Teaser Novel nucleic acid-based therapeutic approaches as a promising non-invasive solution for the treatment of cervical cancer caused by Human Papillomavirus.

Cervical cancer and HPV infection: ongoing therapeutic research to counteract the action of E6 and E7 oncoproteins

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Cervical cancer is the fourth most common cancer among women worldwide and its development is mainly associated with human papillomavirus infection, a highly sexually transmissible virus. The expression of E6 and E7 viral oncoproteins deregulates cell repairing mechanisms through impairment of tumor suppressor protein functions, such as p53 or retinoblastoma protein. Although the implementation of new preventive vaccines has decreased the infection rate and cervical cancer progression, there are still many women who are affected by this pathology. Nowadays, the main treatment often requires the use of invasive techniques. From well-established strategies, like DNA vaccines and gene therapy, to innovative gene silencing technologies; different methodologies are currently under scrutiny that target the E6 and E7 oncoproteins and/or their modes of action.

Introduction

The connection between human papillomavirus (HPV) and cancer development, more specifically cervical cancer, was first established in 1983 by Harald zur Hausen and co-workers [1]. At the time, it had already been possible to correlate the development of certain carcinomas in animals with the infection of specific viruses – leading scientists to pursue the same line of thought and study HPV in cancer biopsies, given that HPV DNA had been reported in genital warts [2]. Hence, for the first time, zur Hausen was able to identify HPV type 16 and demonstrated its presence in several malignant tumor biopsies [1]. Later on, zur Hausen's research group found the presence of HPV-16 and HPV-18 DNA in cervical cancer cell lines, as well as in cervical carcinoma biopsies [3]. The first steps to understand the development of cervical cancer were taken, and the groundbreaking work performed by the German scientist was later recognized in the form of a Nobel prize. Ever since such scientific discoveries were performed, many efforts have been made toward the establishment of new detection, screening, preventive and therapeutic methodologies against cervical cancer, mainly by exploring the crucial role of HPV in the development of this pathology.

Although the incidence of cervical cancer has been slowly decreasing with the advances in this field, it still represents one of the most common cancers among women nowadays and it is

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mandatory to search for new methodologies that will help to overcome this problem. To fully understand the ultimate treatment for this disease, it is first necessary to unveil the background of cervical cancer, to decode the mechanisms responsible for the development and progression of HPV infection and to explore different and promising therapeutic approaches.

Cervical cancer worldwide

Cancers are some of the deadliest pathological conditions globally. In particular, cervical cancer is considered the fourth most common cancer in women, accounting for 266 000 deaths in 2012 [4]. When consulting data collected and provided by GLOBOCAN 2018, it is possible to infer that the cervical cancer burden is higher in less developed countries [5]. Actually, in Eastern and Central Africa, this cancer is known as the primary cancer found in women. This might be strongly associated with the fact that less developed countries present very poor health resources in comparison to developed countries, perhaps leading to a late diagnosis [6]. In addition, the recent distribution of several HPV vaccines worldwide probably contributed to a decrease in HPV infection, and a consequent decrease in the number of the cervical cancer cases in countries with easy access to vaccination, such as developed countries. Given its severe implication in the development of cervical cancer, it is therefore very important to understand the biological mechanisms by which HPV operates within the cervical epithelium and further develop suitable strategies that might prevent or overturn its infection and tumorigenicity.

HPV infection and cervical cancer development

According to World Cancer Reports, 'persistent epithelial infection with one or more oncogenic types of HPV may lead to the development of precancerous lesions . . . which may progress to invasive cervical cancer' [6]. As a matter of fact, between 79% and 100% of invasive cervical cancer cases worldwide account with the presence of DNA belonging to high-risk HPV types, from which 70% are related to HPV-16 and HPV-18 [7,8]. Even though HPV is directly associated with cervical cancer, other cancers have also been found to be correlated with HPV infection such as anal, vaginal, vulvar, penile and head and neck cancers [9]. Indeed, there are about 200 HPV genotypes currently documented; however, only some present carcinogenic potential and are known to have the ability of inducing cancer [10]. The fact that different HPV types are related to different clinical symptoms has led scientists to classify each HPV type according to the severity of the clinical outcomes, namely by low, intermediate and high risk. The high-risk types encompass HPV that can induce the development of tumor cells [11]. The severity of the symptoms is usually associated with the affinity presented by E6 and E7 oncoproteins within each HPV type toward the target proteins: tumor suppressors p53 and retinoblastoma protein (pRB), respectively.

HPV is a small double-stranded DNA virus, belonging to the family *Papillomaviridae*, and one of the most common sexually transmissible viruses worldwide. It presents a nonenveloped icosahedral structure and its genome contains eight open reading frames (ORFs) [12]. These ORFs are responsible for coding eight proteins, which can be divided into early stage (E1, E2, E4-E7) and late stage (L1 and L2). As the name implies, early proteins are the proteins to be first expressed upon virus infection of the host cell and are associated with the infection itself and the possible transformation of infected cells. By contrast, late proteins constitute the viral capsid and contribute to the spreading of the infection through the host system, via the release of virus particles within the superficial epithelial cells [12].

Figure 1a illustrates the normal cell repairing pathway. Viral infection with HPV begins when the virus can penetrate the cervical epithelium through micro-abrasions. Then, the E1 and E2 protein expression leads to regulation of the viral replication within infected cells, resulting on the expression of other early-stage proteins. At this point, E5, E6 and E7 oncoproteins begin to be expressed, contributing to cell survival and uncontrolled proliferation, as presented in Fig. 1b [13–15]. Such a carcinogenic mechanism is strongly dependent on the HPV ability in mainly expressing E6 and E7 viral oncoproteins. Both proteins are known to interfere with cell-cycle regulation, affecting the signaling pathways for cell repair and apoptosis [13,14].

The E6 protein can induce tumor suppressor p53 degradation, hindering its function as an apoptosis signaling cascade regulator, through E6AP protein binding. The latter is unable to bind itself to p53, in normal circumstances. However, in the presence of E6 protein, E6AP and E6 form a protein complex that can recognize and bind to p53. Such attachment can impair transcriptional activation or repression of p53-responsive promoters by preventing its binding to specific DNA

development of new biotechnological platforms to obtain biopharmaceuticals (mainly plasmid DNA, minicircle DNA and RNA) with potential therapeutic applications. In particular, the purification of plasmid DNA and RNA is being largely investigated through the design and development of specific technologies. Moreover, considering the formulation of the bioproducts enabling their stabilization, protection and delivery to cellular models. The main therapeutic areas addressed in these studies have been cancer and neurodegenerative diseases, through the evaluation of gene therapy or gene silencing approaches.

Ângela Sousa received her PhD in biochemistry at the Universidade da Beira Interior (UBI), Portugal, in 2011, and was awarded two postdoctoral grants in 2012 and 2015. From 2017 to 2018 was Invited Auxiliary Professor and since september 2018 is auxiliary researcher at UBI. Her main scientific interests include biotechnological processes for the biosynthesis, isolation and purification of biopharmaceuticals (plasmids, minicircle DNA and RNA), with particulated or monolithic chromatographic supports modified with amino acids and derivative ligands, to be used as DNA vacines or gene therapy. Her work is also focused on the development of nanosystems for plasmid targeted delivery to dendritic or cervical cancer cells, as well as the study of the therapeutic action of nucleic-acid-based vectors in the treatment of cancer.

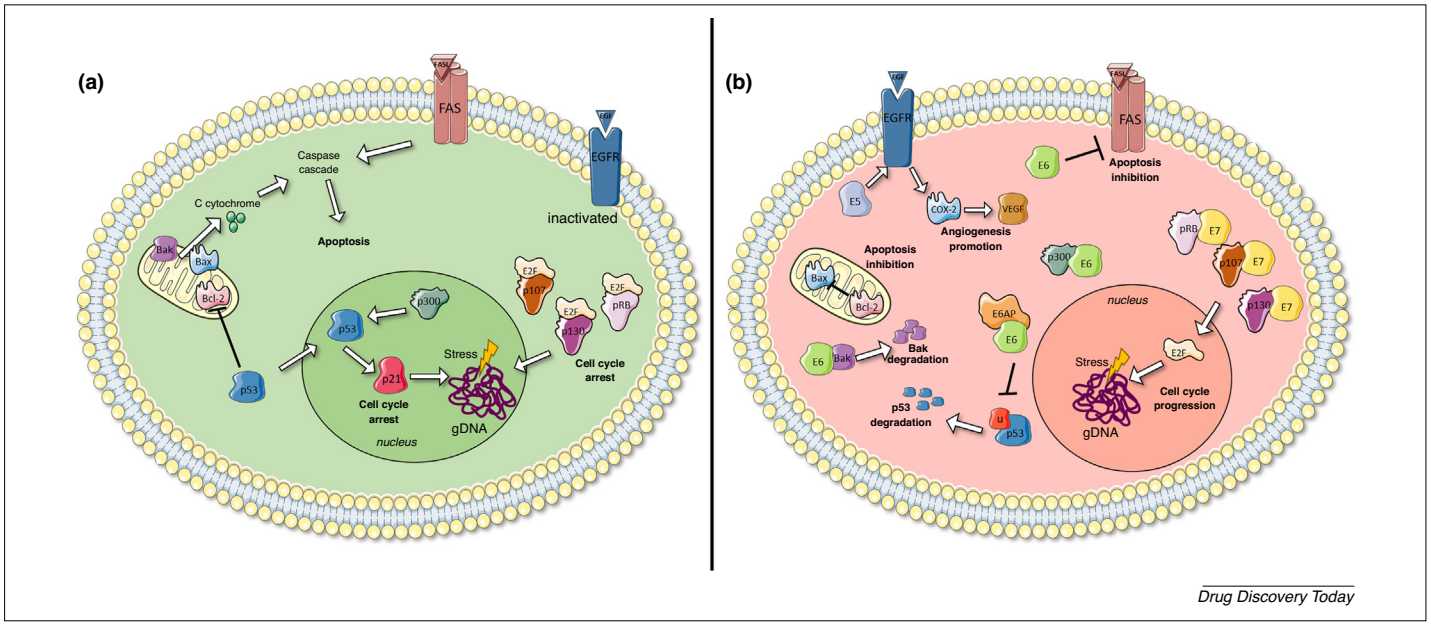

FIGURE 1

Schematic representation of cell-repairing systems in normal cells vs cells infected with human papilloma virus (HPV). **(a)** Normal cell. When a stressful event leads to cell damage, different mechanisms are triggered to induce cell quiescence or death. Tumor suppressor protein p53 is expressed at high levels and different targets are either activated or repressed. In the nucleus, p300 acetylates p53 and induces its ability to act as a transcription promoter for p21 (between other targets) expression. Then, p21 triggers a series of events that will result in cell-cycle arrest. p53 represses the transcription of Bcl-2, an antiapoptotic protein. Thus, Bak and Bax apoptotic proteins induce C cytochrome production, which will in turn trigger the caspase cascade, leading to apoptosis induction. FAS receptors are also able to trigger the caspase cascade when triggered by FASL binding. Additionally, pRB, p107 and p130 bind to E2F transcription factor, impeding its action as a cell-cycle promoter. **(b)** HPV-infected cell. Upon production of E5, E6 and E7 HPV oncoproteins, the cell repairing systems are altered. E5 presence leads to inappropriate activation of EGFR, triggering a series of events that culminate in VEGF production, thus promoting angiogenesis, one of the hallmarks of cancer progression. E6 production leads to the repression of several signaling systems. E6 can form a complex with E6AP able to mark p53 for degradation, thus decreasing the levels of this tumor suppressor protein. Such degradation of p53 leaves Bcl-2 expression deregulated, leading to Bax inhibition. Moreover, E6 can mark Bak for degradation, an important proapoptotic protein, and can bind to p300, thus inhibiting its p53 activation mechanism. Besides, E6 has the ability to bind to FAS and accelerate its degradation. All these mechanisms triggered by E6 result in apoptosis protection. E7 has the ability to bind to tumor suppressors pRB, p107 and p130, which are regulator proteins of the E2F transcription factor. This binding frees E2F, which in turn can continuously induce cell-cycle progression. Overall, HPV protein interference leads to the uncontrolled proliferation of cells. Abbreviations: E6AP, E6AP ubiquitin-protein ligase; EGF, epidermal growth factor; EGFR, EGF receptor; FAS, Fas receptor; FASL, Fas ligand; gDNA, genomic DNA; VEGF, vascular endothelial growth factor; pRB, retinoblastoma protein.

motifs and ultimately lead p53 to degradation through ubiquitination, resulting in a decrease in p53 levels [13,16]. The inactivation of p53 function favors the continuous replication of damaged DNA and abnormal cell survival, which would otherwise be repaired or eliminated by apoptosis induction if p53 was expressed in normal levels (Fig. 1a) [13]. Moreover, E6 has been shown to be able to interact with other proteins that also regulate cell signaling pathways. For instance, E6 can also interact with p300, a promoter that activates p53 through acetylation in an E6AP-independent manner [17]. Also, E6 can target for degradation of other proteins involved in the signaling cascade for apoptosis, namely Bak, the adaptor molecule Fas-associated death domain (FADD) and procaspase 8 [17]. Taking these interactions altogether, the disruptive effect that E6 can exert on the regulation of cell-cycle proliferation and repair is notorious. By contrast, E7 protein is involved in the impairment of tumor suppressor pRB function. As portrayed in Fig. 1a, whereas p53 activates apoptosis, pRB functions mainly rely on the inactivation of transcription factors, such as E2F. This molecule, when freely available, stimulates cellular cycle transition to S phase. Thus, upon viral infection, E7 is expressed and binds to pRB, disrupting its complex with E2F and contributing to continuous cell proliferation (Fig. 1b) [14,18]. Moreover, E7 oncoprotein is also involved with the interference of p107 and p130 functions, two proteins that also regulate cell-cycle proliferation through E2F transcription factor binding. The ultimate inhibition of pRB, p107 and p130 by E7 viral protein contributes to the uncontrolled cell proliferation and progression to malignant transformation seen in HPV-infected cervical epithelium [14,19]. Although E6 and E7 proteins are considered the main causative agents for HPV-infected cell transformation, E5 also presents carcinogenic activity. This protein has the ability to enhance cell proliferation through interaction with epidermal growth factor (EFG), a known stimulator of cell growth, therefore contributing to tumor progression (Fig. 1b) [15].

With the evolution of HPV infection, normal cervical tissue begins to lose its normal features and cells become more undifferentiated. The progression of the disease leads to the establishment of cervical intraepithelial neoplasia (CIN), which can be mild (CIN1) or moderate (CIN2) and characterized by cytologic abnormalities in the tissue. Without suitable treatment and as a result of persistent infections, CIN2 can evolve into high-grade lesions such as severe CIN (CIN3) or invasive carcinoma [20]. Therefore, given

the timeline of HPV infection and possible progression into cervical cancer, it is crucial to detect and treat the HPV infection before its evolution into more serious conditions.

HPV infection and cervical cancer: prevention and therapy

With the understanding of the HPV role in the development of cervical cancer, and other genital diseases, researchers have focused on developing suitable strategies to detect and prevent the disease. Suitable screening techniques are currently available, such as Papanicolaou stain or HPV DNA testing, allowing physicians to detect HPV infection at an early stage and prevent its evolution into invasive cervical cancer [21]. In 2002 a monovalent vaccine was studied, which consisted in the development of HPV-16 L1 capsid virus-like particles (VLPs) [22]. Scientists were able to verify a decrease in the incidence of HPV-16 infection in the population vaccinated with this particular vaccine, suggesting this vaccine was able to confer protection against this particular type of HPV and reduce the risk of developing cervical cancer [22–24]. Considering the fact that there is a wide range of HPV types associated with serious infections that can ultimately lead to the onset of cancer, HPV vaccine research began to focus on developing new vaccines that could provide protection against multiple HPV types. In 2005, a Phase II clinical trial was published regarding the administration of a quadrivalent HPV vaccine, which could supposedly confer protection against four HPV types, responsible for 70% of cervical cancer cases (HPV-16 and HPV-18) and 90% of genital warts (HPV-6 and HPV-11) [25]. According to the data, vaccinated women presented a 90% reduction of combined incidence of persistent infection or disease with the four HPV types, in comparison to women assigned with the placebo [25]. Given the success demonstrated in Phase II and Phase III clinical trials [25–29], this quadrivalent vaccine was later approved and licensed by the FDA, followed by its launch under the name of Gardasil®, by Merck. Simultaneously, a bivalent vaccine was developed with the main goal of preventing cervical cancer, therefore containing only L1 VLPs of HPV-16 and HPV-18 [30,31]. Some studies revealed that the bivalent vaccine could elicit higher antibody production than the quadrivalent vaccine, suggesting that it could persist longer [32]. The bivalent vaccine was launched as Cervarix® by GlaxoSmithKline in Europe and the USA in 2007 and 2009, respectively. Cervarix® and Gardasil® have been shown to be protective over 10 years (time that has elapsed since the initial clinical trials) [33,34]. More recently, Merck developed a new vaccine named Gardasil®-9 which confers protection against nine HPV types (HPV−6, −11, −16, −18, −31, −33, −45, −52 and −58) that, together, have been found in 85% of invasive cervical cancers worldwide [7,35,36]. By expanding the vaccination coverage worldwide, cervical cancer and other HPV-associated cancer rates are expected to decrease. However, to increase the protection and possibly eradicate these high-risk HPV types, vaccination of men must also be considered, given that HPV can also be responsible for the development of anal or penile cancers [37]. As a matter of fact, some countries have implemented a gender-neutral vaccination system with this purpose. Nevertheless, modern antivaccination movements, led by organizations who believe in a cause–effect relationship between immunization and development of severe adverse reactions, has been contributing to a decrease in the adherence to vaccination programs through the influence of general opinion [38,39]. Also, considering that HPV virions can infect the epithelium through the existence of micro-abrasions, it is very important to vaccinate individuals at an early age, preferably before initiating an active sexual life, given these vaccines do not exert any therapeutic effect against an ongoing HPV infection. Therefore, it is also necessary to focus on the development of suitable cervical cancer treatment options.

Currently, the main guidelines for cervical cancer treatment at an early disease state describe the use of surgery to remove the cancerous tissue, sometimes accompanied by chemotherapy or radiotherapy [40]. Surgery can vary from conization to radical hysterectomy, sometimes implying loss of fertility. The coupling with radiotherapy is very common in the treatment of several cancers, presenting satisfactory results in numerous cases and currently representing the most suitable treatment for local and nonmetastatic cancer [40]. When the treatment turns out to be ineffective or it is necessary to expand its range to the entire body, owing to tumor metastasis, chemotherapy is the next logical step [41]. Chemotherapy involves the administration of cytotoxic substances that can interfere with cell-cycle proliferation, therefore preventing tumor growth and leading to cancer cell death [41]. However, given that radiotherapy and chemotherapy treatments are unable to differentiate cancerous tissue from healthy tissue, there is a significant cytotoxicity associated that usually leads to the appearance of side effects and can cause significant discomfort to the patient [41]. Therefore, the design and development of anticancer therapeutics that are efficient and targeted toward the unhealthy tissue is imperative.

Cervical cancer and HPV infection: ongoing therapeutic research

DNA is, without doubt, a crucial molecule in all living organisms owing to its coding functions within the cell, providing the necessary information for protein synthesis as well as for regulation of cell mechanisms. With advances in biotechnology, DNA recombinant technology was born and continues to grow. The possibility for rearranging different genes and manipulating their functions has led scientists to dedicate their research to the development of new gene-based therapies. RNA has also been gaining attention because of the discoveries made, regarding its ability to control gene expression, allowing modification and regulation of cell signaling pathways. With the knowledge acquired through the study of these molecules, researchers were able to propose and explore innovative strategies for the treatment of different pathologies. Currently, several of these cutting-edge technologies are under study to prevent or treat cervical cancer.

DNA vaccines

DNA vaccines have been categorized as the third generation of vaccines, promising humoral and cellular immune responses, with low cost production, thermostability and easy distribution in comparison to conventional vaccines [42]. Such immunological technology is based on the delivery of genetic information with the intent of inducing an immune response against a specific antigen. After inoculation, the treated individual should be capable of producing a strong immune response against the antigen for which genetic information was previously delivered. The antigen

chosen to be encoded in the DNA vaccine must be related with a given pathology [43]. Such an immune response is triggered mainly as a result of the transfection of antigen-presenting cells (APCs) and non-APCs that will later express the antigen encoded in the DNA vaccine. As depicted in Fig. 2, there are two pathways that can lead to antigen presentation. In Fig. 2a, cells transfected with the DNA vaccine can directly present the antigen to $CD8^+$ T lymphocytes, through major histocompatibility complex (MHC) class I [43,44]. This activation leads to the maturation of cytotoxic T lymphocytes (CTLs), effector cells, which can identify cells that present the target antigen through MHC class I. Once these cells are found by the CTLs, cell death is triggered via different pathways. Such a pathway represents the therapeutic effect of the DNA vaccine [45]. By contrast, antigen production by non-APCs can lead to APCs taking up the antigen exogenously, resulting in the presentation of the latter to $CD4^+$ T lymphocytes via MHC class II, as portrayed in Fig. 2b. Such activated lymphocytes can activate B cells, which are white blood cells that can produce antibodies against specific antigens. The secretion of such antibodies can lead to neutralization of the microbe responsible for the production of the target antigen (in this case, the virus) [46]. Also, memory B cells are formed from this activation. Such cells are responsible for the induction of a strong immune response when reinfection occurs, thus representing the preventive effect of the DNA vaccine [46]. The combination of both pathways leads to the emergence of a strong humoral and cellular immune response, contrarily to prophylactic vaccines [43,44].

Although one of the advantages of DNA vaccines over prophylactic vaccines is the broad range of the elicited immune response, this is not the only great feature presented by this technology. DNA vaccines are much more economic to manufacture than prophylactic vaccines, considering the simplicity of its production process [47]. DNA vaccines can be easily obtained in large quantities and high-grade purity, owing to the fact that DNA is a molecule easier to manipulate than the peptides from which prophylactic vaccines are made [48]. This also accounts the fact that DNA is more stable, facilitating its transportation and storage. Moreover, DNA vaccines have the potential of providing longer *in vivo* exposure to antigens, which could lead to the increase of immunogenicity and to a decrease in the number of required immunizations [49].

Given that DNA vaccines can exert preventive and therapeutic effects, they are being currently explored in the treatment of cancer. Many cancers exhibit specific antigens that could be used in the development of a DNA vaccine to elicit a specific immune response against those antigens [50]. Thus, given all the advantages inherent to DNA vaccines, much effort has been devoted to developing suitable DNA vaccines for cervical cancer. Considering the main role that E6 and E7 oncoproteins display in cancer development, therapeutic DNA vaccines are currently being developed by using these oncoproteins as antigenic agents. So far, there are many research studies that have been conducted with the aim of developing a suitable DNA vaccine against HPV. From codon optimization to the inclusion of different fusion proteins to facilitate the antigen-presenting process, many approaches have been studied for this matter [51–53]. Such approaches are crucial to induce a strong immune response, because they can significantly alter the immunogenicity for a given DNA vaccine [54]. Codon optimization is used to increase antigen production, principally in heterologous expression systems. This need emerges with the fact that different sets of nucleotides can code for the same amino acid, thus constituting a codon. However, not all organisms are known to make use of all the existing codons. As a matter of fact, a preference for some codons has been shown for different organisms. This can lead to an ineffective translational

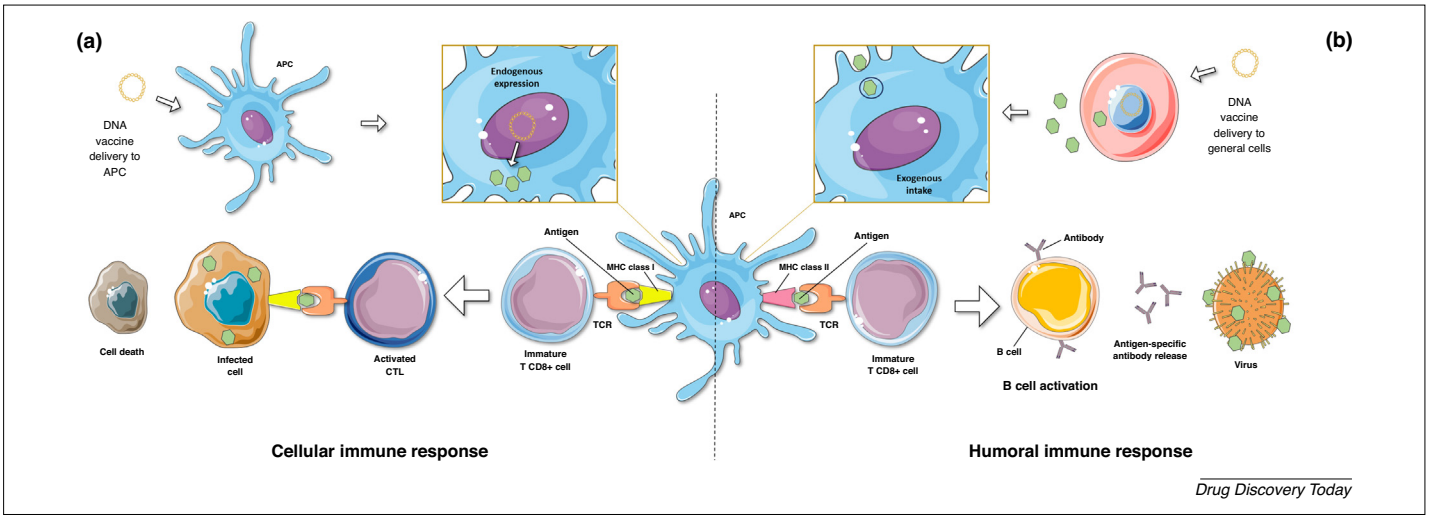

FIGURE 2

Antigen presentation schematics. **(a)** Endogenous antigen presentation. APCs transfected with DNA vaccine can endogenously produce the target antigen. This antigen will be presented by MHC class I to naive $CD8^+$ T cells, activating them as CTLs. Then, CTLs identify cells expressing MHC class I coupled with the antigen that is used for their activation. When such identification is successful, the cell presenting the antigen is destroyed by CTLs as a result of the activation of different paths that mainly lead to apoptosis induction. **(b)** Exogenous antigen presentation. APCs can take exogenous antigens, produced by other cells transfected with the DNA vaccine, and present them by MHC class II to naive $CD4^+$ T cells, activating them. Then, these cells activate B cells into the producing antigen-specific antibodies. These antibodies are released and can trigger different pathways that lead to virus neutralization. Abbreviations: APC, antigen-presenting cell; CTL, cytotoxic T Lymphocyte; MHC, major histocompatibility complex; TCR, T cell receptor.

process and result in decreased protein expression [43]. Thus, codon optimization might be necessary to improve antigen expression and increase immune response. Adjuvants can also be used to improve the outcome of DNA vaccines. These adjuvants might come in as separate molecules or can be included in the antigen design as fusion proteins [43]. Whereas the former might be obtained through the concomitant administration of a plasmid encoding these adjuvants, for example, the latter are constructed by genetically engineering the gene encoding the antigen to produce a protein fused to one, or more, proteins. Such engineering can be used to take advantage of different protein domains. For instance, it can be used to increase the uptake or the stability of a given antigen [43,52]. Table 1 lists a few selected examples of proteins that have been included in the design of different DNA vaccines and their functions in the optimization of this strategy. Some of these proteins have been included in the design of DNA vaccines that have reached clinical trials, such as HSP70, a heat shock protein that facilitates antigen presentation and concomitantly exhibits antiproliferation effects, or the more recently studied interleukin (IL)-10 receptor, which can inhibit the action of IL-10, which can interfere with triggering of the immune response [53,55]. As mentioned above, different molecules with different roles can be used to optimize the therapeutic effect of a DNA vaccine, altering significantly the performance of the immunological response.

Although several DNA vaccines have been designed in the past years toward E6 and E7 antigens, few have reached clinical trials for cervical cancer. Cornelia Trimble and her research group have been dedicating their research to developing and improving DNA vaccines for cervical cancer treatment, having published a Phase I clinical trial (NCT00121173) regarding the use of pNGVL4a-Sig/E7 (detox)-HSP70 in 2009 [53]. This DNA vaccine encodes the HPV-16 E7 oncoprotein modified, so that it is unable to bind to pRB. The DNA vaccine was also coupled to a signal peptide to facilitate the release from cells through a secretory pathway, coupled to HSP70 [53]. In this study it was found that 33% of the patients presented histologic regression. Later on, in 2014, another Phase I study (NCT00788164) was conducted with this particular vaccine, where a robust, localized and effective immune response was detected in the treated patients [56]. Recently, a Phase II trial began to explore the effect of this vaccine coupled with TA-CIN vaccine, a protein vaccine that comprises L2/E6/E7 proteins from HPV-16 (NCT03911076). In 2015, Trimble's research group conducted a Phase II clinical trial (NCT01304524) in patients with CIN2/3 by using VGX-3100, a DNA vaccine consisting of an equal mixture of two plasmid DNAs, each containing the optimized sequence for an

TABLE 1

Examples of proteins that have been selected to improve DNA vaccine therapeutic action in recent years

Year	Protein	Function	Antigens used	Targeted HPV type	Clinical trials (cervix-related diseases)	Refs
2008–2017	CRT	Ca^{2+}-binding protein able to associate with ER transportation molecules, aiding in the antigen presentation through MHC class I	E6, E7, E6/E7	HPV-16 HPV-11	NCT00988559	[59,100,101]
2009–2014	Flt3L	Growth factor able to expand DC and increase population of lymphocytes, improving $CD8^+$ T cell and NK cell activation	E7, E6/E7	HPV-16 HPV-18	NCT02139267, NCT02596243, NCT01634503, NCT02100085, NCT03206138, NCT02411019, NCT03444376	[51,102,103]
2009	HSP70	Heat-shock protein able to improve the uptake of the linked antigen by APC and aid in the antigen presentation through MHC class I and class II, eliciting proinflammatory responses	E7	HPV-16	NCT03911076, NCT00788164, NCT00121173	[53]
2017	IL-2	Interleukin able to induce T and B cell proliferation as well as immunoglobulin synthesis, eliciting strong and robust immune response	E7	HPV-16	–	[104]
2011–2017	TTFC	Foreign molecule able to elicit $CD4^+$ T helper response and provide more stability to the antigen, improving its half life	E6, E7	HPV-16	–	[52,105]
2018	MLT	Immunomodulatory hormone able to induce lymphocyte activation and exert proapoptotic effects	E7	HPV-16	–	[106]
2019	SA-4-1BBL	Artificial ligand for TNF receptor that promotes the proliferation and survival of CD8 + T cells and induces production of different proinflammatory cytokines	E7	HPV-16	–	[107]
2019	IL-10 receptor	Inhibits IL-10, which is an interleukin that interferes with antigen presentation and reduces $CD8^+$ T responses	E7	HPV-16	–	[55]

Abbreviations: APC, antigen-presenting cells; CRT, calreticulin; DC, dendritic cells; ER, endoplasmic reticulum; Flt3L, Fms-related tyrosine kinase 3 ligand; gp, glycoprotein; HSP, heat-shock protein; IL, interleukin; NK, natural killer; MLT, melatonin; TNF, tumor necrosis factor; TTFC, tetanus toxin fragment C.

E6/E7 fusion protein of HPV-16 or HPV-18 [57]. This particular vaccine had the advantage of possibly being offered as a treatment for two of the HPV strains most associated with cervical cancer: HPV-16 and HPV-18. The results were shown to be promising, given that histologic regression was observed in 49.5% of treated patients, without major adverse reactions. These findings suggested that this DNA vaccine could be a suitable alternative to more-invasive techniques such as surgery [57]. Following this line of thought, a Phase III clinical trial, entitled REVEAL 1 – Evaluation of VGX-3100 and Electroporation for the Treatment of Cervical HSIL (NCT03185013), was initiated in 2017 by Inovio Pharmaceuticals with the intent of further proving the safety and effectiveness of the VGX-3100 vaccine. This study is expected to end in 2021 and bring support to the commercialization of this vaccine [58].

In 2016, Trimble and co-workers also published data regarding a Phase I clinical trial (NCT00988559) that was conducted with pNGVL4a-CRT/E7 (detox), a DNA vaccine specifically designed to target HPV-16 E7 oncoprotein. This pDNA molecule expresses the E7 protein, with a modification that prevents its ability to transform healthy cells, coupled to calreticulin, a heat-shock protein similar to HSP70 [59]. In the conducted study, 30% of the patients, who exhibited CIN2/3, demonstrated histologic regression and well tolerability toward the vaccine administration [59]. However, in comparison to VGX-3100, this DNA vaccine is limited to HPV-16 infections, which can be considered as a disadvantage by not targeting HPV-18 infections as well, limiting its coverage.

Another DNA vaccine that has reached clinical trials, this time developed by Kim and co-workers, is the GX-188E HPV DNA vaccine. This DNA vaccine was designed to express shuffled HPV-16 E6 and E7 proteins, to prevent the interaction with tumor suppressors p53 and pRB, coupled to an extracellular domain of Flt3L and the signal sequence of tissue plasminogen activator (tpa) [51]. The latter sequences were included to promote antigen presentation and to enhance the release of the fused protein through the secretory pathway, respectively [51]. In this Phase I study (NCT01634503), which was also conducted in patients with CIN2/3, 78% of treated patients demonstrated histologic regression and 89% displayed enhanced HPV-specific CD8 T cell response, suggesting this DNA vaccine can be a promising noninvasive therapeutic strategy for patients with cervical dysplasia [51]. A Phase Ib/II study is currently being carried out to assess the effect of GX-188E coupled with pembrolizumab, a monoclonal antibody, in patients with advanced and nonresectable HPV-positive cervical cancer (NCT03444376).

Overall, DNA vaccine research is evolving accompanied by very interesting results, suggesting that this therapeutic pathway can be promising to cervical cancer treatment. Nevertheless, researchers have generally found that the majority of DNA vaccines developed so far do not present the high-level immunogenicity observed in mouse models, thus justifying the lack of DNA vaccines approved for human use [48,60]. New methodologies are currently under study to improve the clinical outcome of DNA vaccines. These methodologies derive from the outstanding progress that has been observed in the past years for this field of studies [60]. Moreover, cases like VGX-3100, which stands out as the current HPV DNA vaccine with most promising results, are proof that the first DNA vaccine to be commercially available for humans might be launched in the near future.

Gene therapy

Gene therapy has been in the research spotlight for years. The gene therapy premise involves the delivery of a foreign DNA sequence to the host cells with the intent of altering the function of a given molecule that could play an important part in disease development [61]. The potential of this field for the treatment of 'incurable' diseases resultant from inherited mutations has led researchers to focus their efforts on the search for the next 'miracle' cure. Although its popularity has been affected owing to the threat posed by some severe adverse reactions that occurred in some initial clinical studies and as a result of the lack of significant therapeutic results, researchers have been able to turn the tables through the knowledge acquired during the past decades [62,63]. In recent years, more efforts have been made in the development of gene therapy strategies suitable for cancer treatment. As a matter of fact, the majority of the gene therapy clinical trials performed to date have been directed toward cancer treatment (65%) [64]. Therefore, there has been an increased interest in studying tumor suppressor proteins in the development of such strategies. For instance, pRB is a tumor suppressor protein, the action of which is inhibited owing to HPV viral activity, more precisely the expression of oncoprotein E7. As stated before, its function is mainly involved in regulating cell-cycle proliferation through the inactivation of transcription factor E2F. In 2001, Ip and co-workers designed a gene therapy study to evaluate the therapeutic potential of an adenovirus (AV) expressing pRB [65]. However, their findings suggested that pRB is not a suitable candidate for the development of a cervical cancer therapy because no inhibitory effect was found in the cell growth of three different cervical cancer cell lines, as opposed to an osteosarcoma cell line [65]. Moreover, they evaluated the use of a p53-expressing AV in the same cell lines and apoptosis induction was detected through flow cytometry [65]. The main reason for such different outcomes is probably related to the fact that p53 activates a signaling pathway responsible for apoptosis induction, whereas pRB is mainly involved in regulating cell proliferation, leading to G1 phase arrest and cell senescence but not necessarily contributing to cell death [18]. Additionally, a few reports state that E2F activates apoptosis p53-independently, and normal function can be impaired in the presence of overexpressed pRB [66]. Nevertheless, the poor results obtained with the pRB-based gene therapy protocol for the treatment of cervical cancer led this strategy to fall into oblivion.

By contrast, the p53 gene has been considered the main tumor suppressor gene chosen for the development of gene therapy strategies, sometimes combined with other conventional therapies such as chemotherapy or radiotherapy [64]. Although 50% of the cancer cases worldwide result from mutations in the p53 gene, rendering the protein unable to regulate cell proliferation and to activate the apoptosis cascade [67], in cervical cancer p53 is degraded because of the presence of E6 viral oncoprotein [13]. Restoring p53 levels could be a suitable strategy for inducing tumor cell apoptosis, which could be accomplished by transfecting malignant cells with a p53 expression vector. The use of such a strategy has been controversial according to the literature, given that some researchers claim that this type of gene therapy might

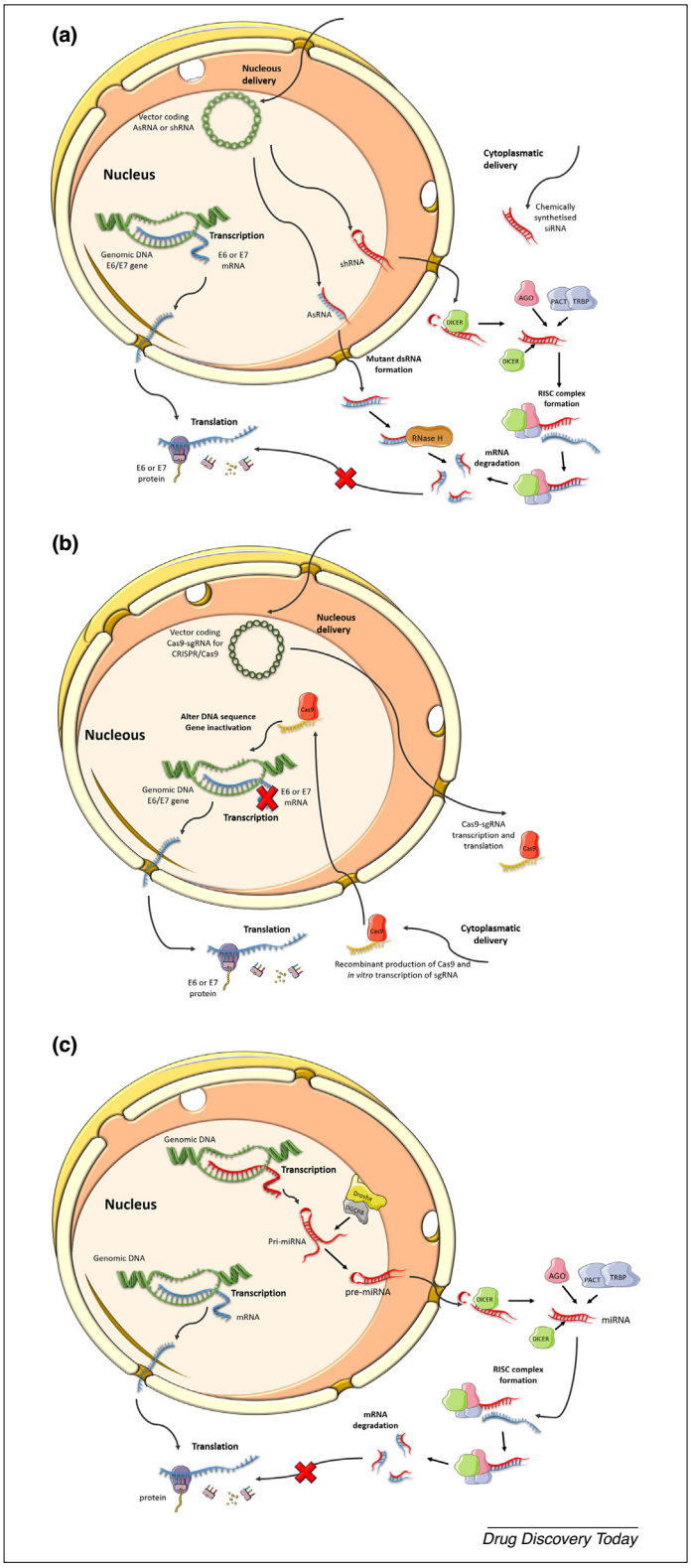

FIGURE 3

Schematic representation of different gene silencing strategies. **(a)** Artificial RNA-based therapies. Such therapies include AsRNA, shRNA and siRNA. AsRNA and shRNA must be delivered to the nucleus via a vector encoding these molecules, whereas siRNA molecules are delivered directly to the cytoplasm. Upon excretion to the cytoplasm, AsRNA binds to the target mRNA, forming a mutant dsRNA that will be marked to degradation by RNase H. By contrast, shRNA will be excreted and processed by Dicer before forming the RISC complex. shRNA and siRNA follow the same process after RISC complex formation, leading to target mRNA identification and degradation. Such a strategy might be used to mark E6 and E7 mRNA to degradation. **(b)** CRISPR/cas9 systems. Cas9-sgRNA can be delivered to the nucleus, via an encoding vector, or directly delivered to the cytoplasm, when produced *in vitro*. Its assembly and nucleus entering will allow editing of the target sequence to which the sgRNA was designed, leading to genomic DNA definitive alteration. Such genomic editing might be used to delete the human papilloma virus (HPV)-integrated genome, preventing the translation of E6 and E7 mRNA, thus preventing the protein production. **(c)** miRNA biogenesis. miRNAs

not be completely effective owing to E6-mediated degradation of p53 protein resulting from *de novo* synthesis [68]. To surpass this problem and avoid the E6 direct action on the p53 expressed by the gene therapy vector, in 1998, Prabhu and co-workers published a study regarding the development of an AV that could express p73β, a homolog for p53 protein that displays similar functions to the p53 tumor suppressor [68]. This protein can induce apoptosis in a p53-dependent manner but is usually expressed in low levels, because its activation is generally a response to stress. In the study, HPV-E6-expressing cells were transfected with either p53- or p73β-expressing AV. Cellular proliferation was only suppressed in cells transfected with p73β-AV, as opposed to cells transfected with p53-AV. In addition, p73β-AV displayed apoptosis induction in transfected cells, suggesting that the E6 mechanisms responsible for p53 degradation are unable to interfere with p73β signaling [68]. In 2003, such findings were sustained by a study conducted by Das and co-workers, where they developed a nonreplicating p73β-AV, for biosafety purposes, and compared its efficiency with a p53-AV, recording very similar data to those reported previously by Prabhu [68,69]. In 2006, Das *et al.* gave continuity to the research and published a study based on the use of nonreplicating p73β-AV *in vitro* and *in vivo* [70]. The growth inhibition of HPV-positive cervical cancer cells was accomplished with administration of this biopharmaceutical product, followed by apoptosis induction, in *in vitro* and *in vivo* models. Overall, these results suggested that p73β might be a promising strategy for scientists to pursue a next generation HPV-induced cancer treatment. However, no new information regarding the use of this vector has been released to date.

Despite the studies developed by Prabhu and Das, other researchers kept on exploring the use of p53 gene therapy in HPV-induced cervical cancer and good results have been obtained. As a matter of fact, Gendicine, the first known gene therapy product to be released for cancer treatment, the approval of which was restricted to China, consists of a recombinant AV that expresses human p53 protein [71,72]. Twelve years since its market release, >30 000 patients have received this biopharmaceutical drug as cancer treatment. No serious adverse reactions have been reported so far, except high fevers in the first hours upon receiving the treatment [73]. Nevertheless, its approval in other countries was controversial because the methodology used for data collection in China was not comparable to that used by the FDA [73]. In the particular case of cervical cancer, it was demonstrated that the coadministration of Gendicine with radiotherapy resulted in the significant increase of 5-year survival rate in comparison to radiotherapy alone, suggesting that the use of this gene therapy approach can have a synergistic effect in the treatment of this cancer [72]. Although there are interesting results published regarding the use of p53 expressing AV in cervical cancer, the fact that E6-positive cells can interfere with the production of p53 can be hiding the full potential of this method. To overcome this issue, the use of a gene therapy strategy targeting the overexpression of p53 or pRB concomitantly with the silencing of E6 or E7 oncoproteins could be very promising in unravelling the full potential of this approach in the future.

Gene silencing

With the fast and innovative advances in the gene therapy field, a new approach has been taken into consideration: why not directly silence the expression of proteins involved in the disease emergence? Since the discovery of antisense RNA technology in 1981 by Tomizawa and co-workers [74], followed by the accidental discovery of RNA interference by Guo *et al.* [75] and the study of its implication in gene expression by Fire *et al.* [76], such a strategy became easier to pursue. Thus, many scientists have been exploring the implementation of this technology in different diseases. In cervical cancer induced by HPV, the most obvious pathway to follow is the downregulation of E6 and E7 oncoproteins. With this in mind, several HPV oncoprotein silencing studies have been performed in the past years. In Fig. 3 the main technologies used for this purpose are represented: antisense RNA (AsRNA), small interfering RNA (siRNA), short hairpin RNA (shRNA), the clustered regularly interspaced short palindromic repeats associated with Cas9 (CRISPR/cas9) system and microRNA. In next subsections, the promising roles of these technologies will be discussed for the treatment of HPV-induced cancer.

Artificial RNA-based therapies

Artificial RNA-based therapies encompass antisense RNA (AsRNA), a single-stranded antisense RNA complementary to the target mRNA; siRNA, a double-stranded RNA complementary to mRNA; and shRNA, a double-stranded RNA that originates siRNA. As schematized by Fig. 3a, these systems can interfere with protein expression by binding to the mRNA of the target protein through perfect complementarity, targeting this molecule to degradation. In 1988, the first attempt at silencing the expression of E6 and E7 oncoproteins was performed, when von Knebel Doeberitz and co-workers transfected a C4-1 cell line, a cervical carcinoma bearing HPV-18, with a plasmid containing HPV-18 E6 and E7 AsRNA sequences [77]. Here, they were able to verify that cell growth was affected by such an experiment, given that, in the presence of dexamethasone, transfected cells revealed a reduction in its growth rate comparatively to nontransfected cells. Since then, many studies have been conducted regarding the silencing of E6 and E7 oncoproteins in an independent manner or simultaneously. It is noteworthy to mention that E6 and E7 mRNAs are bicistronically transcribed from the same promoter in HPV-infected cells. Thus, some silencing sequences can be designed to simultaneously target both oncoproteins.

are naturally produced by the cellular organism and are known to regulate different cellular pathways. Thus, these molecules are first transcribed in the nucleus, from the cell's genomic DNA, presenting a primary miRNA transcript named pri-miRNA. In the nucleus, this transcript is processed by Drosha and DGCR8 proteins, resulting in a precursor miRNA known as pre-miRNA. Following this, the pre-miRNA molecule is exported to the cytoplasm, where it is processed by DICER, as mature miRNA, to integrate into the RISC complex, an RNA-induced silencing complex formed by DICER, AGO, PACT and TRBP. Such a complex is able to identify the mRNA targets of the miRNA to which it is bound, leading to the degradation of these mRNAs. Abbreviations: AGO, argonaute; AsRNA, antisense RNA; Cas9, CRISPR associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; DGCR8, protein DiGeorge syndrome critical region gene 8; dsRNA, double-stranded RNA; miRNA, microRNA; mRNA, messenger RNA; PACT, kinase R-activating protein; RISC, RNA-induced silencing complex; sgRNA, single guide RNA; shRNA, short-hairpin RNA; siRNA, small interfering RNA; TRBP, Tar RNA-binding protein.

In 2000, Choo and co-workers developed a retrovirus for an AsRNA strategy targeted against HPV-16 E7 oncoprotein, with the intent of studying its impact in the tumorigenicity of HPV-16-positive cervical cancer cells [78]. In that study, cell growth inhibition was observed, from a decrease in E7 oncoprotein levels. However, such inhibition was unable to result in tumor cell death, rather resulting in cell differentiation, a cell feature that is lost in cancer progression [78]. By contrast, Cho and co-workers conducted a study in 2002 by using a plasmid encoding an AsRNA for HPV-16 E6 oncoprotein in the same cell model used in Choo's research [79]. They were able to verify that, after transfection, cell growth was inhibited and an apoptotic morphology was acquired by the cells, which was confirmed by an increase in p53 levels [79]. These results suggested that E6 silencing leads to a more preeminent function in tumor suppression than E7 silencing.

Curiously, Yamato and co-workers took advantage of the bicistronic transcript and conducted a study with two synthetic siRNAs, designed to target different splicing variants of HPV-18 E6 mRNA. One of them was able to knockdown E6 and E7 expression and the other could successfully knockdown E6 levels but was unable to inhibit E7 as efficiently as the former [80]. Their findings showed that the isolated E6 suppression exerted a more significant tumor cell death effect comparatively to concomitant suppression of E6 and E7, suggesting that targeting E6 oncoprotein could be crucial when developing a siRNA-based strategy for cervical cancer induced by HPV [80]. Although these results seem to be in accordance with the investigation previously conducted by either Choo and Cho's research groups [78,79], it goes against the work developed by Jiang and Milner [81]. In 2002, the researchers published a study reporting the comparative use of synthetic siRNAs against either E6 or E7 HPV-16 oncoproteins. However, in contrast to what Choo *et al.* and Yamato *et al.* found in their studies [78,80], Jiang and Milner reported a reduced cell growth derived from E6 silencing and apoptosis induction from E7 inhibition [81]. These apparently contradictory results can be explained by the differences shown in study design, namely because different types of HPV were studied, distinct methods were used for silencing, the bicistronic nature of the transcription of which E6 and E7 mRNA are originated from, and considering the diverse splicing variants that can arise from such process. Moreover, as presented in Fig. 3a, the artificial RNA-based therapies integrate different pathways before interfering with E6 and E7 oncoprotein translation, which can result in different mRNA degradation levels. The delivery of these molecules, which can occur directly to the nucleus, through the use of an expression vector (such as shRNA), or to the cytoplasm after chemical synthesis (like siRNA molecules), can result in different outcomes, because it can lead to different levels of the intended RNA molecule [82]. In addition, different RNA structures present different stability and resistance to degradation, which can also affect the therapeutic outcome of these molecules [83]. Still, the use of shRNA appears to be more advantageous given that it can express, in a continuous form, silencing molecules that naturally integrate the endogenous miRNA pathway, leading to a stronger and more effective silencing effect than chemically synthetized siRNA [84]. Nevertheless, many studies have been dedicated in the past decade to the concomitant silencing of E6 and E7 oncoproteins with artificial RNA-based therapies. The most recent studies that have been performed by using AsRNA, siRNA or shRNA for simultaneous E6 and E7 silencing, as well as any major findings, are represented in Table 2. However, several obstacles have been found that can impair the implementation of these techniques. These limitations rely on the fact that RNA molecules are very unstable and can be rapidly degraded because of host RNase action [83]. Also, exogenous RNA molecules can present motifs that trigger immune responses owing to interferon stimulation, which can result in the development of severe adverse reactions [85]. To overcome this problem, many efforts have been focused on chemically altering the RNA structure to make it more stable and disable the stimulation of interferon [85]. Moreover, the development of suitable delivery strategies that protect the RNA molecule and direct it to the target tissue is crucial to increase the bioavailability of these biopharmaceuticals, and is currently the main goal of researchers working in this field [83,85]. Thus, further studies are necessary before proceeding with this strategy to clinical trials.

TABLE 2

Recent E6 and E7 silencing studies performed for AsRNA, shRNA and siRNA

Year	Silencing type	Antigen silencing	TargetedHPV type	Findings	Refs
2007	AsRNA	Simultaneous	HPV-16	p53 expression was significantly increased, followed by apoptosis and senescence detection in SiHa cells	[108]
2013	shRNA	Simultaneous	HPV-16	Tumor suppressor protein levels were increased in HPV-positive cell models and treated mice demonstrated lower tumor growth and higher survival rates than control	[109]
2016	siRNA	Simultaneous	HPV-16 HPV-18	Growth of subcutaneously injected HPV-positive cancer cells was suppressed in mice through systemic administration of siRNA by actively targeted polyion complex micelles	[110]
2017	siRNA	Simultaneous	HPV-16 HPV-18	Coupled with cisplatin or paclitaxel, tumor growth was suppressed in mice owing to synergistic effect between siRNA and chemotherapeutics	[111]
2018	shRNA	Simultaneous	HPV-16	Almost complete eradication of tumor mass in mice without adverse reactions	[112]
2018	AsRNA	Separate	HPV-16	AsRNA-E7 demonstrated higher inhibitory effects in tumor growth than AsRNA-E6	[113]

Abbreviations: AsRNA, antisense RNA; shRNA, short hairpin RNA; siRNA, small interfering RNA.

Gene editing with CRISPR/cas9 systems

More recently, CRISPR/cas9 systems have been described as the new cutting-edge technology of gene silencing strategies. The technology consists of a programmable RNA-guided endonuclease system that can be used to edit defective genes [86]. This technology, as represented in Fig. 3b, allows disruption of the DNA sequence of a target gene with the help of an RNA-guide strand, rendering the gene unable to be expressed.

Its application in HPV-induced cancers is more recent, Hu and co-workers developed a study, in 2014, regarding the use of CRISPR technology for the disruption of HPV-16 E7 gene in HPV-16-positive human cancer cell lines [87]. Apoptosis induction and decreased E7 oncoprotein levels were found in HPV-16-positive cancer cell lines such as CaSki and SiHa as opposed to HPV-16-negative cancer cell lines (C33A and HEK293). These findings highlighted the promising future of CRISPR/cas9 in HPV-induced cancer treatment. In a different approach, Zhen and co-workers used this system for the disruption of the HPV-16 E6/E7 promoter coupled with cisplastin, a chemical drug, in the HPV-16-positive cancer cell line SiHa. In this study, it was possible to verify that cell viability was lower in cells transfected with CRISPR/cas9 in comparison with cells only treated with cisplatin. However, when combining both strategies, a synergistic effect was detected, suggesting CRISPR/Cas was able to sensitize cells to cisplatin [88]. This study showed that CRISP/cas9 could be used concomitantly with chemotherapeutic drugs to potentiate its effect. Nevertheless, suitable delivery systems should be considered given that chemotherapeutic drugs are known to affect healthy tissues. Later on, Kennedy and co-workers also developed a CRISPR/cas9 system targeting E6 or E7 genes that was able to decrease the content of these oncoproteins and thus activate the p53 or pRB signaling pathway, respectively, in HPV-16- and HPV-18-positive cancer cell lines [89]. The CRISPR/cas9 systems used in this study were designed specifically for each type of protein (E6 and E7) and each type of HPV (16 and 18), resulting in four different CRISPR/cas9 systems.

More recently, Zhu *et al.* developed nanoparticles for the delivery of plasmid DNA encoding either CRISPR/cas9 or shRNA specific for E7 silencing [90]. Their findings suggested that shRNA is more effective in silencing the target protein owing to its functional status right upon plasmid transcription, whereas CRISPR/cas9 technology requires protein translation to function. Also, considering the permanent knockout condition of CRISPR/cas9 technology, and considering the possible off-targets that this technology can present, the use of shRNA is considered safer [90]. Nevertheless, despite the overall popularity and promising features of this technology, new findings suggest that its utilization could lead to large rearrangements in the genome, posing a severe threat to the regular cell function and perhaps leading to the emergence of pathological conditions [91]. Therefore, new studies must be performed accompanied by exhaustive genome examination, to guarantee the safe use of CRISPR/cas9 in humans.

microRNAs: an emergent silencing alternative

Lately, the study of microRNA profiling in cancer has been very important in detecting these molecules, because their expression levels can be used as prognostic markers. Several studies have revealed that, in different tissues, microRNAs can present significant alterations in the expression pattern and therefore contribute to a phenotypic cancer state. For instance, microRNA upregulation can contribute to the downregulation of proteins where function is fundamental for cell growth regulation, and *vice versa* [92]. Besides the fact that miRNA levels provide crucial information regarding the cellular mechanisms that can contribute to the onset of the disease, it can also indicate useful pathways to explore when trying to find a cure. These small noncoding RNAs that present 19–25 nucleotides are naturally expressed from miRNA-encoding genomic sequences [93,94]. First, as portrayed in Fig. 3c, its expression renders a long primary transcript (pri-miRNA), which will be cleaved by a complex formed by Drosha and protein DiGeorge syndrome critical region gene 8 (DGCR8) [93]. The processing of pri-miRNA by this complex results in a long hairpin molecule, the precursor of miRNA (pre-miRNA). As presented in Fig. 3c, this whole process occurs in the nucleus. Then, pre-miRNA is exported into the cytoplasm so that it can be cleaved by Dicer, which is coupled with Tar RNA-binding protein (TRBP) and kinase R-activating protein (PACT) [93]. This process presents a double-stranded mature miRNA. The antisense miRNA strand consists in the guide-strand for RNA-induced silencing complex (RISC), which will be loaded onto this complex once Dicer processing is complete. Then, the complex is able to identify target mRNAs that present some complementarity to the miRNA sequence [93]. Different outcomes can result according to the complementarity between RNA molecules, namely: degradation, destabilization or translation repression of target mRNA [93,95]. The main difference between siRNA and miRNA systems is the fact that miRNA can regulate hundreds of different target molecules whereas siRNA is only able to interfere with the expression of molecules with 100% complementarity [93,95]. This feature might be seen as a threat, given that miRNA interaction with off-targets can lead to severe cell deregulation. However, considering that miRNAs can regulate molecules and processes within similar functions and signaling pathways, the exploration of these molecules in therapy could also pose as an advantage in comparison to siRNA technology, because it could be used to simultaneously regulate different oncotargets. For instance, the research work developed by Wei and co-workers demonstrated that miR-17-5p was able to target tumor protein p53-induced nuclear protein 1 (TP53INP1) in cervical cancer cells – a protein that is usually upregulated in this type of tumor and is responsible for regulation of p53 transcriptional activity [96]. In this study, a plasmid expressing pri-miR-17-5p was able to inhibit the proliferation and the viability of cervical cancer cells.

Another microRNA where the tumor-suppressive function has been studied in cervical cancer is miR-125. *In vivo* studies performed by Cui *et al.* demonstrated that overexpression of miR-125b would decrease the ability of injected HeLa cells to form tumors in mice [97]. Moreover, Fan and co-workers were able to prove that miR-125a overexpression was able to suppress the metastasis of cervical cancer cells by targeting STAT3, leading to tumor growth inhibition and impairing metastasis formation in mice [98]. In this study, it was also possible to verify that the presence of HPV viral oncoproteins E6 and E7 would suppress the p53-mediated activation of miR-125a, suggesting that the downregulation of this microRNA is caused by the presence of the viral oncoproteins. Although these studies provide remarkable path-

ways that could be used for the treatment of cervical cancer, it still suggests that HPV infection might offer resistance to such treatments. Nevertheless, Jung *et al.* were able to prove that overexpression of miR-375 was able to directly downregulate E6 and E7 expression in HPV-16- and HPV-18-positive cervical cancer cell lines, rescuing p53 and pRB pathway signaling [99]. Moreover, the downregulation of E6AP, CIP2A and 14-3-3ζ oncogenic proteins was also seen in this study, contributing to a reduction of 35% telomerase activity and ultimately leading to cell-cycle arrest and inhibition of tumor cell proliferation [99]. The fact that this particular microRNA is able to silence E6 and E7 expression while downregulating other oncogenic proteins that are upregulated in cervical cancer cells suggests that the role of miR-375 in HPV-positive cervical cancer might be crucial. The exploration of therapeutic approaches that involve the expression of microRNAs able to regulate HPV-16 and HPV-18 transcripts and promote tumor suppressor protein expression, such as miR-375, can pose as a promising option in the future search for the ultimate HPV-induced cervical cancer treatment. Nevertheless, such a deed might still be remote, considering the amount of study and research that is necessary before exploring this strategy in humans.

Concluding remarks and future perspectives

The HPV impact in women's health is undeniable. It is crucial to invest in the prevention of this infection through the development of vaccines that cover the high-risk HPV types. It is also necessary to make sure these are affordable and can be easily distributed in less developed countries, which are currently the most affected by this virus. Given that vaccination has become a controversial issue recently, owing to increasing popular belief in a link between vaccines and severe adverse reactions, one must consider that full vaccination coverage and HPV eradication might be difficult to achieve in the near future. Therefore, suitable noninvasive and effective strategies should be explored to provide a comfortable and burden-free cervical cancer treatment to patients, as opposed to the currently available treatments. Here, we discussed three approaches that are currently being explored by researchers as a possible pathway that can lead to the discovery of such treatment. Each of these technologies: DNA vaccines, gene therapy and gene silencing, present their advantages and drawbacks. Currently, DNA vaccines stand as the most likely strategy to be implemented more rapidly for HPV treatment, when comparing their achievements in clinical trials with the remaining approaches. Nevertheless, the potential of gene therapy and gene silencing is undeniable and is currently under intensive study. Thus, the exploration and combination of these approaches poses as a fruitful and promising strategy in the future of HPV-induced cervical cancer treatment.

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References

1 Dürst, M. *et al.* (1983) A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc. Natl. Acad. Sci. U. S. A* 80, 3812–3815
2 zur Hausen, H. (1974) *Condylomata acuminata* and human genital cancer. *Cancer Res.* 36, 794
3 Schwarz, E. *et al.* (1985) Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* 314, 111–114
4 Bray, F. *et al.* (2013) Global estimates of cancer prevalence for 27 sites in the adult population in 2008. *Int. J. Cancer* 132, 1133–1145
5 Ferlay, J. *et al.* (2018) Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Cancer Epidemiol.* 144, 1941–1953. http://dx.doi.org/10.1002/ijc.31937
6 Stewart, B.W. and Wild, C.P. (2014) *World Cancer Report 2014*. doi: 92832042982014
7 de Sanjose, S. *et al.* (2010) Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. *Lancet Oncol.* 11, 1048–1056
8 Rogovskaya, S.I. *et al.* (2013) Human papillomavirus prevalence and type-distribution, cervical cancer screening practices and current status of vaccination implementation in Russian Federation, the Western countries of the former Soviet Union, Caucasus Region and Central Asia. *Vaccine* 31 . http://dx.doi.org/10.1016/j.vaccine.2013.06.043
9 Stern, P.L. *et al.* (2012) Therapy of human papillomavirus-related disease. *Vaccine* 30 . http://dx.doi.org/10.1016/j.vaccine.2012.05.091
10 van Doorslaer, K. *et al.* (2017) The papillomavirus episteme: a major update to the papillomavirus sequence database. *Nucleic Acids Res* 45, D499–506
11 Chan, P.K.S. *et al.* (2012) Laboratory and clinical aspects of human papillomavirus testing. *Crit. Rev. Clin. Lab. Sci.* 49, 117–136
12 Doorbar, J. *et al.* (2015) Human papillomavirus molecular biology and disease association. *Rev. Med. Virol.* 25, 2–23
13 Vande Pol, S.B. and Klingelhutz, A.J. (2013) Papillomavirus E6 oncoproteins. *Virology* 445, 115–137
14 Roman, A. and Munger, K. (2013) The papillomavirus E7 proteins. *Virology* 445, 138–168
15 DiMaio, D. and Petti, L.M. (2013) The E5 proteins. *Virology* 445, 99–114. http://dx.doi.org/10.1016/j.virol.2013.05.006
16 Thomas, M. *et al.* (1999) The role of the E6-p53 interaction in the molecular pathogenesis of HPV. *Oncogene* 18, 7690–7700. http://dx.doi.org/10.1038/sj.onc.1202953
17 Tomaić, V. (2016) Functional roles of E6 and E7 oncoproteins in HPV-induced malignancies at diverse anatomical sites. *Cancers* 8, 95. http://dx.doi.org/10.3390/cancers8100095
18 Duronio, R.J. and Xiong, Y. (2013) Signaling pathways that control cell proliferation. *Cold Spring Harb. Perspect. Biol.* 5, a008904. http://dx.doi.org/10.1101/cshperspect.a008904
19 Pang, C.L. and Thierry, F. (2013) Human papillomavirus proteins as prospective therapeutic targets. *Microb. Pathog* 58, 55–65. http://dx.doi.org/10.1016/j.micpath.2012.11.002
20 Shah, S.S. *et al.* (2016) Current technologies and recent developments for screening of HPV-associated cervical and oropharyngeal cancers. *Cancers* 8, 85. http://dx.doi.org/10.3390/cancers8090085
21 Ronco, G. *et al.* (2014) Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. *Lancet* 383, 524–532
22 La, K. *et al.* (2002) A controlled trial of a human papillomavirus type 16 vaccine. *N. Engl. J. Med.* 2002, 1645–1651
23 Mao, C. *et al.* (2006) Efficacy of human papillomavirus-16 vaccine to prevent cervical intraepithelial neoplasia: a randomized controlled trial. *Obstet. Gynecol.* 107, 18–27
24 Rowhani-Rahbar, A. *et al.* (2009) Longer term efficacy of a prophylactic monovalent human papillomavirus type 16 vaccine. *Vaccine* 27, 5612–5619

25 Villa, L.L. *et al.* (2005) Prophylactic quadrivalent human papillornavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomiseddouble-blind placebo-controlled multicentre Phase II efficacy trial. *Lancet Oncol* 6, 271–278

26 Villa, L.L. *et al.* (2006) High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up. *Br. J. Cancer* 95, 1459–1466

27 Garland, S.M. *et al.* (2007) Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. *N. Engl. J. Med.* 356, 1928–1943

28 FUTURE II Study Group (2007) Quadrivalent vaccine against human papillomavirus to prevent high-grade cervical lesions. *N. Engl. J. Med.* 356, 1915–1927

29 Ault, K.A. (2007) Effect of prophylactic human papillomavirus L1 virus-like-particle vaccine on risk of cervical intraepithelial neoplasia grade 2, grade 3, and adenocarcinoma *in situ*: a combined analysis of four randomised clinical trials. *Lancet* 369, 1861–1868. http://dx.doi.org/10.1016/S0140-6736(07)60852-6

30 Harper, D.M. *et al.* (2006) Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. *Lancet* 367, 1247–1255

31 Paavonen, J. *et al.* (2009) Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): final analysis of a double-blind, randomised study in young women. *Lancet* 374, 301–314

32 Einstein, M.H. *et al.* (2014) Comparison of long-term immunogenicity and safety of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine and HPV-6/11/16/18 vaccine in healthy women aged 18-45 years: end-of-study analysis of a Phase III randomized trial. *Hum. Vaccines Immunother* 10, 3435–3445

33 Naud, P.S. *et al.* (2014) Sustained efficacy, immunogenicity, and safety of the HPV-16/18 AS04-adjuvanted vaccine: final analysis of a long-term follow-up study up to 9.4 years post-vaccination. *Hum. Vaccines Immunother* 10, 2147–2162

34 Nygård, M. *et al.* (2015) Evaluation of the long-term anti-human papillomavirus 6 (HPV6), 11, 16, and 18 immune responses generated by the quadrivalent HPV vaccine. *Clin. Vaccine Immunol.* 22, 943–948

35 Joura, E.A. *et al.* (2015) A 9-valent HPV vaccine against infection and intraepithelial neoplasia in women. *Obstet. Gynecol. Surv.* 70, 446–448

36 Luxembourg, A. *et al.* (2015) Phase III, randomized controlled trial in girls 9–15 years old to evaluate lot consistency of a novel nine-valent human papillomavirus L1 virus-like particle vaccine. *Hum. Vaccines Immunother.* 11, 1306–1312

37 van Damme, P. *et al.* (2016) A Phase III clinical study to compare the immunogenicity and safety of the 9-valent and quadrivalent HPV vaccines in men. *Vaccine* 34, 4205–4212

38 Lefèvre, H. *et al.* (2018) HPV vaccination rate in French adolescent girls: an example of vaccine distrust. *Arch. Dis. Child.* 103, 740–746. http://dx.doi.org/10.1136/archdischild-2017-313887

39 Larson, H.J. *et al.* (2014) Tracking the global spread of vaccine sentiments: the global response to Japan's suspension of its HPV vaccine recommendation. *Hum. Vaccines Immunother.* . http://dx.doi.org/10.4161/21645515.2014.969618

40 Marth, C. *et al.* (2017) Cervical cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol* 28, iv72–83

41 Pérez-Herrero, E. and Fernández-Medarde, A. (2015) Advanced targeted therapies in cancer: drug nanocarriers, the future of chemotherapy. *Eur. J. Pharm. Biopharm.* 93, 52–79

42 Hasson, S.S.A.A. *et al.* (2015) The past, current and future trends in DNA vaccine immunisations. *Asian Pac. J. Trop. Biomed.* 5, 344–353

43 Li, L. and Petrovsky, N. (2016) Molecular mechanisms for enhanced DNA vaccine immunogenicity. *Expert Rev. Vaccines* 15, 313–329

44 Lee, J. *et al.* (2018) Engineering DNA vaccines against infectious diseases. *Acta Biomater.* 80, 31–47

45 Yang, B. *et al.* (2014) DNA vaccine for cancer immunotherapy. *Hum. Vaccines Immunother.* . http://dx.doi.org/10.4161/21645515.2014.980686

46 Lichtor, T. and Glick, R.P. (2012) Immunogene therapy. *Adv. Exp. Med. Biol.* 746, 151–165

47 Almeida, A.M. *et al.* (2015) Optimization of supercoiled HPV-16 E6/E7 plasmid DNA purification with arginine monolith using design of experiments. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 978–979, 145–150

48 Hancock, G. *et al.* (2018) Therapeutic HPV vaccines. *Best Pract. Res. Clin. Obstet. Gynaecol.* 47, 59–72

49 Gulce-Iz, S. and Saglam-Metiner, P. (2019) Current state of the art in DNA vaccine delivery and molecular adjuvants: Bcl-xL anti-apoptotic protein as a molecular adjuvant. *Immune Response Act. Immunomodulation IntechOpen* . http://dx.doi.org/10.5772/intechopen.82203 https://www.intechopen.com/books/immune-response-activation-and-immunomodulation/current-state-of-the-art-in-dna-vaccine-delivery-and-molecular-adjuvants-bcl-xl-anti-apoptotic-prote

50 Yang, B. *et al.* (2014) DNA vaccine for cancer immunotherapy. *Hum. Vaccines Immunother.* 10, 3153–3164

51 Kim, T.J. *et al.* (2014) Clearance of persistent HPV infection and cervical lesion by therapeutic DNA vaccine in CIN3 patients. *Nat. Commun* 5 . http://dx.doi.org/10.1038/ncomms6317

52 Samuels, S. *et al.* (2017) HPV16 E7 DNA tattooing: safety, immunogenicity, and clinical response in patients with HPV-positive vulvar intraepithelial neoplasia. *Cancer Immunol. Immunother.* . http://dx.doi.org/10.1007/s00262-017-2006-y

53 Trimble, C.L. *et al.* (2009) A Phase I trial of a human papillomavirus DNA vaccine for HPV16+ cervical intraepithelial neoplasia 2/3. *Clin. Cancer Res* 15, 361–367

54 Chandra, J. *et al.* (2017) DNA vaccine encoding HPV16 oncogenes E6 and E7 induces potent cell-mediated and humoral immunity which protects in tumor challenge and drives E7-expressing skin graft rejection. *J. Immunother.* 40, 62–70

55 Silva, J.R. *et al.* (2019) Expression of a soluble IL-10 receptor enhances the therapeutic effects of a papillomavirus-associated antitumor vaccine in a murine model. *Cancer Immunol. Immunother.* 68, 753–763

56 Maldonado, L. *et al.* (2014) Vaccination: intramuscular therapeutic vaccination targeting HPV16 induces T cell responses that localize in mucosal lesions. *Sci. Transl. Med.* 6, 221ra13. http://dx.doi.org/10.1126/scitranslmed.3007323

57 Trimble, C.L. *et al.* (2015) Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled Phase 2b trial. *Lancet* 386, 2078–2088. http://dx.doi.org/10.1016/S0140-6736(15)00239-1

58 Inovio Pharmaceuticals (2019) *Inovio Completes Enrollment of VGX-3100 Phase 3 Trial (REVEAL 1) for the Treatment of HPV-Related Cervical Pre-cancer*. 2019 Available at: http://ir.inovio.com/news-and-media/news/press-release-details/2019/Inovio-Completes-Enrollment-of-VGX-3100-Phase-3-Trial-REVEAL-1-for-the-Treatment-of-HPV-Related-Cervical-Pre-cancer/default.aspx

59 Alvarez, R.D. *et al.* (2016) A pilot study of pNGVL4a-CRT/E7(detox) for the treatment of patients with HPV16 + cervical intraepithelial neoplasia 2/3 (CIN2/3). *Gynecol. Oncol.* 140, 245–252

60 Hobernik, D. and Bros, M. (2018) DNA vaccines – how far from clinical use? *Int. J. Mol. Sci.* 19, 3605

61 Wang, W. *et al.* (2013) Non-viral gene delivery methods. *Curr. Pharm. Biotechnol.* 14, 46–60

62 Naldini, L. (2015) Gene therapy returns to centre stage. *Nature* 526, 351–360

63 Sheridan, C. (2011) Gene therapy finds its niche. *Nat. Biotechnol.* 29, 121–128

64 Ginn, S.L. *et al.* (2018) Gene therapy clinical trials worldwide to 2017: an update. *J. Gene Med.* 20, e3015

65 Ip, S.M. *et al.* (2001) pRb-expressing adenovirus Ad5-Rb attenuates the p53-induced apoptosis in cervical cancer cell lines. *Eur. J. Cancer* 37, 2475–2483. http://dx.doi.org/10.1016/S0959-8049(01)00308-2

66 Hunt, K.K. *et al.* (1997) Adenovirus-mediated overexpression of the transcription factor E2F-1 induces apoptosis in human breast and ovarian carcinoma cell lines and does not require p53. *Cancer Res.* 57, 4722–4726

67 Valente, J.F. *et al.* (2018) p53 as the focus of gene therapy: past, present and future. *Curr. Drug Targets* 19 . http://dx.doi.org/10.2174/1389450119666180115165447

68 Prabhu, N.S. *et al.* (1998) p73beta, unlike p53, suppresses growth and induces apoptosis of human papillomavirus E6-expressing cancer cells. *Int. J. Oncol.* 13, 5–14

69 Das, S. *et al.* (2003) Efficient growth inhibition of HPV 16 E6-expressing cells by an adenovirus-expressing p53 homologue p73β. *Oncogene* 22, 8394–8402. http://dx.doi.org/10.1038/sj.onc.1206908

70 Das, S. and Somasundaram, K. (2006) Therapeutic potential of an adenovirus expressing p73 beta, a p53 homologue, against human papilloma virus positive cervical cancer *in vitro* and *in vivo*. *Cancer Biol. Ther.* 5, 210–217

71 Guan, Y.S. *et al.* (2005) Successful management of postoperative recurrence of hepatocellular carcinoma with p53 gene therapy combining transcatheter arterial chemoembolization. *World J. Gastroenterol* 11, 3803–3805. http://dx.doi.org/10.3748/wjg.v11.i24.3803

72 Su, X. *et al.* (2016) Effect and safety of recombinant adenovirus-p53 transfer combined with radiotherapy on long-term survival of locally advanced cervical cancer. *Hum. Gene Ther.* 27, 1008–1014

73 Zhang, W.-W. *et al.* (2018) The first approved gene therapy product for cancer Ad-p53 (Gendicine): 12 years in the clinic. *Hum. Gene Ther.* 29, 160–179. http://dx.doi.org/10.1089/hum.2017.218

74 Tomizawa, J. *et al.* (1981) Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. *Proc. Natl. Acad. Sci. U. S. A.* 78, 1421–1425. http://dx.doi.org/10.1073/pnas.78.3.1421

75 Guo, S. and Kemphues, K.J. (1995) par-1, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* 81, 611–620. http://dx.doi.org/10.1016/0092-8674(95)90082-9

76 Fire, A. *et al.* (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811. http://dx.doi.org/10.1038/35888

77 von Knebel Doeberitz, M. *et al.* (1988) Correlation of modified human papilloma virus early gene expression with altered growth properties in C4-1 cervical carcinoma cells. *Cancer Res.* 48, 3780–3786

78 Choo, C.K. *et al.* (2000) Retrovirus-mediated delivery of HPV16 E7 antisense RNA inhibited tumorigenicity of CaSki cells. *Gynecol. Oncol.* 78, 293–301. http://dx.doi.org/10.1006/gyno.2000.5916

79 Cho, C.W. *et al.* (2002) HPV E6 antisense induces apoptosis in CaSki cells via suppression of E6 splicing. *Exp. Mol. Med.* 34, 159–166. http://dx.doi.org/10.1038/emm.2002.23

80 Yamato, K. *et al.* (2006) Induction of cell death in human papillomavirus 18-positive cervical cancer cells by E6 siRNA. *Cancer Gene Ther.* 13, 234–241. http://dx.doi.org/10.1038/sj.cgt.7700891

81 Jiang, M. and Milner, J. (2002) Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. *Oncogene* 21, 6041–6048. http://dx.doi.org/10.1038/sj.onc.1205878

82 Tang, S. *et al.* (2006) Short-term induction and long-term suppression of HPV16 oncogene silencing by RNA interference in cervical cancer cells. *Oncogene* 25, 2094–2104. http://dx.doi.org/10.1038/sj.onc.1209244

83 Castanotto, D. and Rossi, J.J. (2009) The promises and pitfalls of RNA-interference-based therapeutics. *Nature* 457, 426–433. http://dx.doi.org/10.1038/nature07758

84 Rao, D.D. *et al.* (2009) siRNA vs. shRNA: similarities and differences. *Adv. Drug Deliv. Rev.* 61, 746–759. http://dx.doi.org/10.1016/j.addr.2009.04.004

85 De Vincenzo, J.P. (2012) The promise, pitfalls and progress of RNA-interference-based antiviral therapy for respiratory viruses. *Antivir. Ther.* 17, 213–225. http://dx.doi.org/10.3851/IMP2064

86 Zhen, S. *et al.* (2014) *In vitro* and *in vivo* growth suppression of human papillomavirus 16-positive cervical cancer cells by CRISPR/Cas9. *Biochem. Biophys. Res. Commun.* 450, 1422–1426. http://dx.doi.org/10.1016/j.bbrc.2014.07.014

87 Hu, Z. *et al.* (2014) Disruption of HPV16-E7 by CRISPR/Cas system induces apoptosis and growth inhibition in HPV16 positive human cervical cancer cells. *Biomed. Res. Int.* 2014 . http://dx.doi.org/10.1155/2014/612823

88 Zhen, S. *et al.* (2016) *In vitro* and *in vivo* synergistic therapeutic effect of cisplatin with human papillomavirus16 E6/E7 CRISPR/CAS9 on cervical cancer cell line. *Transl. Oncol.* 9, 498–504. http://dx.doi.org/10.1016/j.tranon.2016.10.002

89 Kennedy, E.M. *et al.* (2014) Inactivation of the human papillomavirus E6 or E7 gene in cervical carcinoma cells by using a bacterial CRISPR/Cas RNA-guided endonuclease. *J. Virol.* 88, 11965–11972. http://dx.doi.org/10.1128/JVI.01879-14

90 Zhu, D. *et al.* (2018) Nanoparticles based on poly (β-amino ester) and HPV16 targeting CRISPR/shRNA as potential drugs for HPV16 related cervical malignancy. *Mol. Ther.* 26, 2443–2455. http://dx.doi.org/10.1016/J.YMTHE.2018.07.019

91 Kosicki, M. *et al.* (2018) Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. *Nat. Biotechnol.* 36, 765–771. http://dx.doi.org/10.1038/nbt.4192

92 Li, J. *et al.* (2017) Deregulated miRNAs in human cervical cancer: functional importance and potential clinical use. *Fut. Oncol.* 13, 743–753. http://dx.doi.org/10.2217/fon-2016-0328

93 Pereira, P. *et al.* (2017) Current progress on microRNAs-based therapeutics in neurodegenerative diseases. *Wiley Interdiscip. Rev. RNA* 8, e1409. http://dx.doi.org/10.1002/wrna.1409

94 Konicke, K. *et al.* (2018) The microRNA landscape of cutaneous squamous cell carcinoma. *Drug Discov. Today* 23, 864–870. http://dx.doi.org/10.1016/j.drudis.2018.01.023

95 Díaz-González, S.D.M. *et al.* (2015) Utility of microRNAs and siRNAs in cervical carcinogenesis. *Biomed. Res. Int.* 2015 . http://dx.doi.org/10.1155/2015/374924

96 Wei, Q. *et al.* (2012) miR-17-5p targets TP53INP1 and regulates cell proliferation and apoptosis of cervical cancer cells. *IUBMB Life* 64, 697–704. http://dx.doi.org/10.1002/iub.1051

97 Cui, F. *et al.* (2012) miR-125b inhibits tumor growth and promotes apoptosis of cervical cancer cells by targeting phosphoinositide 3-kinase catalytic subunit delta. *Cell. Physiol. Biochem.* 30, 1310–1318. http://dx.doi.org/10.1159/000343320

98 Fan, Z. *et al.* (2015) miR-125a suppresses tumor growth, invasion and metastasis in cervical cancer by targeting STAT3. *Oncotarget* . http://dx.doi.org/10.18632/oncotarget.4457

99 Jung, H.M. *et al.* (2014) miR-375 activates p21 and suppresses telomerase activity by coordinately regulating HPV E6/E7, E6AP, CIP2A, and 14-3-3ζ. *Mol. Cancer* 13 . http://dx.doi.org/10.1186/1476-4598-13-80

100 Kim, D. *et al.* (2008) Enhancement of DNA vaccine potency through coadministration of CIITA DNA with DNA vaccines via gene gun. *J. Immunol.* 180, 7019–7027. http://dx.doi.org/10.4049/jimmunol.180.10.7019

101 Ahn, J. *et al.* (2017) Immunologic responses to a novel DNA vaccine targeting human papillomavirus-11 E6E7. *Laryngoscope* 127, 2713–2720. http://dx.doi.org/10.1002/lary.26737

102 Seo, S.H. *et al.* (2009) Optimal induction of HPV DNA vaccine-induced CD8+ T cell responses and therapeutic antitumor effect by antigen engineering and electroporation. *Vaccine* 27, 5906–5912. http://dx.doi.org/10.1016/j.vaccine.2009.07.033

103 Zurkova, K. *et al.* (2009) The expression of the soluble isoform of hFlt3 ligand by recombinant vaccinia virus enhances immunogenicity of the vector. *Oncol. Rep.* 21, 1335–1343. http://dx.doi.org/10.3892/or_00000359

104 Sun, Y. *et al.* (2017) Coinjection of IL2 DNA enhances E7-specific antitumor immunity elicited by intravaginal therapeutic HPV DNA vaccination with electroporation. *Gene Ther* 24, 408–415. http://dx.doi.org/10.1038/gt.2017.38

105 Oosterhuis, K. *et al.* (2011) Preclinical development of highly effective and safe DNA vaccines directed against HPV 16 E6 and E7. *Int. J. Cancer* 129, 397–406. http://dx.doi.org/10.1002/ijc.25894

106 Baghban Rahimi, S. *et al.* (2018) Enhancement of therapeutic DNA vaccine potency by melatonin through inhibiting VEGF expression and induction of antitumor immunity mediated by CD8+ T cells. *Arch. Virol.* 163, 587–597

107 Garza-Morales, R. *et al.* (2019) A DNA vaccine encoding SA-4-1bbl fused to HPV-16 E7 antigen has prophylactic and therapeutic efficacy in a cervical cancer mouse model. *Cancers* 11, 96

108 Sima, N. *et al.* (2007) Antisense targeting human papillomavirus type 16 E6 and E7 genes contributes to apoptosis and senescence in SiHa cervical carcinoma cells. *Gynecol. Oncol* 106, 299–304. http://dx.doi.org/10.1016/j.ygyno.2007.04.039

109 Zhou, J. *et al.* (2013) Inhibition of cervical cancer cell growth *in vitro* and *in vivo* by lentiviral-vector mediated shRNA targeting the common promoter of HPV16 E6 and E7 oncogenes. *Antiviral Res.* 98, 305–313. http://dx.doi.org/10.1016/j.antiviral.2013.03.010

110 Nishida, H. *et al.* (2016) Systemic delivery of siRNA by actively targeted polyion complex micelles for silencing the E6 and E7 human papillomavirus oncogenes. *J. Control. Release* 231, 29–37. http://dx.doi.org/10.1016/j.jconrel.2016.03.016

111 Rajasekaran, N. *et al.* (2017) Effect of HPV E6/E7 siRNA with chemotherapeutic agents on the regulation of TP53/E2F dynamic behavior for cell fate decisions. *Neoplasia* 19, 735–749. http://dx.doi.org/10.1016/j.neo.2017.07.005

112 Sato, N. *et al.* (2018) Eradication of cervical cancer in vivo by an AAV vector that encodes shRNA targeting human papillomavirus type 16 E6/E7. *Int. J. Oncol* 52, 687–696. http://dx.doi.org/10.3892/ijo.2018.4245

113 Kojima, Y. *et al.* (2018) Adenovirus-mediated transfer of HPV 16 E6/E7 antisense RNA combined with cisplatin inhibits cellular growth and induces apoptosis in HPV-positive head and neck cancer cells. *Cancer Gene Ther* 25, 274–283. http://dx.doi.org/10.1038/s41417-018-0024-3