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Oligonucleotide Applications for the Therapy and Diagnosis of Human Papillomavirus Infection

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1. Introduction

Cervical cancer is the best known example of a common human malignancy with a proven infectious etiology. Clinical, epidemiological and molecular analyses have long indicated that persistent infection with high-risk human papillomaviruses (HPVs) is causally associated with cervical cancer. Although new prophylactic vaccines and highly sensitive HPV typing methods are currently available, cervical cancer continues as the most common tumor in developing countries, where most of the annual half a million new cases occur (Arbyn et al., 2011). Besides socio-cultural issues restraining professional gynecological care, the relatively high cost of these technologies has limited their availability where they are most needed. Thus, there is a pressing need for affordable and readily available detection and therapeutic tools for HPV infection and cervical cancer. In the last two decades, novel diagnostic and therapeutic approaches based on synthetic oligonucleotides and genomic information have developed into promising tools to fight human disease.

2. HPV and cervical cancer

Genital dysplasia and cervical cancer are associated with persistent infection of a subset of HPVs referred as high-risk, including HPV types 16, 18, 31, 33, 45, 52 and 58 (Clifford et al., 2003). High-risk HPVs normally replicate in keratinocytes from stratified squamous epithelia of their hosts where the 8-kb double-stranded circular DNA genome is usually retained in an episomal form. The highly conserved high-risk HPV genomes consist of six common early genes (E1, E2, E4, E5, E6 and E7) and two late genes (L1 and L2) coding for the capsid proteins (Figure 1). The early genes contribute to cellular transformation, viral regulation and DNA replication (Moody & Laimins, 2010). In addition, the HPV genome also comprises a highly variable non-coding regulatory region, the long control region (LCR), which contains the viral origin of replication and regulatory elements targeted by several cellular transcription factors and the viral E2 gene (Hebner & Laimins, 2006).

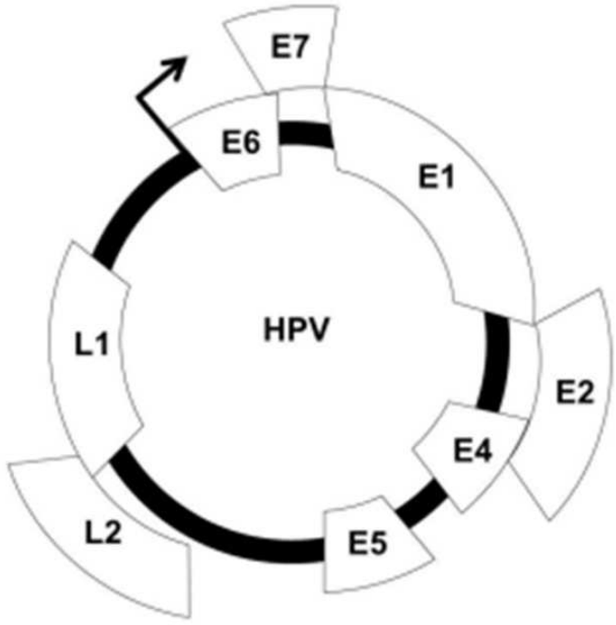

Fig. 1. Map of the HPV genome. Early (E) and ate (L) genes are indicated. The arrow indicates the early promoter.

2.1. HPV targets for diagnostic and therapeutic oligonucleotides

The relationship between cervical cancer and high-risk HPVs is related to the integration of viral DNA to the host genome with the disruption of the viral regulator E2 (Pett & Coleman, 2007; Vernon et al., 1997), and to the production of viral proteins E6 and E7 which are sufficient and necessary to acquire and maintain a transformed phenotype (Pirisi et al., 1988; Xue et al., 2010). Because E2 is usually absent in cervical tumor cells and E6/E7 genes are retained and expressed in most tumors, these features are often referred to as the hallmarks of cervical cancer (Alvarez-Salas & DiPaolo, 2007). In the absence of E2 protein, high-risk HPV E6 and E7 genes are continuously transcribed from a single promoter resulting in polycistronic mRNA containing both transcripts (Wang et al., 2011). The E6 and E7 protein products interact and functionally neutralize key cellular regulatory proteins, so that cell proliferation continues.

High-risk HPV E6 protein targets numerous cellular pathways to insure viral DNA replication and is a key oncogene in HPV associated neoplasias. E6 was first shown to interact with the p53 tumor suppressor protein (Werness et al., 1990) and the E6-AP complex to act as a ubiquitin protein ligase (Huibregtse et al., 1991) inducing the specific ubiquitination and degradation of p53 (Scheffner et al., 1993). Thus, high-risk HPV E6 results in blockage of p53-mediated apoptosis. Interestingly, E6 has been found interacting with the extrinsic apoptotic factors TNFR-1, FADD and caspase-8 (Filippova et al., 2002; Tungteakkhun et al., 2010) suggesting alternative apoptosis inhibitory functions. Although E6-mediated degradation of p53 is considered a key event for the onset of cellular transformation, it is clear that E6 possess other p53-independent transforming and anti-apoptotic activities, such as telomerase activation (Gewin et al., 2004; Klingelhutz et al., 1996; Oh et al., 2001). Many other cellular targets of high-risk E6 proteins have now been

described, including PDZ domain-containing targets such as the human homologue of the tumor suppressor DLG (discs large protein) (Gardiol et al., 1999), MUPP1 (Lee et al., 2000) and MAGUK (membrane-associated guanylate kinase) proteins (Glaunsinger et al., 2000), and a number of transcription regulators (Etscheid et al., 1994; Zimmermann et al., 1999), disrupting cell adhesion, polarity, epithelial differentiation and reducing immune recognition of HPV infected cells (Howie et al., 2009).

The E7 protein plays a vital role in the viral life cycle by disrupting the tight link between differentiation and proliferation, thus allowing viral replication in normal keratinocytes that would be otherwise withdrawn from the cell cycle (McLaughlin-Drubin & Munger, 2009). E7 protein from high-risk HPVs targets pRB and disrupts the E2F-mediated transcriptional regulation resulting in the up-regulation of genes required for G1/S transition and DNA synthesis (Duensing et al., 2001; Munger & Phelps, 1993). HPV-16 E7 can directly bind the G1/S transition antagonists E2F1 (Hwang et al., 2002) and E2F6 (McLaughlin-Drubin et al., 2008) thus ensuring that the infected cells remain in an S-phase-competent state allowing HPVs to bypass negative growth signals. The steady-state level and metabolic half-life of pRB are decreased in HPV-16 E7-expressing cells, because E7 can induce the degradation of pRB through the ubiquitin-proteasome system (Berezutskaya et al., 1997; Boyer et al., 1996). High-risk HPV E7 proteins also contribute to cell cycle dysregulation through the abrogation of the growth inhibitory activities of $p21^{CIP1}$ and $p26^{KIP1}$ (Funk et al., 1997; Jones et al., 1997; Zerfass-Thome et al., 1996). Other functions associated to high-risk HPV expression include epigenetic reprogramming through induction of KDM6A and KDM6B histone demethylases (McLaughlin-Drubin et al., 2011), trophic sentinel signaling abrogation and autophagy induction (Zhou & Munger, 2009), induction of genomic instability (Duensing et al., 2000), and disruption of *Anoikis* signaling through interaction with p600 (Huh et al., 2005).

3. Oligonucleotide applications to cervical cancer and HPV infection

Although the independent E6 and E7 functions may cause genomic instability, cell immortalization and transformation by themselves, the unregulated expression of both proteins is considered the major contribution of HPVs to cervical cancer development. The demonstration of the existence of stable molecular targets in high-risk HPVs has justified the development of small oligonucleotides for cervical cancer detection and treatment. High-risk HPV-16 and 18 express E6 and E7 proteins from a single polycistronic mRNA (Schneider-Gadicke & Schwarz, 1986; Smotkin et al., 1989), suggesting that targeting of either E6 or E7 mRNA would likely impede both E6/E7 translation resulting in similar growth arrest phenotypes. Several groups have identified that inhibition of these genes translation resulted in tumor growth suppression confirming E6/E7 as attractive targets for cervical cancer therapy (Alvarez-Salas et al., 1998; Shillitoe, 2006; Venturini et al., 1999).

In the last decades, novel therapeutic approaches based on genomic information developed into promising tools to fight human disease. Therapeutic oligonucleotides are short DNA or RNA molecules designed to disrupt expression or function of disease-related genes. Approaches to therapeutic oligonucleotide technology include: 1) Blocking of gene transcription by triplex-forming oligodeoxyribonucleotides (TFOs); 2) Translation inhibition by AS-ODNs, small interfering RNAs (siRNAs) and ribozymes; 3) Inhibition of protein function by nucleic acid aptamers and 4) Immunostimulatory oligonucleotides (IM-ONS)

(Alvarez-Salas, 2008). Diagnostic oligonucleotides refer to the application of DNA or RNA oligonucleotides for diagnostic purposes. Diagnostic oligonucleotide technologies comprise oligonucleotides designed for 1) Priming polymerase chain reaction (PCR or RT-PCR) detection, 2) Hybridization-based technologies (hybrid capture or microarrays) and 3) Binding with target proteins (aptamers). Here, we will only discuss diagnostic oligonucleotides used as aptamers for HPV detection because PCR and hybridization technologies have been extensively used for HPV diagnostics for decades and the subject has been comprehensively analyzed elsewhere (Stanley, 2010).

3.1 Antigene technology

TFOs can hybridize with particular sequences in double-stranded DNA (dsDNA) through the formation of Hoogsteen or reverse-Hoogsteen hydrogen bonds between the TFOs and homopurine stretches found in the major groove of the target DNA (Letai et al., 1988; Moser & Dervan, 1987). TFOs have potential for manipulating gene structure and function in living cells, inhibiting transcription by interfering with regulatory protein binding or blocking mRNA elongation (Carbone et al., 2003) (Figure 2A). Although finding appropriate targets for TFO action in genomic DNA may be an issue, TFO technology has a potential advantage over oligonucleotide-based control of translation (antisense and siRNA technologies) because there are generally one to two targets per cell as compared with the hundreds to thousands copies of mRNA targets (Vasquez & Glazer, 2002).

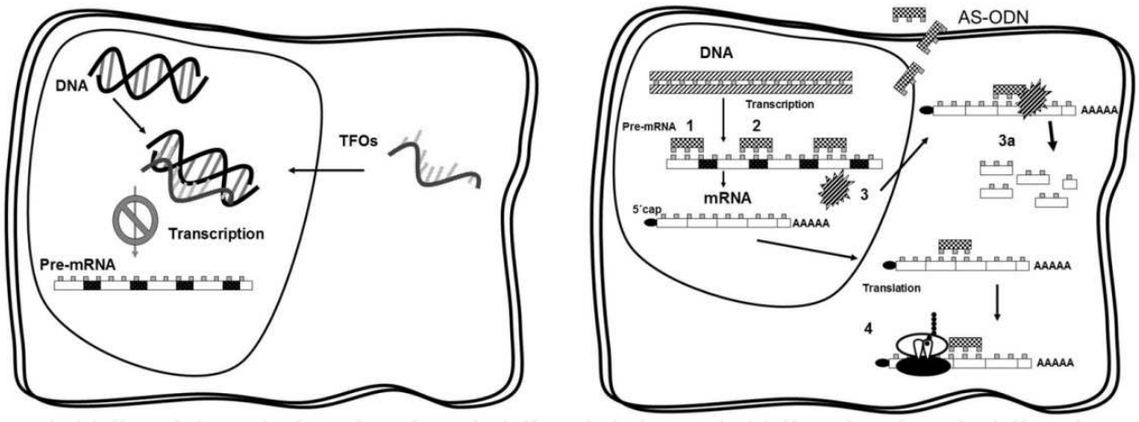

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Fig. 2. A) Antigene technology. Triplex-forming oligonucleotides (TFOs) hybridize with DNA to block transcription. B) Antisense oligonucleotides (AS-ODNs) gene silencing. As-ODNs hybridize with the 5′-UTR (1) or within the coding region (2) of the target mRNA. Formation of DNA-RNA heteroduplexes induces RNaseH (3) activity over the RNA target (3a) producing degradation and thus inhibiting translation. Stable hybridization with the target 5′-UTR mRNA can inhibit ribosome anchorage (4).

3.1.1 Antigene technology on cervical cancer

Several natural triplex-forming sites have been identified within the HPV-16 genome (Malkov et al., 1993). However, only a few attempts have been reported on the use of TFOs against HPV. An initial report for the low-risk HPV-11 established an intramolecular triplex

DNA formation using a TFO directed to a 22nt-long homopurine stretch within the LCR (nt 21-42) overlapping a Sp-1 and a E2 binding site plus the origin of replication. Although the stability of the triplex DNA formation was comprehensively demonstrated, no functional effect on HPV-11 transcription/replication was reported (Hartman et al., 1992). Other report also established formation of pyrimidine-purine-pyrimidine or pyrimidine-purine-purine triplex DNA with the HPV-16 fragment nt 554-685 under favorable conditions using complementary pyrimidine or purine TFOs. The observed DNA triplexes exhibited remarkable sequence specificity (Cherny et al., 1993).

Optical DNA melting experiments and co-migration assay were used to establish stable triplex DNA formation at homopurine-homopyrimidine-rich target sites present in the HPV-16 E7 gene (nt 656-673). The target sequence was specifically recognized by several 17-mer purine TFOs designed to form antiparallel or parallel triplex DNA helices (Popa et al., 1996). Despite the reported success on triplex DNA formation using TFOs and different HPV targets, there are no reports on the application of TFOs to inhibit HPV transcription suggesting that intracellular conditions may not allow effective antigene-based therapies.

3.2 The antisense approach

Antisense inhibition uses DNA sequence information to synthesize an oligonucleotide complementary to a target mRNA and specifically inhibit or modify translation by three main mechanisms: A) Translational arrest by inhibiting ribosome binding at the 5'-UTR (Crooke, 1999); B) Induced degradation of the target mRNA by RNaseH (Agrawal et al., 1990) (Figure 2B), and C) Translational modulation by exon-skipping (Du & Gatti, 2009). The aim of all antisense approaches is to reduce the quantity of the target protein in order to revert or prevent progression of a disease process.

Two main classes of antisense oligonucleotides have been used to silence or modulate gene expression providing that the target sequences are exposed (in a single stranded form lacking secondary structure or protein binding). One class makes use of AS-ODNs complementary to the target mRNA. AS-ODN hybridization to its complementary target mRNA by Watson–Crick base pairing should provide enough specificity and affinity to produce translational arrest (physical blockage of ribosome binding) of the target mRNA. Additionally, the formation of DNA-RNA heteroduplexes leads to the activation of RNaseH thus inducing cleavage of the target mRNA (Bonham et al., 1995). The second antisense oligonucleotide technology class consists of small, catalytic RNA or DNA molecules. The catalytic core of these molecules produces cleavage of a target RNA once the catalytic moiety has hybridized with a Watson-Crick complementary sequence (Benitez-Hess & Alvarez-Salas, 2006). This mechanism may be contrasted with AS-ODNs that require cellular RNaseH activity following hybridization in order to cleave the target mRNA.

3.2.1 AS-ODNs applications as therapeutic moieties

Early use of unmodified AS-ODNs showed that they were highly unstable in biological fluids due to the presence of exonucleases thus limiting their use as therapeutic moieties. Later, several nucleotide analogues were introduced to increase ODN stability. To avoid changes in hybridization specificity, modifications were limited to the phosphate and ribose

backbone of DNA/RNA oligonucleotides. First generation modified AS-ODNs consisted of sulfur-substituted DNA on the free oxygen molecules constituting a phosphodiester bond. Phosphorothioated ODNs (PS-ODNs) display a high degree of stability in biofluids while retaining the ability to form RNaseH substrates leading to efficient, highly specific degradation of the target mRNA (Agrawal & Zhang, 1997). RNaseH activity is mostly nuclear and thus would likely produce cleavage of the pre-mRNA within the nucleus before splicing (Wagner et al., 1993). However, the strongly polyanionic nature of PS-ODNs cause *in vivo* issues regarding affinity, specificity, cellular uptake, biodistribution and toxicity thus limiting their therapeutical use (Akhtar & Agrawal, 1997). Nevertheless, PS-ODNs have been the most extensively studied AS-ODNs in various animal models and humans leading to oligonucleotide-based drugs such as Fomivirsen (Vitravene™) or Affinitak (Patil et al., 2005).

The use of other modifications such as methylphosphonates, ethylphosphonates or 2'-*O*-methyl, confer high affinity for target sequences and extraordinary stability in biofluids, but they do not activate RNaseH (Mercatante & Kole, 2000). Thus, these modifications are better suited for modulating gene expression or exon-skipping approaches rather than gene silencing (Sierakowska et al., 2000). Second-generation AS-ODNs provide molecules with RNaseH activity but minimal off-target effects. Mixed-backbone oligodeoxynucleotides (MBOs) contain strategically placed segments of phosphorothioated backbones (able to induce RNaseH activity on the target mRNA) mixed with segments of either modified oligodeoxyribonucleotides or oligoribonucleotides (reducing off-target effects). The advantages of MBOs over PS-ODNs are increased biological activity, reduced polyanionic- and CG-dinucleotide-related side effects and increased *in vivo* stability (Agrawal & Zhao, 1998). A third-generation AS-ODNs contains DNA/RNA oligonucleotides with 2'-*O*-methyl modification in addition to a phosphorothioate core and methylphosphonate ends further reducing toxicity. Improved stability, high specificity and low toxicity characterize these molecules, allowing for efficient destruction of target mRNA at nanomolar concentrations (Sternberger et al., 2002).

3.2.2 Antisense technology on cervical cancer therapy

Earlier reports on high-risk HPV E6 and E7 functions used plasmid-borne full-length antisense RNA to show that inhibition of HPV-18 E6/E7 expression results in growth arrest in C4-1 cells (von Knebel Doeberitz & Gissmann, 1987; von Knebel et al., 1988). Later, plasmid-borne antisense RNA delivery was shown to induce apoptosis in CaSki cells via up-regulation of p53 and apoptosis induction (Cho et al., 2002). Delivery of antisense RNA using adenoviral vectors and retroviral vectors on SiHa and CaSki cells resulted in the reduction of HPV16 E7 protein expression and cell proliferation. These changes were accompanied by cell cycle arrest, up-regulation of RB, down-regulation of E2F-1 and BCL-2 and dose-dependent and retarded tumor growth of CaSki cells, a cervical cancer line with multiple copies of HPV-16 (Choo et al., 2000; Hayashi et al., 1997). More recently, non-neuroinvasive HSV-1 vectors, lacking the $\gamma_1$34.5 gene, were used to express antisense RNA complementary to the first 100nt of the HPV-16 E7 gene. These recombinant viruses down-regulated E7 protein expression in CaSki cells in a dose-dependent manner (Kari et al., 2007). Overall, these results confirmed the validity of targeting high-risk HPV E6/E7 for cervical cancer therapy. Nevertheless, due to the difficulties

of administrating plasmids, large antisense RNA molecules or even infectious viruses to patients, the use of small antisense moieties such as AS-ODNs, catalytic oligonucleotides or siRNAs might be a better alternative.

Pioneering attempts on E6/E7 targeting by AS-ODNs directed antisense PS-ODNs to the translational start site of E6/E7 mRNA (Steele et al., 1993; Tan & Ting, 1995). *In vivo* testing on CaSki, SiHa and HeLa cells (all cervical cancer cell lines containing high-risk HPV) showed that these PS-ODNs produced cell growth inhibition. Nevertheless, no data was provided to show growth inhibition due to a true antisense mechanism. Later, a rational approach to antisense exposed regions was applied to HPV-16 E6/E7 using fast-hybridizing RNA segments obtained from partially digested E6/E7 mRNA (Kronenwett & Sczakiel, 1997). A selection of AS-ODNs directed against such exposed sequences resulted in growth inhibition of cultured SiHa cells (Venturini et al., 1999).

Our group developed AS-ODNs covering a so-called "antisense window" within a stable HPV-16 E6 (nt 410-445) region (Alvarez-Salas et al., 1995). Two short antisense PS-ODNs complementary to nt 410-445 produced efficient growth inhibition of monolayer and agar-growth HPV-16-containing tumor cell lines in a dose-dependent manner. One of such PS-ODNs also inhibited tumor growth in nude mice (Alvarez-Salas et al., 1999; Marquez-Gutierrez et al., 2007). Interestingly, the combined use of both AS-ODNs resulted in the additive but not synergistic growth inhibition suggesting that they can be applied together to overcome issues related to genital HPV genomic variability (Marquez-Gutierrez et al., 2007). Nevertheless, the high doses used in these studies (within the micromolar range) suggest that further modifications are required to improve therapeutical efficiency avoiding off-target effects. Overall, current AS-ODN applications to high-risk HPV E6/E7 gene silencing as a therapy for cervical cancer appears promising and relatively safe, providing that the tested PS-ODNs are administered locally to control off-target issues commonly observed with phosphorothioated moieties. Nevertheless, second and third-generation AS-ODNs remain to be clinically tested as the advancement of other gene silencing technologies (i.e. siRNA and shRNAs) overcame an otherwise very effective molecular therapy.

3.3 Therapeutic catalytic oligonucleotides

Small ribozymes and DNAzymes are oligonucleotides possessing, at the very least, enzymatic RNA cleavage and ligation activities (Haseloff & Gerlach, 1988; Santoro & Joyce, 1998). Ribozymes were initially described as catalytic RNA moieties found in the self-splicing group I introns from the unicellular algae *Tetrahymena* (Kruger et al., 1982), and within the RNA active site of *Escherichia coli* RNaseP (Guerrier-Takada et al., 1983). However, the relatively large size of these two ribozymes precluded gene modulating/silencing applications. Later, small catalytic RNA cores from naturally occurring ribozymes were isolated from the circular genomes of certain pathogenic plant RNA viroids (Haseloff & Gerlach, 1989). In particular, two catalytic moieties have been intensely used as therapeutic agents; the hammerhead and hairpin ribozymes. By simply altering the native substrate recognition sequences, natural *cis*-cleaving ribozymes can be engineered to recognize and cleave any target RNA in *trans* by Watson-Crick hybridization (Michienzi & Rossi, 2001) (Figure 3A). Consequently, ribozymes received considerable attention as potentially valuable tools for the inhibition of virus replication, modulation of tumor progression, and analysis of cellular gene function (Morrissey et al., 2002).

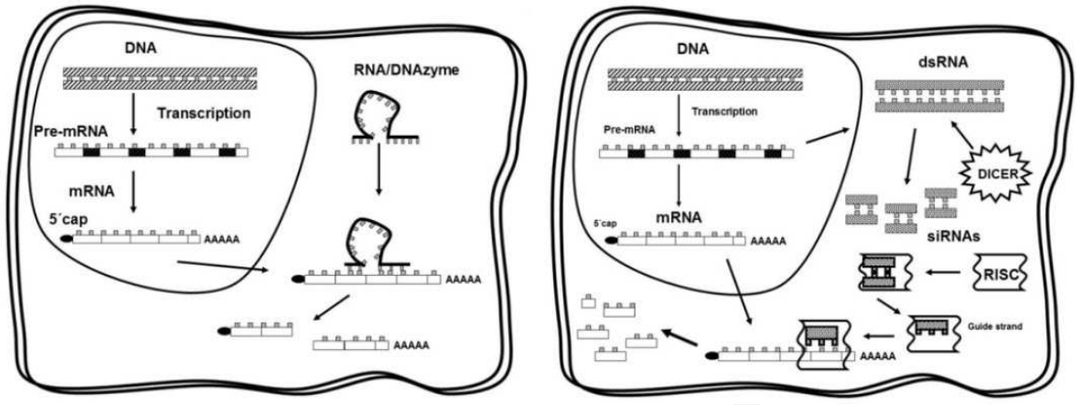

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Fig. 3. A) Catalytic oligonucleotides mode of action. Ribozymes or DNAzymes hybridize and cleave the target mRNA. B) RNA interference (RNAi) mechanism. Double-stranded RNA (dsRNA) found in a given mRNA are recognized and cleaved by DICER to yield small interfering RNAs (siRNAs) that in turn are incorporated into the RISC complex to cleave the mRNA from which they were derived. By administrating synthetic siRNAs the RISC complex may be manipulated to target a particular mRNA.

3.3.1 The hammerhead ribozyme

Hammerhead ribozymes are small (30-40nt) catalytic RNA moieties composed of three basic components including a highly conserved catalytic domain flanked by two base-pairing sequences and a complementary sequence within the target RNA containing the sessile phosphodiester bond. Natural catalytic centers can be formed within contiguous RNA sequences or by sequences several nucleotides apart (Epstein & Gall, 1987; Hutchins et al., 1986). Providing that the target RNA is single-stranded, hammerhead ribozymes can cleave any substrate RNA containing the triplet 5´-NUX-3´, where U is conserved, N is any nucleotide and X can be C, U or A (Vaish et al., 1998). Cleavage occurs 3′ to the 5′-NUX-3′ triplet (Uhlenbeck, 1987), generating 5′ hydroxyl termini and a 2′,3′-cyclic phosphate at the cleavage site (Hutchins et al., 1986; Prody et al., 1986).

3.3.2 The hairpin ribozyme

Hairpin ribozymes are about 60nt long and efficiently catalyze a reversible, site-specific cleavage reaction. Structurally, hairpin ribozymes are composed by the substrate recognition domain A located aside the catalytic domain B and the target RNA containing the target site. The established cleavage requirements for heterologous substrates indicate that the substrate sequence must contain a 5′-BN*GUC-3′ motif (where * is the site of cleavage) (Anderson et al., 1994; Hampel et al., 1990). Cleavage occurs at the 5′ side of guanosine and yields two products: the 3′-product containing the 5′-hydroxyl terminus newly formed within a terminal G, and the 5′-product containing the 2′,3′-cyclic phosphate (Yu & Burke, 1997).

3.3.3 Catalytic DNA

DNAzymes are small (~30nt) catalytic DNA oligonucleotides capable of cleaving target RNA molecules in a sequence specific manner (Baum & Silverman, 2008). These molecules

represent a new generation of catalytic oligonucleotides artificially obtained through *in vitro* selection procedures by their capacity to catalyze the *cis*-cleavage of a target RNA sequence. Similar to ribozymes, DNAzymes hybridize substrate RNAs by Watson-Crick pairing and cleavage results in RNA fragments containing 2′,3′-cyclic phosphate and 5′-hydroxyl ends, suggesting a common mechanism (Santoro & Joyce, 1998). Therefore, the same widely tested design rules for AS-ODNs and ribozymes can be readily applied for therapeutic DNAzymes. However, unlike ribozymes, usage of DNAzymes as therapeutic moieties has the advantage of simpler and cost-effective synthesis, easier administration and higher stability in biofluids (Dass et al., 2008). So far, the 10-23 DNAzyme is the most commonly used in therapeutic applications. The 10-23 moiety structure resembles that of a hammerhead ribozyme consisting of a 15nt catalytic core flanked by two target recognition arms complementary to the target RNA (Santoro & Joyce, 1997). Cleavage is strictly dependent on Mg++ and is specifically produced at a RY (R, purine; Y, pyrimidine) junction within the target RNA (Cairns et al., 2003; Faulhammer & Famulok, 1997). Although the 10-23 DNAzyme is active at 37°C it may require nucleotide modifications to keep high activity under physiological conditions (Takamori et al., 2005). Other DNAzymes with different structural and catalytic features have been successfully used as sensors for ions, molecules and even proteins (Ali et al., 2011; Zhang et al., 2011). Thus, DNAzymes offer a wide array of possible therapeutical and diagnostic applications.

3.3.4 Hammerhead ribozymes and HPV

Early reports on ribozyme use against HPV assumed that translational start regions within mRNA are poorly structured thus allowing accessibility to antisense moieties. These reports were limited to cell-free experimentation and characterization of ribozyme cleavage on HPV target transcripts. Hammerhead ribozymes directed to HPV-16 nt 110 and 558 and expressed from plasmids showed efficient cleavage of HPV-16 E6/E7 transcripts in cell-free tests in different conditions of ionic strength, Mg^{++} concentration and temperature. Evaluation of the simultaneous activity of both ribozymes suggested a cooperative effect to disrupt HPV-16 E6/E7 gene expression (Lu et al., 1994). Similarly, targeting HPV-16 E6/E7 genes by hammerhead ribozymes specific to nt 240 and 597 showed that target size is important for catalytic activity (He et al., 1993). Chen *et al.*, developed hammerhead ribozymes targeting HPV-18 E6/E7 genes (nt 120, 309 and 671) that were initially tested by incubation of total RNA from HeLa and Vero cells with *in vitro* transcribed ribozymes resulting in the efficient and specific degradation of HPV-18 transcripts from HeLa cells. In a more unorthodox approach *E. coli* was used to co-expresses both the ribozymes and a 1266nt fragment from HPV-18 (including E6 and E7 genes). Ribozymes were induced by infection with a helper phage (T7/M13) resulting in the progressive down-regulation of the HPV-18 target. Finally, ribozyme anti-tumor activity was tested on HeLa cells resulting in a cell growth reduction, increased serum dependency, and reduced foci formation in soft agar (Chen et al., 1996; Chen et al., 1995) .

3.3.5 Hairpin ribozymes and HPV

Hairpin ribozymes are usually more efficient at 37°C than hammerhead ribozymes and co-factors are not a strict requirement for activity because the catalytic mechanism appears to rely on structural components (Walter et al., 1998). However, efficient cleavage by hairpin

ribozymes requires a specific sequence within the target RNA (5′-GUC-3′ motif). We performed a comprehensive search for hairpin ribozyme target sites within HPV-16 E6/E7 genes showed six potential target sites. A hairpin ribozyme directed to site 434 (R434) was *in vivo* tested for *cis*-cleavage using a plasmid-borne HPV-16 E6/E7 transcripts containing ribozymes at the 3′-UTR. Only R434-containig transcripts caused significant delay in the growth rate of transfected cells and inhibited E6/E7 immortalization (Alvarez-Salas et al., 1998). Although this report demonstrated the feasibility of hairpin ribozyme use as a therapeutic antisense moiety for cervical cancer, the larger size of hairpin ribozymes limited their application as plasmid-borne moieties. Thus, we developed multiplex expression systems (triplex) based on hairpin ribozymes for R434. Such systems resulted in the complete release of multiple independent catalytic units from a single transcript by a self-processing mechanism, allowing individual R434 activity and increasing efficiency of degradation of E6 RNA (Aquino-Jarquin et al., 2008; Aquino-Jarquin et al., 2010). The measured activity of a single R434 unit resulted in 30% inhibition of HPV-16 E6/E7 mRNA in SiHa cells, suggesting that multiple ribozymes directed against the same or other target sites might result in complete inhibition (Aquino-Jarquin et al., 2008).

It is worth mentioning that notwithstanding the relative success on inhibiting HPV E6/E7 by ribozymes, the catalytic features from both hammerhead and hairpin ribozymes dramatically drop within the cellular environment. Intracellular variables affecting activity include Mg^{++} availability, co-localization with the target, nuclease action and protein binding (Benitez-Hess & Alvarez-Salas, 2006). Plasmid-borne ribozymes usually contain stem-loop or tRNA structures to extend intracellular life that may get targeted by several RNA-processing mechanisms thus impeding activity (Alvarez-Salas L.M., *unpublished data*). Alternatively, because of their small size (~30nt) hammerhead ribozymes may be synthesized including modified nucleotides to escape nuclease and RNA processing mechanisms but this alternative usually brings deleterious off-target effects that may mislead data interpretation and limit clinical application. The therapeutical use of ribozyme in cervical cancer has never approached to the simple, efficient and relatively safe use of AS-ODNs. Therefore, because the proven high ribozyme specificity and catalytic features for HPV transcripts it is likely that ribozymes may be better suited for diagnostic usage in cervical cancer.

3.3.6 DNAzymes on cervical cancer therapy

DNAzymes have become one of the most versatile oligonucleotide technologies available. In the last few years, DNAzymes have been used as therapeutical agents, computing DNA, biochemical analysis tools and sensors (Baum & Silverman, 2008; Stojanovic et al., 2005). However, there are only a few reports on DNAzyme application in the fields of cervical cancer and HPV. Our first report on a modified 10-23 DNAzyme directed to HPV-16 E6/E7 mRNA "antisense window" showed efficient down-regulation of E6/E7 transcripts both *in vitro* and *in vivo* resulting in specific inhibition of proliferation and cell death in a dose-dependent manner (Reyes-Gutierrez & Alvarez-Salas, 2009). To improve efficiency and intracellular stability the DNAzyme was modified with locked nucleic acids (LNAs) producing more thermostable DNAzyme-RNA complexes resulting in better cleavage efficiency (Benitez-Hess et al., 2011). In our hands, DNAzyme technology merged the relative simplicity and affordability of DNA synthesis and modification with the expected

benefits of enzymatic catalysis yielding a therapeutical moiety superior in performance and specificity when compared to AS-ODNs and even siRNAs at the nanomolar range.

3.4 The small RNA revolution: siRNAs, shRNAs and miRNAs

The mechanism of RNA interference (RNAi) is a natural and wide-spread gene knockdown phenomenon induced by the formation of double-stranded RNA (dsRNA) segments in most mRNAs (Elbashir et al., 2001; Fire et al., 1998; Sharp, 1999). The RNAi process occurs in the cytoplasm where dsRNA regions within mRNAs are digested into double-stranded 21–23nt fragments, with a 2nt 3'-overhand, by the RNaseIII-like enzyme DICER (Bernstein et al., 2001). Subsequently, these small fragments, now called small interfering RNAs or siRNAs, are denatured by a helicase and one strand (leader or guide strand) is then incorporated into an RNA-induced silencing complex (RISC) that includes DICER, AGO2, TRBP and other members of the *Argonaute* family (Cullen, 2006; Hammond et al., 2001; Meister et al., 2004; Nykanen et al., 2001). The activated RISC complexes containing the guide strand hybridize and cleave the homologous mRNA from which they were derived. Therefore, there are no genes coding for particular siRNAs (Bartel, 2004). Recent reports suggest that siRNA activity exhibit serious dependence on the target site, as is the case for other antisense-based technologies, which might significantly limit the convenient use of siRNA (Miyagishi et al., 2003). Target recognition by activated RISC complexes appear to rely on the perfect matching between the target sequence and a 2-8nt region within the guide strand known as "seed" region (Lin et al., 2005).

By using synthetic siRNAs administrated exogenously, the RNAi machinery can be manipulated to silence a given mRNA (Figure 3B). These siRNAs can bypass the earlier steps in the RNAi pathway and can be loaded directly onto the RISC complex. Both synthetic siRNAs and vector-borne delivery of the equivalent short hairpin RNAs (shRNAs) have been used as potential therapeutic moieties for cervical cancer. Because shRNAs rely on the vector used, we will only focus in the developments of siRNAs as true therapeutic oligonucleotides. Although powerful, the widely reported gene silencing effects of siRNAs do not appear specific in many instances as down-regulation of unintended targets with partial sequence complementarities and stimulation of innate immune by type I interferon (IFN-I) and/or inflammatory cytokine responses have been often reported (Jackson et al., 2003; Judge et al., 2005; Kalali et al., 2008).

On the other hand, miRNAs are also small 20–25nt long non-coding dsRNAs with very specific functions modulating gene expression by hybridizing to complementary sequences present in the 3′ UTR of many protein-coding mRNAs (Bartel, 2004). Unlike siRNAs, the miRNAs are coded in the genome of most metazoans (including humans) by independent loci or within intronic regions of other genes (Cullen, 2004; Kim & Kim, 2007). They are initially transcribed by the RNA polymerase II as primary miRNAs (pri-miRNAa) (Lee et al., 2004), which are cleaved into pre-miRNA hairpins by the RNaseIII-like nuclease DROSHA and PASHA/DGCR8 (Gregory & Shiekhattar, 2005). Pre-miRNAs are exported from the nucleus by the EXPORTIN-5 (Yi et al., 2003). Intronic pre-miRNAs are generated as a product of splicing of the host gene (Berezikov et al., 2007). Once in the cytoplasm, the pre-miRNA hairpins merge to the RNAi pathway and are cleaved by DICER to produce mature miRNAs that are incorporated into RISC and interact with their targets (Valencia-Sanchez et al., 2006).

3.4.1 RNAi therapeutics on HPV

Early attempts to silence high-risk HPV gene expression using siRNAs indicated selective E6/E7 mRNA. E6 silencing by a siRNA directed to HPV-16 nt 224-242 induced accumulation of cellular p53 protein, transactivation of the cell cycle control p21 gene and reduced cell growth but no apoptosis. Surprisingly, E7 silencing produced by a siRNA to nt 662-680 induced apoptotic cell death. HPV-negative cells appeared unaffected by the anti-viral siRNAs (Jiang & Milner, 2002). Because the polycistronic expression of HPV-16 E6 and E7 both siRNAs would have similar effect on E6/E7 expression and thus similar phenotypes (Butz et al., 2000). Although no explanation has been provided for this puzzling result, it is likely that the reported observations may be related to off-target effects.

Later it was reported that vector-borne and synthetic siRNAs directed against the HPV-18 E6 gene (nt 385-403) restored dormant tumor suppressor pathways in HPV-positive cancer cells that are otherwise inactive in the presence of E6. This ultimately resulted in massive apoptotic cell death, selectively in HPV-positive tumor cells (Butz et al., 2003). More recently a siRNA molecule targeting the E7 region of the bicistronic HPV-18 E6 and E7 mRNA (nt 142-160) reduced expression of E6 and E7 in HeLa cells. Application of siRNAs against E6 and E7 also inhibited cellular DNA synthesis and induced morphological and biochemical changes characteristic of cellular senescence. These results demonstrate that reducing E6 and E7 expression is sufficient to cause HeLa cells to become senescent thus establishing that targeting of E6/E7 mRNA affects synthesis and functions of both E6 and E7 (Hall & Alexander, 2003). The simultaneous targeting of HPV-18 E6/E7 has also been reported to induce apoptosis and reduce proliferation of HeLa cells (Qi et al., 2010). Interestingly, it has been shown that cellular apoptosis induced by siRNA directed to HPV-18 E6 in HeLa cells relies on the p53 and ubiquitin proteolysis pathway thus inhibiting cell proliferation and promoting cell apoptosis. Anti-oncogene and upper regulation of immunization-related genes produced regression of the malignant phenotype after E6 inhibition (Min et al., 2009).

Other synthetic siRNA decreased the levels of HPV-16 E6/E7 mRNA and induced nuclear accumulation of p53 in SiHa cells. The siRNA also suppressed monolayer and anchorage-independent growth associated with p21CIP1/WAF1 induction and hypophosphorylation of retinoblastoma protein. Furthermore, SiHa cells treated with the anti-E6 siRNA prior to subcutaneous injection, formed tumors in NOD/SCID mice that were significantly smaller than in those treated with a control siRNA (Yoshinouchi et al., 2003). Thus, sequence-specific targeting of high-risk HPV E6/E7genes, siRNAs may be developed into novel therapeutics that can efficiently inhibit growth of cervical cancer cells. Nevertheless, *in vivo* delivery of siRNAs is still a major obstacle to their clinical use.

As with other oligonucleotide-based therapeutical approaches, *in vivo* delivery of siRNA is mostly affected by ribonuclease degradation, rapid renal excretion and nonspecific uptake by the reticuloendothelial system (Whitehead et al., 2009). Additionally, siRNAs are polyanions that do not readily cross the cell membrane. In turn to avoid these issues, siRNAs are often synthesized with phosphorothioated or 2′-O-modified bases and encapsulated in delivery systems allowing enhanced stability in biofluids and cell uptake, escape immune recognition, and improve pharmacokinetics by avoiding excretion and renal filtration (Lorenz et al., 2004; Sorensen et al., 2003). Encapsulated siRNAs still need to co-localize with the appropriate target cell/tissue type leading to more complicated therapeutic strategies. Thus, several considerations regarding biodistribution, extracellular

and intracellular transport must be addressed in addition to potency and biostability before clinical use of siRNAs. Nevertheless, the recent success in clinical trials using siRNA to treat age-related macular degeneration (Bevasiranib), respiratory syncytial virus infection (ALN-RSV01) and the targeted *in vivo* gene silencing via systemic delivery of siRNA using transferrin-tagged, cyclodextrin-based nanocapsules for human cancer therapy (CALAA-01) have demonstrated the therapeutic feasibility of siRNAs (Shim & Kwon, 2010).

In the last few years, siRNA use for silencing high-risk HPV E6/E7 has been widely reported although emphasis shifted from design and targeting to delivery and specificity, as highly active siRNAs are now commercially available. Many reports showed successful E6/E7 inhibition, but most of them were limited to cell culture, lacking of toxicity controls (Jonson et al., 2008; Lea et al., 2007; Sima et al., 2008; Yamato et al., 2008). A recent report, undertook a more comprehensive approach by designing and testing nine different siRNAs against either the E6 or E7 genes of HPV-16 or HPV-18 in several combinations. The siRNAs were tested on CaSki or HeLa cell lines resulting in significant cell growth and colony formation inhibition in both cell lines with a significant increase in apoptosis. The siRNAs had no effect in HPV-negative C33-A cells, demonstrating a lack of off-target effects. In addition, a xenograft study showed that intratumor injection of the siRNAs reduced tumor growth in BALB/c nude mice (Chang et al., 2010).

The transient nature of antisense technology (including siRNAs) forced research on long expression using vector-borne shRNAs. Although many reports have established the feasibility of this approach (Bai et al., 2006; Bousarghin et al., 2009; Gu et al., 2011), it is clear that prolonged siRNA expression may lead to dysfunction of the RNAi pathway (Tang et al., 2006) or other intracellular effects (Koivusalo et al., 2006) due to the sudden rise in p53 and pRB proteins after siRNA treatment (Sima et al., 2008). Thus, siRNA treatment has been used to enhance already established therapies for cervical cancer such as paclitaxel (Liu et al., 2009), cisplatin (Wu et al., 2011) and TRAIL (Eaton et al., 2011) with sometimes mixed results depending on the condition of p53 expression (Koivusalo et al., 2005).

As noted above, nucleotide modifications at specific positions enable oligonucleotides to avoid intracellular nuclease degradation and meddling with the endogenous RNAi pathway, but they also help to overcome off-targeting issues in siRNAs (Jackson et al., 2006). In addition, DNA inclusion in the seed region of the guide strand and its complementary sequence within the siRNA, so-called a double-stranded RNA–DNA chimera (dsRNA-DNA), abolishes off-target effects sacrificing some silencing activity (Ui-Tei et al., 2008). Application of dsRNA-DNA chimeras from previously reported and highly active siRNAs to nt 497, 573 and 752 within HPV-16 E6/E7 mRNA (Yamato et al., 2008), resulted in reduced cytotoxicity in two of three chimeric siRNAs (497 and 752), but not in the other (573), correlating with their reported off-target effects. Silencing activity was marginally affected in chimeric siRNAs 497 and 573 and moderately in 752. Chimeric siRNA 497 induced E6/E7-specific growth suppression of cervical cancer cells and E6/E7-immortalized human keratinocytes (Yamato et al., 2011).

The delivery of antisense moieties has also attracted much attention in siRNA research. Unlike AS-ODNs, there is a stringent requirement for transfection of siRNAs that has limited its applications as powerful cell culture inhibitors. To become truly useful therapeutic moieties, *in vivo* delivery methods have been developed for cervical cancer treatment using siRNAs besides the obvious use of vector-borne shRNAs. These approaches

vary from the traditional direct intratumor injection (Fujii et al., 2006) to more innovative methods such as dendrosomal nanoparticles delivery (Dutta et al., 2010), encapsidation in HPV-16 virus-like particles (Bousarghin et al., 2005), encapsulation in lipidic particles (Wu et al., 2011) and coating of quantum dots (Zhao et al., 2011). All of these approaches showed the feasibility of *in vivo* siRNA treatment for cervical cancer. No clinical reports have been published so far.

3.4.2 MicroRNAs (miRNAs) in cervical cancer

The participation of miRNAs as regulatory molecules in differentiation, apoptosis, and proliferation strongly suggested a role in cervical cancer. Although miRNAs are not therapeutic or diagnostic oligonucleotides in strict sense, they can be used as synthetic moieties to block key biological processes leading to malignant transformation. Because the HPV life cycle is linked to epithelial differentiation and requires actively proliferating keratinocytes, it has been hypothesized that HPV proteins may modulate miRNA expression. Interestingly, high-risk HPVs do not encode for any known miRNA (Cai et al., 2006; Lui et al., 2007), although they may control expression of cellular miRNAs to regulate the activities of cellular proteins through expression of viral regulatory proteins (i.e. E5, E7 and E7) (Greco et al., 2011; Wang et al., 2009; Zheng & Wang, 2011). In the last few years, many alterations in cellular miRNA patterns in cervical cancer tissue or cervical cancer cells have been reported, suggesting that knowledge of differential miRNA expression may have a significant diagnostic and prognostic value (Lui et al., 2007).

High-risk HPV E6 may exert modulation of miRNA expression through p53 down regulation. Cervical cancer cells containing high-risk HPVs show reduced expression of miR-34a, a p53 effector with tumor-suppressor abilities. Reduction of miR-34a expression in HPV-containing human keratinocytes correlated with expression of viral E6. Furthermore, siRNA knockdown of viral E6 expression in high-risk HPV-containing cervical cancer cell lines lead to increased expression of p53 and miR-34a and accumulation of miR-34a in G0/G1 phase cells. Ectopic expression of miR-34a in HPV-containing and HPV-negative cells resulted in substantial cell proliferation inhibition and moderate apoptosis, suggesting HPV modulation of cellular miRNA expression (Wang et al., 2009). The HPV-16 E6 was also found to decrease expression of miR-23b in SiHa and CaSki cells by repressing the promoter and increased expression of its cellular target, the urokinase-type plasminogen activator (uPA), a known inductor of cell migration in cervical cancer cells. The link between HPV-16 E6 and miR-23b transcription was associated to the presence of a p53 binding site within the miR-23b promoter, suggesting a cell migration modulatory role for E6 (Au Yeung et al., 2011).

High-risk HPV E7 expression in human keratinocytes modulated expression of human miR-203 and its downstream target, ΔNp63. Although the underlying mechanism is not fully understood, E7 is sufficient for blocking miR-203 expression probably by modulation of the mitogen-activated protein kinase (MAPK) pathway signaling. The p63 family, is related to the p53 tumor suppressor. ΔNp63 isoform is expressed at high levels in proliferating undifferentiated basal keratinocytes, and its expression is down-regulated in differentiated non-proliferating cells. Down-regulation of ΔNp63 has been associated to regulation of epithelial proliferation and differentiation. Thus, inhibition of miR-203 allows HPV productive replication in differentiating cells (Melar-New & Laimins, 2010). Interestingly,

expression of the high-risk HPV-16 E5 protein (considered an overall enhancer of E6/E7 activities) resulted in rapid (96 hours) alteration of miR-146a, miR-203 and miR-324-5p and their target genes in transfected keratinocytes, suggesting a miRNA regulatory role for E5 (Greco et al., 2011).

Genomic microarray analyses in normal and cervical cancer tissues using the same miRNA array platform showed increased expression miR-15b, miR-16, miR-17-5p, miR-20a, miR-20b, miR-21, miR-93, miR-106a, miR-155, miR-182, miR-185, and miR-224 and decreased expression of miR-29a, miR-34a, miR-126, miR-127, miR-145, miR-218, miR-424, miR-450, and miR-455) in cervical cancer tissues (Li et al., 2010; Wang et al., 2008). Further confirmation of miR-126, miR-143/145, miR-155, and miR-424/450 alterations was performed by deep sequencing (Witten et al., 2010). Other studies with customized miRNA arrays and different assay platforms showed increased miR-21 expression in cervical cancer, a common occurrence in cancer cells (Lui et al., 2007). More interestingly, miR-143 and miR-145 showed basically null expression in cancer samples, suggesting the potential value of these miRNAs as tumor markers (Lui et al., 2007; Pereira et al., 2010). Yet another study concluded that infection with high-risk HPV lowered miR-218 expression suggesting a role for miR-218 in the pathogenesis of cervical cancer. Nevertheless, the specific role of all these miRNAs in cervical carcinogenesis and HPV infection is unknown.

The growth inhibitory activity of miR-34c-3p was recently shown by our group in SiHa cells but not in other cell types. Although the inhibitory mechanism is not clear, transfection of a mi34c-3p mimic resulted in specific fast apoptosis induction (24 hours), inhibition of colony formation, cell migration and invasion, suggesting a potential therapeutic use for this miRNA (Lopez & Alvarez-Salas, 2011).

3.5 Aptamers

Aptamers are single-stranded oligonucleotides that, unlike AS-ODNs and siRNAs, function by folding into specific globular structures that dictate high-affinity binding to a variety of targets (Cerchia et al., 2002). They are often referred as functional homologues of the antibodies and are obtained through the use of the systematic evolution of ligands by exponential enrichment (SELEX) procedure. The SELEX method is a PCR-based *in vitro* selection procedure of large oligonucleotide libraries that recapitulates natural evolution resulting in the isolation of specific ligands that bind with high affinity to a wide variety of proteins and cell surface epitopes (Ellington & Szostak, 1990; Tuerk & Gold, 1990). These molecules have been used in flow cytometry, biosensors, affinity probe electrophoresis, capillary electrochromatography, and affinity chromatography (Yan et al., 2005).

Notwithstanding the obvious value of aptamer for diagnostics, the incorporation of modified nucleotides into RNA transcripts resulting in stability in biofluids has considerably increased the use of aptamers as probes to inhibit protein functions (Pagratis et al., 1997). Nuclease-resistant RNA and DNA aptamers to block cell adhesion events gained importance in the last years. Wang *et al*., selected RNA aptamers that bind to infectious human cytomegalovirus and inhibit viral infection *in vitro*, showing the feasibility of the SELEX technique for the evolution of novel compounds that protect cells against infection by pathogens such HPV (Wang et al., 2000). Furthermore, combinatorial synthesized nuclease-resistant RNA and DNA aptamers are promising candidates for use in diagnostic and therapeutic onsets.

3.5.1 Aptamers on HPV detection and therapy

Even though oligonucleotide aptamer technologies have been available for a number of years, it is only beginning to be established for HPV detection. During its life cycle, HPV expresses proteins according the cellular differentiation program that is modified after E2 disruption and the onset of malignant transformation (Pett & Coleman, 2007; Xue et al., 2010). Such features offer several protein targets for the detection of HPV infection and molecular diagnosis of cervical cancer by using aptamers as diagnostic oligonucleotides. The first aptamer directed against high-risk HPV proteins was obtained using a modified SELEX in which unspecific sequences were eliminated applying an antidote-like strategy (Toscano-Garibay et al., 2011). This RNA aptamer effectively recognized the viral protein in a purified form with affinity comparable to other aptamers that bind small proteins. In addition, the interacting mechanism was common to those observed for little targets; it folds into two hairpin structures and wraps E7 making contact with independent sites located on the CR1 and CR3 protein domains. Even though its behavior with infected cell extracts showed a cross-recognition between at least two types of HPV, this aptamer constitutes an important step towards the design of reliable and affordable detection methods.

A second set of aptamers obtained against E7 has established the effect of a single nucleotide changes on the function of aptamers over the protein activity. By changing only one nucleotide (U>C) anti-E7 aptamers prevented the formation of pRB-E7 complexes, meanwhile the replacement of two bases conducted to inactive sequences (Nicol et al., 2011). These observations suggest that following a mutation-by-mutation planning process or even using error prone PCR, some of the obtained aptamers could improve intracellular stability to impede the activity E7 proteins and eventually become a complement for therapies against cervical cancer.

4. Conclusion

Over the last few years, small oligonucleotides have been proved as feasible alternatives to HPV infection and cervical cancer therapy. The most common and successful approaches appoint to antisense technology in the form of siRNAs and AS-ODNs against different target sequences within high-risk HPV E6/E7 mRNA. Above all, siRNA technology shows a higher capacity than AS-ODNs to inhibit HPV expression. However, siRNA-induced inhibition of high-risk HPV E6/E7 is still far from practical use, limiting research to cell culture applications. Several issues regarding the transient nature of siRNA-mediated inhibition and the associated and always difficult to control off-target effects have undermined the clinical application of this otherwise powerful technology. The ability to efficiently and stably produce and deliver sufficient amounts of siRNA to the proper target tissues still requires further refinement although recent advancements in siRNA delivery (encapsulation) and the use of modified nucleotides in synthetic siRNAs may finally allow clinical testing for cervical cancer. The use of vectors-borne shRNAs appears as a more distant solution due to the multiple ethical and biological issues arising from the use of viral vectors and the still impractical non-viral approaches (i.e. liposomes, dendrosomes, quantum dots, etc.).

Even though off-targeting and delivery issues might be overcome the intracellular presence of any antisense moiety or siRNA must confront the role of innate immune responses. AS-

ODNs have been shown to induce distinct classes of innate responses that can mislead data interpretation by masking true antisense effects in the clinical setup. In particular, the presence of CpG dinucleotides along the AS-ODN sequence that activate the immune system through Toll-like receptor 9 (TLR9), resulting in cytokine release and antitumor cytotoxicity (Kandimalla et al., 2005; Sivori et al., 2004). Transfection of unmodified siRNAs and shRNAs trigger a similar response through TLR3 and TLR7 (Judge et al., 2005). In fact, a whole new class of oligonucleotides known as IM-ONs that is virtually unexplored in the HPV and cervical cancer setup as therapeutical moieties or vaccine adjuvants.

The involvement of miRNAs in cervical carcinogenesis has opened a new dimension in HPV research. Although many reports establish the alteration of a myriad of miRNAs, it is becoming clear that most of these are artifacts. A more stringent protocol should be used to establish participation of miRNAs in cervical cancer including functional assays in HPV-positive and HPV-negative cervical cells and *in situ* detection in normal and tumor cervical tissues. Nevertheless, HPV modulation of miRNA expression is firmly established. The use of aptamers in cervical cancer diagnosis and HPV detection is promising as the natural history of HPV infection offers a plethora of targets previously addressed with antibodies. As SELEX-derived oligonucleotides grow in diversity and specificity, new and cost-effective aptamer-based technologies will provide fast and reliable prevention and early screening strategies that will compete with massive vaccination programs in the future. No use of aptamers in cervical cancer therapy has been reported, but the use of AS-ODNs and aptamers currently accepted by the FDA and the advent of new RNA therapeutic targets (i.e. miRNAs), suggest that the best options for the clinical application of oligonucleotides against cervical cancer are yet to come.

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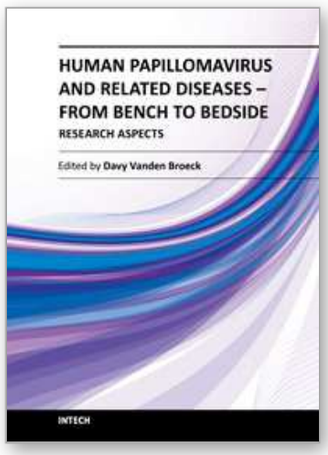

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Cervical cancer is the second most prevalent cancer among women worldwide, and infection with Human Papilloma Virus (HPV) has been identified as the causal agent for this condition. The natural history of cervical cancer is characterized by slow disease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

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