E6E7SH fusion polypeptide, giving rise to a polypeptide referred to as HPV16 E6E7E2SH, which comprises SEQ ID NO: 5. The
30 sequence encoding this construct is provided as SEQ ID NO: 6.

For control purposes, we also constructed sequences encoding a polypeptide that contains the wild-type sequences for full-length HPV16 E6 and E7 as a fusion

protein (E6 from aa 1 to 158 directly fused to E7 from aa 1 to 98, named herein E6E7wt).

We also tested the effect of adding leader sequences to the polypeptide. As a
5 non-limiting example, a sequence encoding an IgE leader sequence (see e.g. US
6,733,994) [the sequence of the leader peptide is provided in SEQ ID NO: 7] was
fused at the N-terminus of some of the constructs, e.g. in the E6E7wt construct, which
rendered LSE6E7wt, and in the E2E6E7SH construct, which rendered LSE2E6E7SH.
The effect thereof was significantly ($p < 0.05$) enhanced immunogenicity in
10 comparison to the same antigen without the LS sequence as measured by E7-tetramer
analysis in immunized mice (as can for instance be seen in Fig. 9).

The sequences that encode the E6E7SH polypeptides of the invention, with or
without E2, can for instance be expressed from DNA constructs, from RNA or from
15 viral vectors. Fig.1 demonstrates expression in HEK-293T cells upon transient
transfection with DNA vectors expressing the transgenes as described above. After
transfection, cells were harvested and cell extracts were analyzed by SDS-PAGE and
western blotting with an antibody against HPV16 E7. This experiment demonstrates
expression of the expected fusion proteins of appropriate size upon transfection of the
20 expression vectors.

Adenoviral vectors can be used to express the E6E7, either with or without E2,
and with or without additional sequences to augment the immunogenicity of the
encoded fusion protein.

25 The genes, coding for HPV16 E6E7 wt control or HPV designer sequences
described above were gene optimized for human expression and synthesized, by
Geneart. A Kozak sequence (5' GCCACC 3') was included directly in front of the
ATG start codon, and two stop codons (5' TGA TAA 3') were added at the end of the
respective coding sequence. The genes were inserted in the pAdApt35BSU plasmid
30 and in the pAdApt26 plasmid (Havenga *et al.*, 2006, *J Gen Virol* 87, 2135-43) via
HindIII and XbaI sites.

All adenoviruses were generated in PER.C6 cells by single homologous
recombination and produced as previously described (for rAd35: Havenga *et al.*,
2006, *J Gen Virol* 87: 2135-43; for rAd26: Abbink *et al.*, 2007, *J Virol* 81: 4654-63).

PER.C6 cells (Fallaux *et al.*, 1998, *Hum Gene Ther* 9: 1909-17) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), supplemented with 10mM MgCl₂.

Briefly, PER.C6 cells were transfected with Ad vector plasmids, using
5 Lipofectamine according to the instructions provided by the manufacturer (Life Technologies). Cells were harvested one day after full cytopathic effect (CPE) was reached, freeze-thawed, centrifuged for 5 min at 3,000 rpm, and stored at -20°C. The viruses were plaque purified and amplified in PER.C6 cells cultured in a single well of a multiwell 24 tissue culture plate. Further amplification was carried out in PER.C6
10 cells cultured in a T25 tissue culture flask and subsequently in a T175 tissue culture flask. Of the crude lysate prepared from the cells obtained after the T175 flask, 3 to 5 ml was used to inoculate 24×T1000 five-layer tissue culture flasks containing 70% confluent layers of PER.C6 cells. The virus was purified using a two-step CsCl purification method. Finally, the virus was stored in aliquots at -85°C.

15 Ad35.HPV16-E6E7wt, and Ad35.HPV16-E6E7SH are recombinant adenovirus serotype 35 (Ad35) vectors comprising the codon-optimized nucleotide sequences for the expression of, respectively, a fusion protein of the wild type HPV16 E6 and E7 proteins (E6E7wt), and a designer fusion protein variant as described above (E6E7SH, having the amino acid sequence provided in SEQ ID NO:
20 1). The combined E6 and E7 sequences were placed under the control of a CMV promoter in the E1 region of the E1,E3 deleted adenovirus genome. Ad26.HPV16-E6E7wt, and Ad26.HPV16-E6E7SH are the equivalent vectors based on recombinant adenovirus serotype 26.

25 Similarly, Ad26 and Ad35-based recombinant adenoviral vectors were produced that encode the HPV16 E2E6E7SH (SEQ ID NO: 3) variant. Likewise, Ad26 and Ad35 encoding the HPV16 E6E7E2SH (SEQ ID NO: 5) variant were produced. Also, an Ad35 vector encoding the E2E6E7SH fusion protein with an IgE leader sequence at the N-terminus was produced, named Ad35.HPV16-LSE2E6E7SH.
30 Also a control adenovirus with the E6E7wt fused to the IgE leader sequence at the N-terminus was produced.

The recombinant adenoviruses were produced on PER.C6 cells and purified by centrifugation on cesium chloride gradients.

Further examples of constructs of the invention that were coupled to repressor systems are provided in a later example below.

5 **Example 2. Lack of transforming activity of the designer constructs**

Wild-type HPV16 E6 and E7 proteins have tumorigenic potential, which is apparent as transforming activity in certain assays, such as colony formation in a soft-agar assay (Massimi and Banks, 2005, *Methods Mol Med* 119: 381-395). The E6E7SH polypeptide as described in example 1 comprises the fragments of the E6
10 and E7 proteins in a re-ordered fashion. This is expected to remove the tumorigenic potential, as can be measured for instance by a significantly reduced transforming activity as compared to either of wt E6 and E7 proteins in such assays.

Others reported that gene-shuffled variants of HPV16 E6 and E7 have indeed lost their oncogenic potential (Öhlschläger *et al.*, 2006, *Vaccine* 24: 2880–93; Henken
15 *et al.*, 2012, *Vaccine* 30: 4259-66), demonstrating that gene shuffling destroys the wild-type functions of E6 and E7 proteins.

To assess the loss of tumorigenic properties, we assessed the ability of our E6E7SH constructs to confer the ability to grow in soft agar upon NIH 3T3 cells (as
20 described by e.g. Massimi and Banks, 2005, *Methods Mol Med* 119: 381-395). Transfection of NIH3T3 cells with a plasmid expressing the wild type HPV16 E7 resulted consistently in colony formation. In these assays, expression of wild type HPV16 E6 alone did not cause colony formation above background. This is in line with published observations that E7wt is much more efficient than E6wt in this assay
25 (Sedman *et al.*, 1991, *J Virol* 65: 4860-66). Transfection with our E6E7SH construct did not lead to growth of colonies of cells in soft agar (Fig. 2) in four independent experiments, demonstrating that nucleic acids encoding a polypeptide of the invention, E6E7SH, have lost the transforming capacity that is associated with E7.

30 The tumorigenic potential of E6 and E7 is associated with their ability to reduce the levels of the cellular proteins p53 and pRb respectively. p53 and pRb degradation assays were performed to demonstrate that nucleic acid encoding a polypeptide of the invention, E6E7SH, construct does not have the biological activity associated with the wild-type E6 and E7 at the molecular level. In short, HPV16 E6wt

and our E6E7SH construct were expressed in NCI-H1299 cells that lack endogenous p53 for the p53 degradation assay. For the pRb degradation assay HPV16 E7wt and the E6E7SH construct were expressed in pRb null Saos-2 cells. As can be seen in Fig. 3, co-expression of p53 with E6wt, but not with E6E7SH, leads to reduced p53 levels (panels A and B). Likewise, panels 3C and 3D show that co-expression of pRb with E7wt, but not with E6E7SH, leads to reduced pRB levels. These data demonstrate that nucleic acid encoding a polypeptide of the invention has no ability to form colonies in soft agar and does not contain main biological activities of the wild-type E6 and E7 polypeptides, namely the inactivation of p53 and pRb respectively.

To further demonstrate the safety of nucleic acid constructs encoding polypeptide of the invention, we made use of primary human foreskin keratinocytes that are the natural target cells for HPV mediated transformation. Immortalization of primary human keratinocytes requires the action of both E6 and E7 wild-type (Munger *et al.*, 1989, *J Virol* 63: 4417-21). This assay is probably the physiologically most relevant *in vitro* assay to demonstrate the safety of our constructs (Massimi and Banks, 2005, *Methods Mol Med* 119: 381-395). Cells transduced with lentiviruses expressing wild type E6 and E7 from HPV16 (E6E7wt) induce immortalization in primary keratinocytes as indicated by the extension of their lifespan as compared to non-transduced control cells (Fig. 4) and activation of hTERT, the catalytic subunit of telomerase (data not shown). Expression of the polypeptide of the invention (E6E7SH) is not able to extend the lifespan compared to GFP-transduced or non-transduced keratinocytes. A similar result was obtained in two additional independent donors (data not shown). Taken together these data demonstrate that our constructs have lost the ability to induce immortalization in primary human keratinocytes, that are considered a highly physiological model.

Another construct wherein fragments of HPV16 E6 and E7 were recombined in another order was also incapable of immortalization of primary human foreskin keratinocytes. However, an expanded life span up to approximately 120-150 days was observed for that construct. This indicates some unpredictability in this field, and demonstrates the superiority of the designer molecules according to the invention in this safety-related aspect.

All together the experiments in this example provide strong evidence of the lack of transforming activity of nucleic acids encoding polypeptides according to the invention, and thus a strongly improved safety over HPV16 E6 and E7 wt constructs.

5

Example 3. Immune responses to the E6E7SH designer constructs

We have prepared DNA vectors and adenoviral vectors, as described in example 1.

We used the CB6F1 mouse strain for measuring immune responses, based on initial experiments where mice were immunized with DNA plasmids encoding wild type E2, or E6 or E7, and immunization with HPV16 E2, E6 and E7 antigens induced a broader cellular immune response in CB6F1 than in C57BL/6 mice or Balb/c mice. In a separate experiment mice were immunized with DNA vectors encoding molecules of the invention and cellular immune responses were measured. HPV16 E7-specific immune responses could be measured in mice immunized with DNA plasmids expressing E6E7SH (Fig. 5).

The following data shown in this example are from mouse experiments that were carried out with adenoviral vectors.

20

To evaluate the vaccine induced immunogenicity, CB6F1 mice were immunized with adenovectors (Ad35) expressing E6E7wt, LSE6E7wt, E6E7SH or adenovectors not encoding a transgene (Empty). Two doses were tested for administration to the mice: 5×10^9 viral particles (vp) and 1×10^{10} vp. Two and eight weeks after immunization the mice were sacrificed and isolated splenocytes were stimulated overnight with an HPV16 E7 15mer peptide pool. E7-specific responses at two weeks and at eight weeks were analyzed by IFN γ ELISPOT. The data are presented in Fig. 6.

This shows that immunization of mice with Ad35.HPV16-E6E7SH induces E7-specific immune responses as measured by ELISPOT analysis. In addition, the results in Fig. 6 demonstrates the possibility to enhance the immune response against an adenoviral expressed transgene by adding an N-terminal leader sequence to the transgene.

Next the effect of adding E2 to the E6E7SH polypeptide with respect to immunogenicity was tested. The Ad35 vectors encoded polypeptides that had E2 either fused to the N-terminus (E2E6E7SH) or to the C-terminus (E6E7E2SH).

5 CB6F1 mice were immunized with a dose of 1×10^{10} vp. Fig. 7 (E7-tetramer staining) and Fig. 8 (Panel C, IFN γ ELISPOT) show the immune responses against E7, which for the designer constructs including E2 tends to be higher in comparison to the construct without E2, although the differences were not statistically significant. The response against E2 was higher for adenoviral vectors encoding only E2 compared to
10 the response for adenoviral vectors that had E2 fused to the E6E7SH designer polypeptide (Fig. 8B), with differences being significant for both E2 vs E2E6E7SH and E2 vs E6E7E2SH (p-value: <0.05).

It is concluded that the designer constructs that further include E2 can still provide an immune response against E7, and in addition also provide an immune
15 response against E2, thus increasing the breadth of the immune response over the constructs that do not include E2.

Addition of a leader sequence was shown to result in higher E7-specific responses when fused to the N-terminus of the fusion protein of wild type E6 and E7 (Fig. 6C).
20 Similarly, the effect of the leader sequence on immunogenicity of the E2E6E7SH fusion protein was determined. Therefore, Ad35 vectors encoding the designer polypeptide, with or without N-terminal E2 and an Ad35 vector encoding LSE2E6E7SH were used for immunization of mice and blood samples were taken at two-week intervals to measure E7-specific immune responses (Fig. 9). As shown in
25 Figs. 7 and 8 the presence of E2 at either N- or C-terminally fused to E6E7SH tended to increase the immune responses. Addition of the IgE leader sequence further increased the E7-specific response (Fig. 9B). Over time sustained immune responses were observed for all three adenoviral vectors that encoded designer molecules according to the invention, and the highest response after the immunization
30 corresponded with the highest responses over the duration of the experiment.

It is concluded that the responses that are induced by the designer construct that further includes N-terminal E2 can be increased by addition of specific

sequences, e.g., the IgE leader sequence, that target the encoded protein to specific cellular compartments.

The cellular immune response against the peptide of the invention can be induced with different types of adenoviral vectors. In the previous experiment we used Ad35 vectors, while in the experiment of Fig. 10, mice were immunized with an Ad26 adenoviral vector expressing E2E6E7SH. The data show that also immunization with an Ad26-based vaccine induced E7-specific T-cells. In addition, the results demonstrate that a second immunization with an Ad35 adenoviral vector expressing E2E6E7SH further boosted the cellular immune responses (Fig. 10).

Example 4. Immunogenicity of designer constructs in rhesus macaques.

To evaluate the ability of the adenoviral vectors expressing the designer sequence of the invention to induce immune responses in non-human primates, rhesus macaques were immunized by intramuscular injection with adenovectors (Ad26) expressing E2E6E7SH or adenovectors not encoding a transgene (Empty), with a dose of 1×10^{11} vp. Eight weeks after the immunization the immune responses were boosted by immunization with Ad26 vectors expressing the same antigen. At week 16 the animals received one more injection with the Ad35 vectors expressing the same antigen. Blood samples were taken at several time points and isolated white blood cells were stimulated overnight with a peptide pools corresponding to HPV16 E2, E6 or E7. Specific responses were measured by IFN γ ELISPOT. The data are presented in Fig. 11. In addition at week 10 and week 18 post prime immunization, the cellular immune response specific to peptides spanning the novel junctions in the invention was evaluated. The induction of IFN γ response was in all animals below the limit of detection of < 50 SFU per 1×10^6 PBMC (data not shown).

The data show that immunization of non-human primates with Ad26.HPV16-E2E6E7SH resulted in cellular immune responses against all three HPV16 proteins that are present in the encoded transgene, but not against the novel junctions. Responses could be boosted by the additional immunization with Ad26.HPV16-E2E6E7SH and additional boost at week 16 with the corresponding Ad35 vector further increased the HPV16 E2, E6 and E7-specific immune responses.

A late booster administration at week 72 with Ad26.HPV16-E2E6E7SH again resulted in an increase of the HPV16 cellular immune response, which after a few weeks was declined (not shown).

In a separate experiment (not shown), Rhesus macaques were immunized by intravaginal administration with a combination of two adenoviral vectors, one expressing HPV16 E6E7SH and the other the HPV16 L1 protein. Low but measurable cellular responses were measured in peripheral mononuclear blood cells against both E6 and E7. In these experiments, strong cellular immune responses against L1 were detected.

Example 5. Therapeutic efficacy in a mouse tumor model.

The polypeptide of the invention is capable of inducing HPV16-specific cellular immune response in animals, which can exert a therapeutic effect on cells expressing HPV16 E6 and/or E7. Therapeutic immunization, i.e. immunization after tumor growth has started, can be used to demonstrate efficacy of a therapeutic HPV vaccine candidate. The therapeutic effect of Ad26 and Ad35 vectors was tested in mice that were injected with TC-1 cells (mouse cells expressing HPV16 E6 and E7) (Lin *et al.*, 1996, *Cancer Res* 56: 21-6). TC-1 cells will form solid tumor within a few days to weeks after sub-cutaneous injection in mice. Without vaccine the tumors grew rapidly and reach a pre-determined size of 1000 mm³ within 30 days (panels D and E). Upon reaching this size the mice are sacrificed for ethical reasons.

With a prime-boost immunization scheme with SLPs (used as a positive control; Kenter *et al.*, 2009, *N Engl J Med* 361:1838-47; Zwaveling *et al.*, 2002, *J Immunol* 169:350-8) or adenoviral vectors expressing HPV16-E2E6E7SH, a marked decrease of the growth of TC-1 induced tumors was observed (Fig. 12, panels B and C). Closer inspection of the first 30 days after the prime immunizations (Panels F and G) shows that the immunization with the adenovectors expressing E2E6E7SH have a substantially larger impact on tumor growth than immunization with the SLPs. The initial growth rate is much lower and in most cases the tumors shrunk. In 3 out of 11 mice immunized with the adenoviral vectors, the tumors were completely eradicated, which is reflected in the survival plot (panel H).

In conclusion, immunization with adenoviral vectors expressing a polypeptide of the invention significantly reduced tumor growth or completely eradicated established tumors in a well-established challenge model for HPV16-induced cancer.

5

Example 6: Employment of repressor systems to improve the productivity and genetic stability of adenoviral vectors expressing HPV-derived antigens

It has previously been reported that transgenes inserted into adenovirus vectors under the control of powerful constitutively active promoters can, depending on the properties of the transgene product, negatively impact vector production (Yoshida & Yamada, 1997, *Biochem Biophys Res Commun* 230:426-30; Rubinchik *et al.*, 2000, *Gene Ther* 7:875-85; Matthews *et al.*, 1999, *J Gen Virol* 80:345-53; Edholm *et al.*, 2001, *J Virol* 75:9579-84; Gall *et al.*, 2007, *Mol Biotechnol* 35:263-73). Examples of transgene-dependent vector productivity issues include inefficient vector rescue and growth, low final vector yields, and, in severe cases, rapid outgrowth of viral mutants with defective transgene cassettes. To solve these issues, multiple studies explored the possibility to silence vector transgene expression during vector replication in producer cells (Matthews *et al.*, 1999, *J Gen Virol* 80:345-53; Edholm *et al.*, 2001, *J Virol* 75:9579-84; Gall *et al.*, 2007, *Mol Biotechnol* 35:263-73; Cottingham *et al.*, 2012, *Biotechnol Bioeng* 109:719-28; Gilbert *et al.*, 2014, *J Virol Methods* 208:177-88). In this regard, different repression systems have previously been implemented in the context of Ad vectors and have indeed shown to improve vector productivity and genetic stability for vectors encoding different types of (inhibitory) transgenes.

It was observed that some of the adenovirus vectors described herein, as well as some other adenoviral vectors encoding certain HPV antigen variants, displayed some of the transgene-dependent vector productivity issues described above, and therefore could possibly be further improved in that respect. We therefore sought to investigate whether usage of systems to repress vector transgene expression can improve production characteristics of Ad vectors expressing HPV-derived antigens as those described herein. For this purpose, we implemented two existing repressor-operator systems, i.e. TetR/TetO (Yao & Eriksson, 1999, *Hum Gene Ther* 10:419-22, EP0990041B1) and CymR/CuO (Mullick *et al.*, 2006, *BMC Biotechnol* 6:43), into our adenovirus vector platform. Both the TetR/TetO and the CymR/CuO system have previously been used by others to improve adenovirus vector productivity through

vector transgene silencing during vector replication (Gall *et al.*, 2007, *Mol Biotechnol* 35:263-73; Cottingham *et al.*, 2012, *Biotechnol Bioeng* 109:719-28; Gilbert *et al.*, 2014, *J Virol Methods* 208:177-88). Implementation of these two systems involved the generation of adenoviral vectors expressing genes of interest under the control of either a TetO or a CuO sequence-containing CMV promoter. Furthermore, the implementation entailed the generation of cell lines stably expressing the respective cognate repressors proteins (i.e. TetR or CymR).

Several E1-deleted, Ad26- and Ad35-based vectors were generated in which sequences encoding heterologous polypeptides were operably linked to a CMV promoter containing either TetO or CuO operator sequences. First, certain TetO- or CuO-containing sequences (SEQ ID NO: 11 and SEQ ID NO: 12, respectively) were inserted near the transcription start site (TSS) of the CMV promoter (SEQ ID NO: 13) of pAdapt26 and pAdapt35.Bsu plasmids (Abbink *et al.*, 2007, *J Virol* 81:4654-63; Havenga *et al.*, 2006, *J Gen Virol* 87:2135-43). The operator-containing sequences were inserted at precisely the same positions of the CMV promoter as previously described for the two systems (Yao & Eriksson, 1999, *Human Gene Ther* 10:419-22; EP0990041B1; Mullick *et al.*, 2006, *BMC Biotechnol* 6:43; EP1385946B1). Specifically, relative to the TSS (as originally assigned; Stenberg *et al.*, 1984, *J. Virol.* 49: 190-9), the TetO- and CuO-containing sequences were inserted directly downstream of positions -20 and +7, respectively. In SEQ ID NO: 13, these two positions correspond to positions 716 and 742, respectively. The resulting operator-containing CMV promoters are termed, respectively, CMVTetO and CMVCuO. Next, different transgenes were inserted downstream of the (modified) CMV promoters of the resulting constructs using HindIII and XbaI restriction sites. These transgenes included genes encoding a fusion protein of green fluorescent protein and luciferase (GFP-Luc), LSE2E6E7SH from the present invention, and another polypeptide with some similarity to LSE2E6E7SH (a construct referred to in this example as 'HPVAg'). HPVAg comprises the same leader sequence as present in LSE2E6E7SH, as well as E2, E6, and E7 sequences of HPV16. Using methods as described herein, the resulting modified pAdapt26 and pAdapt35.Bsu plasmids were used for the generation of adenoviral vectors expressing the above mentioned reporter and HPV transgenes under the control of either the CMVTetO or the CMVCuO promoter.

Cell lines expressing either TetR or CymR were generated by stable transfection of PER.C6® cells using, respectively, plasmid pcDNATM6/TR (LifeTechnologies, V1025-20) and a derivative of pcDNATM6/TR in which the TetR-coding sequence (SEQ ID NO: 14, which encodes polypeptide SEQ ID NO: 15) is replaced by a codon-optimized CymR-coding sequence (SEQ ID NO: 16, which encodes polypeptide SEQ ID NO: 17). Stable cell line generation was performed largely as described by the supplier of pcDNATM6/TR using a transient transfection-based assay to screen for cell clones capable of repressing expression of CMVTetO- or CMVCuO-driven genes. The resulting PER.C6/TetR and PER.C6/CymR cell lines were analyzed for their ability to repress transgene expression during vector replication in these cells. Experiments conducted with vectors expressing GFP-Luc under the control of operator-containing CMV-promoters showed at least a 10-fold reduction of luciferase gene expression throughout the complete virus replication cycle in the cell lines expressing the repressor corresponding to the respective operator sequences (data not shown). This confirmed that the PER.C6/TetR and PER.C6/CymR cell lines were capable of repressing vector transgene expression in the context of replicating adenovirus vectors.

The effect of TetR- and CymR-mediated repression of adenovector transgene expression on vector yields was investigated for Ad35-based vectors expressing HPVAg (Fig. 13A). To this end, PER.C6, PER.C6/TetR, and PER.C6/CymR cell lines, seeded at 3×10^5 cells per well in 24-well plate wells, were subjected to quadruplicate infections – at 1000 virus particles per cell and for a duration of three hours – by vectors expressing HPVAg from either CMVTetO or CMVCuO promoters. As controls, parallel infections were performed with corresponding vectors expressing GFP-Luc instead of HPVAg. Four days after infection, crude viral lysates were prepared by subjecting the contents of the wells (i.e. infected cells and medium) to two freeze-thaw cycles. Adenovector titers were subsequently determined by an Ad35 hexon sequence-specific quantitative PCR-based protocol that uses a purified Ad35 vector with known virus particle titer as a standard. The results show that both the TetO- and the CuO-containing HPVAg-encoding Ad35 vectors, compared to the control vectors expressing GFP-Luc, display decreased vector yields on normal PER.C6 cells. By contrast, when produced on cells expressing their cognate repressors (i.e. TetR and CymR, respectively), these same vectors gave yields as high as those obtained with the control vectors. These data indicate that repression of

transgene expression during vector production in producer cells can be beneficial for the productivity of Ad35 vectors carrying HPVAg as a transgene.

The effect that repression of adenovector transgene expression may have on vector yields was also investigated for vectors derived from adenovirus serotype 26 (Ad26) (Fig. 13B). In an assay performed essentially as described above for the Ad35 vectors, Ad26 vectors carrying CMVTetO promoter-controlled transgenes encoding either GFP-Luc, HPVAg, or LSE2E6E7SH were used to infect PER.C6 and PER.C6/TetR cells at 1500 virus particles per cell. Three days later the infections were harvested and virus particle titers determined by an Ad26 hexon sequence-specific quantitative PCR-based method. The results show that on PER.C6 cells the yields for the vectors encoding HPVAg and LSE2E6E7SH are lower than obtained with the control vector encoding GFP-Luc. In contrast, on PER.C6/TetR cells, both these vectors showed titers that are as high as that obtained for the control vector. Together with the results above (for Ad35 vectors), these data indicate that repression of transgene expression during adenovector production increases the yields of vectors expressing HPVAg and LSE2E6E7SH.

We have observed major issues regarding the genetic stability of an adenovirus vector that carried a CMV promoter-driven transgene for HPVAg. For example, it was observed that after several passaging rounds of this vector on PER.C6 the majority of the vector population consisted of a mutant vector that carried a large deletion in the HPVAg coding sequence (data not shown).

We reasoned that employment of a transgene expression repression system, such as one of the two described above, could prevent genetic stability issues associated with transgenes, such as HPVAg that are inhibitory to vector growth. To test this, an Ad35-based vector with CMVCuO promoter-driven HPVAg expression was assessed for transgene cassette stability upon growth of the vector on either PER.C6 or PER.C6/CymR cells (Fig. 14). In brief, vector DNA was transfected into the two different cell lines and resultant viral plaques were allowed to grow under an agarose layer. From each of the two transfections, five viral plaques were isolated and separately passaged further on the same cell line (i.e. as used for the transfection), for ten consecutive viral passages. Transgene integrity was assessed by PCR amplification of the transgene cassette at viral passage number ten (VPN10), and the subsequent analysis of resultant PCR products by gel electrophoresis and Sanger sequencing. In addition, at VPN7, the passaged viral clones were assessed for their

ability to express HPVAg. This was done by using the passaged viral isolates to infect A549 cells at 1000 virus particles per cell, lysing the cells at 48 hours post infection, and subsequently analyzing the expression of HPVAg by western blotting using a monoclonal antibody directed against HPV16 E7 (Santa-Cruz Biotechnology). The results of the gel electrophoresis and sequencing analyses showed that all five viral isolates that had been passaged on PER.C6 each carried either small frameshifting deletions or premature stop mutations within the transgene cassette. By contrast, such deletions or mutations could not be detected in any of the vector isolates that had been passaged on the cell line expressing CymR (PER.C6/CymR). In agreement with these data, all PER.C6/CymR-propagated vector isolates were able to express HPVAg, while all PER.C6-grown vectors completely lost this ability, suggesting defective transgene cassettes for these vectors. In conclusion, our data demonstrate that employment of a repressor system, as for instance the CymR/CuO system, to repress vector transgene expression during vector propagation is an effective means to prevent severe transgene cassette instability, such as that seen for vectors carrying a transgene expressing HPVAg.

References

- Abbink P, Lemckert AA, Ewald BA, Lynch DM, Denholtz M, Smits S, Holterman L, Damen I, Vogels R, Thorner AR, O'Brien KL, Carville A, Mansfield KG, Goudsmit J, Havenga MJ, Barouch DH (2007) Comparative seroprevalence and immunogenicity of six rare serotype recombinant adenovirus vaccine vectors from subgroups B and D. *J Virol* 81:4654-4663
- Ausubel FM (1995) Short protocols in molecular biology : a compendium of methods from Current protocols in molecular biology. Wiley, [Chichester]
- Cottingham MG, Carroll F, Morris SJ, Turner AV, Vaughan AM, Kapulu MC, Colloca S, Siani L, Gilbert SC, Hill AV (2012) Preventing spontaneous genetic rearrangements in the transgene cassettes of adenovirus vectors. *Biotechnol Bioeng* 109:719-728
- Daayana S, Elkord E, Winters U, Pawlita M, Roden R, Stern PL, Kitchener HC (2010) Phase II trial of imiquimod and HPV therapeutic vaccination in patients with vulval intraepithelial neoplasia. *Br J Cancer* 102:1129-1136
- de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A, van Meijgaarden KE, Drijfhout JW, Kenter G, Vermeij P, Melief CJ, Offringa R (2002) Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. *Cancer Res* 62:472-479
- Edholm D, Molin M, Bajak E, Akusjarvi G (2001) Adenovirus vector designed for expression of toxic proteins. *J Virol* 75:9579-9584
- Evans RK, Nawrocki DK, Isopi LA, Williams DM, Casimiro DR, Chin S, Chen M, Zhu DM, Shiver JW, Volkin DB (2004) Development of stable liquid formulations for adenovirus-based vaccines. *J Pharm Sci* 93:2458-2475
- Fallaux FJ, Bout A, van der Velde I, van den Wollenberg DJ, Hehir KM, Keegan J, Auger C, Cramer SJ, van Ormondt H, van der Eb AJ, Valerio D, Hoeben RC (1998) New

- helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum Gene Ther* 9:1909-1917
- Frøkjær S, Hovgaard L (2000) Pharmaceutical formulation development of peptides and proteins. Taylor & Francis, London
- 5 Gall JG, Lizonova A, EttyReddy D, McVey D, Zuber M, Kovesdi I, Aughtman B, King CR, Brough DE (2007) Rescue and production of vaccine and therapeutic adenovirus vectors expressing inhibitory transgenes. *Mol Biotechnol* 35:263-273
- Gao GP, Engdahl RK, Wilson JM (2000) A cell line for high-yield production of E1-deleted adenovirus vectors without the emergence of replication-competent virus. *Hum Gene*
- 10 *Ther* 11:213-219
- Gennaro AR (1990) Remington's pharmaceutical sciences. Mack
- Gilbert R, Guilbault C, Gagnon D, Bernier A, Bourget L, Elahi SM, Kamen A, Massie B (2014) Establishment and validation of new complementing cells for production of E1-deleted adenovirus vectors in serum-free suspension culture. *J Virol Methods*
- 15 208:177-188
- Hamid O, Carvajal RD (2013) Anti-programmed death-1 and anti-programmed death-ligand 1 antibodies in cancer therapy. *Expert Opin Biol Ther* 13:847-861
- Harlow E, Lane D (1988) Antibodies : a laboratory manual. Cold Spring Harbor Laboratory, New York
- 20 Havenga M, Vogels R, Zuijdgheest D, Radosevic K, Mueller S, Sieuwerts M, Weichold F, Damen I, Kaspers J, Lemckert A, van Meerendonk M, van der Vlugt R, Holterman L, Hone D, Skeiky Y, Mintardjo R, Gillissen G, Barouch D, Sadoff J, Goudsmit J (2006) Novel replication-incompetent adenoviral B-group vectors: high vector stability and yield in PER.C6 cells. *J Gen Virol* 87:2135-2143
- 25 Henken FE, Oosterhuis K, Ohlschlager P, Bosch L, Hooijberg E, Haanen JB, Steenbergen RD (2012) Preclinical safety evaluation of DNA vaccines encoding modified HPV16 E6 and E7. *Vaccine* 30:4259-4266
- Hildesheim A, Herrero R, Wacholder S, Rodriguez AC, Solomon D, Bratti MC, Schiller JT, Gonzalez P, Dubin G, Porras C, Jimenez SE, Lowy DR (2007) Effect of human papillomavirus 16/18 L1 viruslike particle vaccine among young women with preexisting infection: a randomized trial. *JAMA* 298:743-753
- 30 Hoganson DK, Ma JC, Asato L, Ong M, Printz MA, Huyghe BG, Sosnowshi BA, D'Andrea MJ (2002) Development of a stable adenoviral vector formulation. *Bioprocess J* 1:43-48
- 35 Hoof I, Peters B, Sidney J, Pedersen LE, Sette A, Lund O, Buus S, Nielsen M (2009) NetMHCpan, a method for MHC class I binding prediction beyond humans. *Immunogenetics* 61:1-13
- Horwitz MS (1996) Adenoviruses. In: Fields BN, Knipe DM, Baines JD (eds) *Virology*. Raven Press Ltd, New York
- 40 Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, Essahsah F, Fathors LM, Offringa R, Drijfhout JW, Wafelman AR, Oostendorp J, Fleuren GJ, van der Burg SH, Melief CJ (2009) Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med* 361:1838-1847
- Kibbe AH (2000) Handbook of pharmaceutical excipients. Pharmaceutical Press, London
- 45 Kovesdi I, Hedley SJ (2010) Adenoviral producer cells. *Viruses* 2:1681-1703
- Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, Wu TC (1996) Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 56:21-26
- Lundegaard C, Lamberth K, Harndahl M, Buus S, Lund O, Nielsen M (2008) NetMHC-3.0:
- 50 accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8-11. *Nucleic Acids Res* 36:W509-512
- Massimi P, Banks L (2005) Transformation Assays for HPV Oncoproteins. In: Davy C, Doorbar J (eds) *Human Papillomaviruses : Methods and Protocols*. Vol 119: Methods in Molecular Medicine Springer, Berlin, pp 381-395

- Matthews DA, Cummings D, Eveleigh C, Graham FL, Prevec L (1999) Development and use of a 293 cell line expressing lac repressor for the rescue of recombinant adenoviruses expressing high levels of rabies virus glycoprotein. *J Gen Virol* 80 (Pt 2):345-353
- McPherson MJ, Hames BD, Taylor GR (1995) PCR 2 : a practical approach. IRL Press at
5 Oxford University Press, Oxford
- Mellman I, Coukos G, Dranoff G (2011) Cancer immunotherapy comes of age. *Nature* 480:480-489
- Mullick A, Xu Y, Warren R, Koutroumanis M, Guilbault C, Broussau S, Malenfant F, Bourget L, Lamoureux L, Lo R, Caron AW, Pilote A, Massie B (2006) The cumate
10 gene-switch: a system for regulated expression in mammalian cells. *BMC Biotechnol* 6:43
- Munger K, Phelps WC, Bubbs V, Howley PM, Schlegel R (1989) The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol* 63:4417-4421
- 15 Ogun SA, Dumon-Seignovert L, Marchand JB, Holder AA, Hill F (2008) The oligomerization domain of C4-binding protein (C4bp) acts as an adjuvant, and the fusion protein comprised of the 19-kilodalton merozoite surface protein 1 fused with the murine C4bp domain protects mice against malaria. *Infect Immun* 76:3817-3823
- Oosterhuis K, Aleyd E, Vrijland K, Schumacher TN, Haanen JB (2012a) Rational Design of
20 DNA Vaccines for the Induction of Human Papillomavirus Type 16 E6- and E7-Specific Cytotoxic T-Cell Responses. *Hum Gene Ther* 23:1301-1312
- Oosterhuis K, Ohlschlager P, van den Berg JH, Toebe M, Gomez R, Schumacher TN, Haanen JB (2011) Preclinical development of highly effective and safe DNA vaccines directed against HPV 16 E6 and E7. *Int J Cancer* 129:397-406
- 25 Oosterhuis K, van den Berg JH, Schumacher TN, Haanen JB (2012b) DNA vaccines and intradermal vaccination by DNA tattooing. *Curr Top Microbiol Immunol* 351:221-250
- Peters B, Tong W, Sidney J, Sette A, Weng Z (2003) Examining the independent binding assumption for binding of peptide epitopes to MHC-I molecules. *Bioinformatics* 19:1765-1772
30
- Prakash SS, Grossman SR, Pepinsky RB, Laimins LA, Androphy EJ (1992) Amino acids necessary for DNA contact and dimerization imply novel motifs in the papillomavirus E2 trans-activator. *Genes Dev* 6:105-116
- Rubinchik S, Ding R, Qiu AJ, Zhang F, Dong J (2000) Adenoviral vector which delivers
35 FasL-GFP fusion protein regulated by the tet-inducible expression system. *Gene Ther* 7:875-885
- Sambrook J, Fritsch E, Maniatis T (1989) *Molecular cloning : a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Sedman SA, Barbosa MS, Vass WC, Hubbert NL, Haas JA, Lowy DR, Schiller JT (1991)
40 The full-length E6 protein of human papillomavirus type 16 has transforming and trans-activating activities and cooperates with E7 to immortalize keratinocytes in culture. *J Virol* 65:4860-4866
- Shenk T (1996) Adenoviridae and their Replication. In: Fields BN, Knipe DM, Baines JD (eds) *Virology*. Raven Press Ltd, New York
- 45 Smahel M, Sima P, Ludvikova V, Vonka V (2001) Modified HPV16 E7 Genes as DNA Vaccine against E7-Containing Oncogenic Cells. *Virology* 281:231-238
- van der Burg SH, Melief CJ (2011) Therapeutic vaccination against human papilloma virus induced malignancies. *Curr Opin Immunol* 23:252-257
- Watson JD (1992) *Recombinant DNA*. Scientific American Books, New York
- 50 Wieking BG, Vermeer DW, Spanos WC, Lee KM, Vermeer P, Lee WT, Xu Y, Gabitzsch ES, Balcaitis S, Balint JP, Jr., Jones FR, Lee JH (2012) A non-oncogenic HPV 16 E6/E7 vaccine enhances treatment of HPV expressing tumors. *Cancer Gene Ther* 19:667-674

- Yan J, Reichenbach DK, Corbitt N, Hokey DA, Ramanathan MP, McKinney KA, Weiner DB, Sewell D (2009) Induction of antitumor immunity in vivo following delivery of a novel HPV-16 DNA vaccine encoding an E6/E7 fusion antigen. *Vaccine* 27:431-440
- 5 Yao F, Eriksson E (1999) A novel tetracycline-inducible viral replication switch. *Hum Gene Ther* 10:419-427
- Yoshida Y, Hamada H (1997) Adenovirus-mediated inducible gene expression through tetracycline-controllable transactivator with nuclear localization signal. *Biochem Biophys Res Commun* 230:426-430
- 10 Yugawa T, Kiyono T (2009) Molecular mechanisms of cervical carcinogenesis by high-risk human papillomaviruses: novel functions of E6 and E7 oncoproteins. *Rev Med Virol* 19:97-113
- Zwaveling S, Ferreira Mota SC, Nouta J, Johnson M, Lipford GB, Offringa R, van der Burg SH, Melief CJ (2002) Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. *J Immunol* 15 169:350-358

Table I. sequences

SEQ ID NO: 1 (HPV16-E6E7SH, amino acid sequence of HPV16 E6/E7 designer polypeptide)

5 MHQKRTAMFQ DPQERPRKLP QLCTELQTTI HDIILECVYC KQQLEDEIDG
 PAGQAE PDRA HYNIVTFCK CDSTLR LCVQ STHVDIRTLE DLLMGT LGIV
 CPICSQKPGT TLEQQYNKPL CDLLIRCINC QKPLCP EEQ RHLDDKKQRFH
 NIRGRWTGRC MSCCRSSRTR RETQM HGDTP TLHEYMLDLQ PETTDLYCYE
 QLNDSS E EED EIDGPAGQAE PDRAHYNIVT FCCQLCTELQ TTIHDIILEC
 10 VYCKQQLLR EVYDFAFRDL CIVYRDGNPY AVCDKCLKFY SKISEYRHYC
 YSLYGT TLEQ QYNKPLCDLL IRCINCQK

SEQ ID NO: 2 (HPV16-E6E7SH, nucleotide sequence encoding amino acid sequence of HPV16 E6/E7 designer polypeptide)

15 ATGCCACCAGA AACGGACCGC CATGTTCCAG GACCCCCAGG AACGGCCCAG AAAGCTGCCC
 CAGCTGTGCA CCGAGCTGCA GACCACCATC CACGACATCA TCCTGGAATG CGTG TACTGC
 AAGCAGCAGC TGAAGATGA GATCGACGGC CCTGCTGGCC AGGCCGAACC CGACAGAGCC
 CACTACAATA TCGTGACCTT CTGCTGCAAG TGCGACAGCA CCCTGCGGGCT GTGCGTGCAG
 20 AGCACCCACG TGGACATCCG GACCCTGGAA GATCTGCTGA TGGGCACCCT GGGCATCGTG
 TGCCCCATCT GCAGCCAGAA GCGCGGCACC ACCCTGGAAC AGCAGTACAA CAAGCCCCTG
 TGCGACCTGC TGATCCGGTG CATCAACTGC CAGAAACCCC TGTGCCCCGA GGAAAAGCAG
 CGGCACCTGG ACAAGAAGCA GCGGTTCCAC AACATCCGGG GCAGATGGAC AGGCAGATGC
 ATGAGCTGCT GCAGAAGCAG CCGGACCAGA CGGGAAACCC AGATGCACGG CGACACCCCC
 25 ACCCTGCACG AGTACATGCT GGACCTGCAG CCCGAGACAA CCGACCTGTA CTGCTACGAG
 CAGCTGAACG ACAGCAGCGA GGAAGAGGAC GAGATTGACG GACCCGCTGG ACAGGCCGAG
 CCTGACCGGG CTCACTATAA CATCGTGACA TTTTGCTGTC AGCTCTGTAC TGA ACTCCAG
 ACAACAATTC ACGATATTAT TCTCGAATGT GTGTATTGTA AACAGCAGCT CCTGCGGAGA
 GAGGTGTACG ACTTCGCCTT CCGGGACCTC TGCATCGTGT ATCGGGACGG CAACCCCTAC
 30 GCCGTGTGCG ACAAGTGCTT GAAGTTCTAC AGCAAGATCA GCGAGTACCG GCACTACTGC
 TACAGCCTGT ACGGAACAAC ACTCGAACAG CAGTATAACA AACC ACTCTG TGATCTGCTG
 ATTCGCTGTA TCAATTGTCA GAAGTGATAA

SEQ ID NO: 3 (HPV16 E2E6E7SH, amino acid sequence of HPV16 E2/E6/E7 designer polypeptide)

METLCQRLNVCQDKILTHYENDSTDLRDHDIDYWKHMRLECAIYYKAREMGFKHINHQVVPTL
AVSKNKALQAIELQLTLETIYNSQYSNEKWTLQDVSLEVYLTAPTGCICKKHGYTVEVQFDGD
5 ICNTMHYTNWTHIYICEEASVTVVEGQVDYGYGLYYVHEGIRTYFVQFKDDAEKYSKNKVWEV
HAGGQVILCPTSVFSSNEVSSPEIIRQHLANHPAATHTKAVALGTEETQTTIQRPRSEPDTG
NPCHTTKLLHRDSVDSAPILTAFNSSHKGRINCNSNTTPIVHLKVDANTLMRLRYRFFKKHCT
LYTAVSSTWHWTGHNVKHKSIAIVTLTYDSEWQRDQFLSQVKIPKTIITVSTGFMSIMHQKRTA
MFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQLEDEIDGPAGQAEPDRAHYNIVTFCC
10 KCDSTLRLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKPGTTLEQQYNKPLCDLLIRCINCQ
KPLCPEEKQRHLDKKQRFHNIRGRWTGRCMSCRSSRTRRETQMHGDTPTLHEYMLDLQPET
TDLYCYEQLNDSSEEEDEIDGPAGQAEPDRAHYNIVTFCCQLCTELQTTIHDIILECVYCKQ
QLLRREVYDFAFRDLCIVYRDGNPYAVCDKCLKFYISKISEYRHYCYSLYGTTLEQQYNKPLC
DLLIRCINCQK
15

SEQ ID NO: 4 (HPV16 E2E6E7SH, nucleotide sequence encoding HPV16 E2/E6/E7 designer polypeptide)

ATGGAACCCCTGTGCCAGCGGCTGAACGTGTGCCAGGACAAGATCCTGACCCACTACGAGAA
20 CGACAGCACCGACCTGCGGGACCACATCGACTACTGGAAGCACATGCGGCTGGAATGCGCCA
TCTACTACAAGGCCAGAGAGATGGGCTTCAAGCACATCAACCACCAGGTGGTGCCACCCTG
GCCGTGTCCAAGAACAAGGCCCTGCAGGCCATCGAGCTGCAGCTGACCCTGGAAACCATCTA
CAACAGCCAGTACAGCAACGAGAAGTGGACCCTGCAGGACGTGTCCCTGGAAGTGACCTGA
CCGCTCCCACCGGCTGCATCAAGAAACACGGCTACACCGTGGAAGTGACGTTTCGACGGCGAC
25 ATCTGCAACACCATGCACTACACCAACTGGACCCACATCTACATCTGCGAAGAGGCCAGCGT
GACCGTGGTGGAAGGCCAGGTGGACTACTACGGCCTGTACTACGTGCACGAGGGCATCCGGA
CCTACTTCGTGCAGTTCAAGGACGACGCCGAGAAGTACAGCAAGAACAAGTGTGGGAGGTG
CACGCTGGCGGCCAGGTCATCCTGTGCCCCACCAGCGTGTTCAGCAGCAACGAGGTGTCCAG
CCCCGAGATCATCCGGCAGCACCTGGCCAATCACCTGCCGCCACCCACACAAAGGCCGTGG
30 CCCTGGGCACCGAGGAAACCCAGACCACCATCCAGCGGCCCAGAAGCGAGCCCCGACACCGGC
AATCCCTGCCACACCACCAAGCTGCTGCACCGGGACAGCGTGGACAGCGCCCCCTATCCTGAC
CGCCTTCAACAGCAGCCACAAGGGCCGGATCAACTGCAACAGCAACACCACCCCCATCGTGC
ACCTGAAGGTGGACGCCAACACCCTGATGCGGCTGCGGTACAGATTCAAGAAGCACTGCACC
CTGTACACCGCCGTGTCCTCCACCTGGCACTGGACCGGCCACAACGTGAAGCACAAGAGCGC
35 CATCGTGACCCTGACCTACGACAGCGAGTGGCAGCGGGACCAGTTCCTGAGCCAGGTCAAAA
TCCCCAAGACCATCACCGTGTCCACCGGCTTCATGAGCATCATGCACCAGAAACGGACCGCC
ATGTTCCAGGACCCCCAGGAACGGGCCAGAAAGCTGCCCCAGCTGTGCACCGAGCTGCAGAC

CACCATCCACGACATCATCCTGGAATGCGTGTACTGCAAGCAGCAGCTGGAAGATGAGATCG
ACGGCCCTGCTGGCCAGGCCGAACCCGACAGAGCCCACTACAATATCGTGACCTTCTGCTGC
AAGTGCGACAGCACCTGCGGCTGTGCGTGCAGAGCACCCACGTGGACATCCGGACCCTGGA
AGATCTGCTGATGGGCACCCTGGGCATCGTGTGCCCCATCTGCAGCCAGAAGCCCGGCACCA
5 CCCTGGAACAGCAGTACAACAAGCCCCTGTGCGACCTGCTGATCCGGTGCATCAACTGCCAG
AAACCCCTGTGCCCCGAGGAAAAGCAGCGGCACCTGGACAAGAAGCAGCGGTTCCACAACAT
CCGGGGCAGATGGACAGGCAGATGCATGAGCTGCTGCAGAAGCAGCCGGACCAGACGGGAAA
CCCAGATGCACGGCGACACCCCCACCCTGCACGAGTACATGCTGGACCTGCAGCCCGAGACA
ACCGACCTGTACTGCTACGAGCAGCTGAACGACAGCAGCGAGGAAGAGGACGAGATTGACGG
10 ACCCGCTGGACAGGCCGAGCCTGACCGGGCTCACTATAACATCGTGACATTTTGTGTGTCAGC
TCTGTACTGAACTCCAGACAACAATTCACGATATTATTCTCGAATGTGTGTATTGTAAACAG
CAGCTCCTGCGGAGAGAGGTGTACGACTTCGCCTTCCGGGACCTCTGCATCGTGTATCGGGA
CGGCAACCCCTACGCCGTGTGCGACAAGTGCCTGAAGTTCTACAGCAAGATCAGCGAGTACC
GGCACTACTGCTACAGCCTGTACGGAACAACACTCGAACAGCAGTATAACAAACCACTCTGT
15 GATCTGCTGATTGCTGTATCAATTGTCAGAAAGTGATAA

SEQ ID NO: 5 (HPV16 E6/E7/E2SH, amino acid sequence encoding
HPV16 E6/E7/E2 designer polypeptide

20 MHQKRTAMFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQLEDEIDGPAGQAEPPDRAHY
NIVTFCCCKDSTLRLCVQSTHVDIRTLEDLLMGTGLGIVCPICSQKPGTTLEQQYNKPLCDLL
IRCINCQKPLCPPEEKQRHLDKKQRFHNIRGRWTGRCMSSCRSSRTRRETQMHGDTPTLHEYM
LDLQPETTDLYCYEQLNDSSEEEDEIDGPAGQAEPPDRAHYNIVTFCCQLCTELQTTIHDIIL
ECVYCKQQLLRREVYDFAFRDLCIVYRDGNPYAVCDKCLKFYISKISEYRHYCYSLYGTTLEQ
25 QYNKPLCDLLIRCINCQKMETLCQRLNVCQDKILTHYENDSTDLRDHIDYWKHMRLECAIYY
KAREMGFKHINHQVVP TLAVSKNKALQAIELQLTLETIYNSQYSNEKWTLQDVSLEVYLTAP
TGCIKKHGYTVEVQFDGDI CN TMHYTNWTHIYICEEASVTVVEGQVDYGLYYVHEGIRTYF
VQFKDDAEKYSKNKVWEVHAGGQVILCPTS VFSSNEVSSPEIIRQH LANHPAATHTKAVALG
TEETQTTIQRP RPSEPDTGNPCHTTKLLHRDSVDSAPILTAFNSSHKGRINCNSNTTPIVHLK
30 VDANTLMRLRYRFKKHCTLYTAVSSTWHWTGHN VKHKS AIVTLTYDSEWQRDQFLSQVKIPK
TITVSTGFMSI

SEQ ID NO: 6 (HPV16 E6/E7/E2SH, nucleotide sequence encoding
HPV16 E6/E7/E2 designer polypeptide

35 ATGCACCAGAAACGGACCGCCATGTTCCAGGACCCCCAGGAACGGCCCAGAAAGCTGCCCCA
GCTGTGCACCGAGCTGCAGACCACCATCCACGACATCATCCTGGAATGCGTGTACTGCAAGC
AGCAGCTGGAAGATGAGATCGACGGCCCTGCTGGCCAGGCCGAACCCGACAGAGCCCACTAC

AATATCGTGACCTTCTGCTGCAAGTGCGACAGCACCTGCGGCTGTGCGTGCAGAGCACCCA
CGTGGACATCCGGACCCTGGAAGATCTGCTGATGGGCACCCTGGGCATCGTGTGCCCCATCT
GCAGCCAGAAGCCCGGCACCACCCTGGAACAGCAGTACAACAAGCCCCCTGTGCGACCTGCTG
ATCCGGTGCATCAACTGCCAGAAACCCCTGTGCCCCGAGGAAAAGCAGCGGCACCTGGACAA
5 GAAGCAGCGGTTCCACAACATCCGGGGCAGATGGACAGGCAGATGCATGAGCTGCTGCAGAA
GCAGCCGGACCAGACGGGAAACCCAGATGCACGGCGACACCCCCACCCTGCACGAGTACATG
CTGGACCTGCAGCCCGAGACAACCGACCTGTACTGCTACGAGCAGCTGAACGACAGCAGCGA
GGAAGAGGACGAGATTGACGGACCCGCTGGACAGGCCGAGCCTGACCGGGCTCACTATAACA
TCGTGACATTTTGTGCTGTCAGCTCTGTACTGAACTCCAGACAACAATTCACGATATTATTCTC
10 GAATGTGTGTATTGTAAACAGCAGCTCCTGCGGAGAGAGGTGTACGACTTCGCCTTCCGGGA
CCTCTGCATCGTGTATCGGGACGGCAACCCCTACGCCGTGTGCGACAAGTGCCTGAAGTTCT
ACAGCAAGATCAGCGAGTACCGGCACTACTGCTACAGCCTGTACGGAACAACACTCGAACAG
CAGTATAACAAACCACTCTGTGATCTGCTGATTCGCTGTATCAATTGTCAGAAGATGGAAAC
CCTGTGCCAGCGGCTGAACGTGTGCCAGGACAAGATCCTGACCCACTACGAGAACGACAGCA
15 CCGACCTGCGGGACCACATCGACTACTGGAAGCACATGCGGCTGGAATGCGCCATCTACTAC
AAGGCCAGAGAGATGGGCTTCAAGCACATCAACCACCAGGTGGTGCCACCCTGGCCGTGTC
CAAGAACAAGGCCCTGCAGGCCATCGAGCTGCAGCTGACCCTGGAACCATCTACAACAGCC
AGTACAGCAACGAGAAGTGGACCCTGCAGGACGTGTCCCTGGAAGTGACCTGACCGCTCCC
ACCGGCTGCATCAAGAAACACGGCTACACCGTGGAAGTGAGTTCGACGGCGACATCTGCAA
20 CACCATGCACTACACCAACTGGACCCACATCTACATCTGCGAAGAGGCCAGCGTGACCGTGG
TGGAAGGCCAGGTGGACTACTACGGCCTGTACTACGTGCACGAGGGCATCCGGACCTACTTC
GTGCAGTTCAAGGACGACGCCGAGAAGTACAGCAAGAACAAGTGTGGGAGGTGCACGCTGG
CGGCCAGGTCATCCTGTGCCCCACCAGCGTGTTCAGCAGCAACGAGGTGTCCAGCCCCGAGA
TCATCCGGCAGCACCTGGCCAATCACCTGCCGCCACCCACACAAAGGCCGTGGCCCTGGGC
25 ACCGAGGAAACCCAGACCACCATCCAGCGGCCCAGAAGCGAGCCGACACCGGCAATCCCTG
CCACACCACCAAGCTGCTGCACCGGGACAGCGTGGACAGCGCCCCTATCCTGACCGCCTTCA
ACAGCAGCCACAAGGGCCGGATCAACTGCAACAGCAACACCACCCCCATCGTGCACCTGAAG
GTGGACGCCAACACCCTGATGCGGCTGCGGTACAGATTCAAGAAGCACTGCACCCTGTACAC
CGCCGTGTCTCCACCTGGCACTGGACCGGCCACAACGTGAAGCACAAGAGCGCCATCGTGA
30 CCCTGACCTACGACAGCGAGTGGCAGCGGGACCAGTTCCTGAGCCAGGTCAAAATCCCCAAG
ACCATCACCGTGTCCACCGGCTTCATGAGCATCTGATAA

SEQ ID NO: 7 (IgE leader peptide amino acid sequence)

35 MDWTWILFLVAAATRVHS

SEQ ID NO: 8 (nucleotide sequence encoding IgE leader peptide)

ATGGACTGGACCTGGATCCTGTTCTGGTGGCTGCCGCAACCCGGGTGCACAGC

SEQ ID NO: 9 (aa HAVT20 leader peptide amino acid sequence)
MACPGFLWALVISTCLEFSMA

5 SEQ ID NO: 10 (nucleotide sequence encoding HAVT20 leader peptide)
ATGGCCTGCCCCGGCTTTCTGTGGGCCCTGGTCATCAGCACCTGTCTGGAATTCAGCATGGC
C

10 SEQ ID NO: 11 (2xTetO-containing sequence)
GAGCTCTCCCTATCAGTGATAGAGATCTCCCTATCAGTGATAGAGATCGTCGAC

SEQ ID NO: 12 (CuO-containing sequence)
AACAAACAGACAATCTGGTCTGTTTGTA

15 SEQ ID NO: 13 (CMV promoter present in pAdApt26 and pAdApt35 plasmids)
TCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGCTATT
GGCCATTGCATACGTTGTATCCATATCATAATATGTACATTTATATTGGCTCATGTCCAACA
TTACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATT
20 AGTTCATAGCCCATATATGGAGTTCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCT
GACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCA
ATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGT
ACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCG
CCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTA
25 TTAGTCATCGCTATTACCATGGTGTATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCG
GTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGC
ACCAAATCAACGGGACTTTCCAAAATGTCTGAACAACTCCGCCCCATTGACGCAATGGGC
GGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGC
CTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTCC
30 GCGGCCGGAACGGTGCATTGGA

SEQ ID NO: 14 (TetR, nucleotide sequence encoding amino acid sequence of TetR polypeptide expressed by pcDNATM6/TR)
ATGTCTAGATTAGATAAAAGTAAAGTGATTAACAGCGCATTAGAGCTGCTTAATGAGGTCGG
35 AATCGAAGGTTTAAACAACCCGTAAACTCGCCCAGAAGCTAGGTGTAGAGCAGCCTACATTGT
ATTGGCATGTAAAAAATAAGCGGGCTTTGCTCGACGCCTTAGCCATTGAGATGTTAGATAGG
CACCATACTCACTTTTGCCCTTTAGAAGGGGAAAGCTGGCAAGATTTTTTACGTAATAACGC
TAAAAGTTTTAGATGTGCTTTACTAAGTCATCGCGATGGAGCAAAAGTACATTTAGGTACAC
GGCCTACAGAAAAACAGTATGAAACTCTCGAAAATCAATTAGCCTTTTTTATGCCAACAAGGT

TTTTCCTAGAGAATGCATTATATGCACTCAGCGCTGTGGGGCATTTTACTTTAGGTTGCGT
ATTGGAAGATCAAGAGCATCAAGTCGCTAAAGAAGAAAGGGAAACACCTACTACTGATAGTA
TGCCGCCATTATTACGACAAGCTATCGAATTATTTGATCACCAAGGTGCAGAGCCAGCCTTC
TTATTCGGCCTTGAATTGATCATATGCGGATTAGAAAAACAACCTTAAATGTGAAAGTGGGTC
5 CGCGTACAGCGGATCCCGGGAATTCAGATCTTATTAA

SEQ ID NO: 15 (TetR, amino acid sequence of TetR polypeptide
expressed by pcDNATM6/TR)

MSRLDKSKVINSALELLNEVGIEGLTTRKLAQKLGVEQPTLYWHVKNKRALLDALAIEMLDL
10 HHTHFCPLEGESWQDFLRNNAKSFRCALLSHRDGAKVHLGTRPTEKQYETLENQLAFLCQQG
FSLLENALYALS AVGHFTLGCVLEDQEHQVAKEERETPTTDSMPPLLRQAIELFDHQGAEPAF
LFGLELIICGLEKQLKCESGSAYS SRSREFRSY

SEQ ID NO: 16 (CymR, nucleotide sequence encoding amino acid
sequence of CymR polypeptide)

ATGTCTCCCAAACGACGGACTCAAGCGGAAAGGGCAATGGAACTCAGGGTAAGCTGATTGC
CGCGGCTCTGGGAGTGCTGCGAGAGAAAGGGTATGCCGGGTTTCGCATAGCCGACGTTCCCTG
GAGCTGCAGGCGTAAGCAGAGGAGCCCAATCTCATCACTTCCGACCAAGCTGGAGCTTTTG
CTGGCTACCTTCGAATGGCTGTACGAGCAGATCACGGAAAGGAGTCGTGCTAGGCTGGCCAA
20 GCTGAAACCCGAGGATGATGTCATTTCAGCAGATGCTGGACGATGCAGCCGAGTTCTTCCTGG
ACGACGACTTCAGCATCAGTCTCGACCTCATCGTAGCCGCAGATCGCGATCCAGCTTTGCGC
GAGGGCATAACAGAGAACAGTCGAGCGGAATCGGTTTGTGGTGGAGGACATGTGGCTTGGTGT
TCTGGTGAGCAGAGGCCTCTCACGGGATGATGCCGAGGACATCCTGTGGCTGATCTTTAACT
CCGTCAGAGGGTTGGCAGTGAGGTCCCTTTGGCAGAAGGACAAAGAACGGTTTGAACGTGTG
25 CGAAACTCAACACTCGAGATTGCTAGGGAACGCTACGCCAAGTTCAAGAGATGA

SEQ ID NO: 17 (CymR, amino acid sequence of CymR polypeptide)

MSPKRRTQAERAMETQGKLIAAALGVLRKGYAGFRIADVPGAAGVSRGAQSHHFPTKLELL
LATFEWLYEQITERSRARLAKLPEDDVIQQMLDDAAEFFLDDDFSISLDLIVAADRPALR
30 EGIQRTVERNRFFVEDMWLGVLVSRGLSRDDAEDILWLIFNSVRGLAVRSLWQKDKERFERV
RNSTLEIARERYAKFKR

SEQ ID NO: 18 (HPV16 E6, aa41-65)

KQQLLRREVYDFAFRDLCIVYRDGN
35

SEQ ID NO: 19 (HPV16 E7 aa 43-77)

GQAEPDRAHYNIVTFCKCDSTLR LCVQSTHVDIR

Claims

1. A nucleic acid molecule encoding a polypeptide comprising SEQ ID NO: 1.
2. A nucleic acid molecule according to claim 1, wherein the encoded polypeptide further comprises a leader sequence.
3. A nucleic acid molecule according to any one of the preceding claims, wherein the encoded polypeptide further comprises at least one epitope of a human papillomavirus (HPV) E2 protein.
4. A nucleic acid molecule according to claim 3, wherein the encoded polypeptide comprises HPV16 E2 protein that has a deletion or mutation in its DNA binding domain.
5. A nucleic acid molecule according to claim 4, wherein the encoded polypeptide comprises SEQ ID NO: 3 or SEQ ID NO: 5.
6. A nucleic acid molecule according to any one of the preceding claims, wherein the nucleic acid sequence is codon-optimized.
7. A nucleic acid molecule according to any one of the preceding claims, comprising SEQ ID NO: 2.
8. A nucleic acid molecule according to any one of the preceding claims, comprising SEQ ID NO: 4 or SEQ ID NO: 6.
9. A vector comprising a nucleic acid molecule according to any one of the preceding claims, wherein a sequence encoding the polypeptide is operably linked to a promoter.
10. A vector according to claim 9, wherein the vector is a recombinant adenovirus.

11. A vector according to claim 9 or 10, wherein the promoter is operably coupled to a repressor operator sequence, to which a repressor protein can bind in order to repress expression of the promoter in the presence of said repressor protein.
12. A vaccine composition comprising a vector according to any one of claims 9-11, and a pharmaceutically acceptable excipient.
13. Use of the vaccine composition according to claim 12 in the manufacture of a medicament for the treatment of HPV, wherein the medicament is to be administered to a subject in need thereof.
14. The use according to claim 13, wherein the medicament according to claim 12 is to be administered to the subject in need thereof more than once.
15. Use of the vaccine composition according to claim 12 in the manufacture of a medicament for the treatment of persistent HPV infection, vulvar intraepithelial neoplasia (VIN), cervical intraepithelial neoplasia (CIN), vaginal intraepithelial neoplasia (VaIN), anal intraepithelial neoplasia (AIN), cervical cancer (such as cervical squamous cell carcinoma (SCC), oropharyngeal cancer, penile cancer, vaginal cancer or anal cancer.
16. A polypeptide comprising SEQ ID NO: 1.
17. A polypeptide according to claim 16, comprising SEQ ID NO: 3 or SEQ ID NO: 5.