

# Molecular Events Towards Wnt Pathway Activation in Cervical Cancer: Changing the Balance on NKD/DVL Signals

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## 1. Introduction

The Wnt signaling pathway is a key regulator of developmental and homeostatic processes, including embryogenesis, stem cell maintenance, cell fate specification, cell polarity, migration, and, when is aberrantly activated, cancer progression. The Wnt pathway is a highly conserved mechanism during evolution, hence homologues of this via can be found in metazoan organisms, showing a crucial role in the developmental processes of metazoan body (Pang et al., 2010).

The aim of the current chapter is to review and analyze recent data pointing to specific alterations in Wnt pathway components occurring during Cervical Cancer (CC) progression. We address an overall discussion about the feasible role of E6/E7 proteins as chromatin remodelers, hence turning off by promoter silencing or turning on, by recruiting co-activators and transcription factors to genes that promote malignant progression. We also present our results, highlighting Wnt aberrant activation in cervical biopsies. Therefore, we propose that cervical neoplasms caused by high-risk human papillomavirus (HPV) activate the Wnt/beta-catenin pathway in order to establish and progress.

## 2. HPV viral cycle and cervical cancer development

Epidemiological and molecular studies have shown a causal relationship between high-risk HPV infection and CC development (Walboomers et al., 1999). However, HPV is a necessary agent, but not sufficient cause of cervical intraepithelial neoplasia and CC. The above

mentioned arises from epidemiological studies that demonstrated that HPV is one of the most common sexually transmitted agents with prevalence between 10-40 percent in women who have no cytological abnormalities (Jacobs et al., 2000; Molano et al., 2002). There is enough evidence suggesting that is very likely that most of people could have an HPV sub-clinical infection, especially increasing the risk at juvenile ages (younger than 25 years old). This is due to many factors, for instance, in the case of older women could be the acquired immunity to HPV from previous exposures, likewise the alcohol consumption and the number of sexual partners can increase specific risk (Burk et al., 1996; Kjaer et al., 1997; Ho et al., 1998; Lazcano-Ponce et al., 2001.).

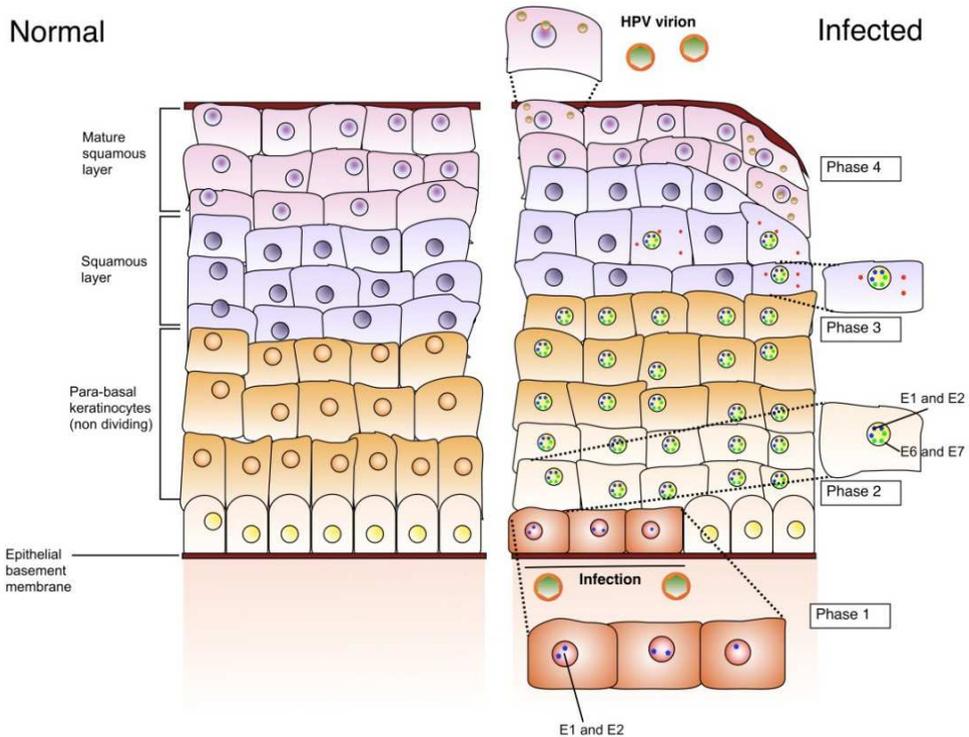
Moreover, other cohort and multi-center studies have shown that the presence of the viral sequence can reach up to 85% in women who have no cytological abnormalities detected by pap-smear (Roteli-Martins et al., 2011). Apparently the median duration of HPV infection is around eight months with a high consistence between different populations (Ho et al., 1998; Franco et al., 1999.; Molano et al., 2002.). Thus, we can conclude that in the CC multistep carcinogenesis process HPV persistent infection is the initial step, however, other factors are involved towards the development of malignant phenotype.

Regarding HPV molecular events towards carcinogenesis, there are three key events during HPV course of infection associated with cancer: 1) viral DNA integration to host genome; 2) expression of viral proteins (namely E1, E2, E4, E5, E6 and E7), and 3) the complex interactions between E2, E6/E7 and cellular proteins (Figure 1). Cervical cancer is a complex disease elicited by the interaction of viral, host, and environmental factors, exerting an influence on the risk of disease progression from early cervical abnormalities to invasive cancer; thus the proper identification of involved factors will lead us to a better knowledge of the natural history of HPV infection.

Once HPV has infected basal cells, the viral genome is actively replicated as episome and early genes (E1-E7) are expressed. E1 and E2 are essential proteins for viral genome replication and viral cycle completion (Matsukura et al., 1989). E1 is an ATP-dependent DNA helicase which unwinds the double-stranded viral DNA and interacts with the -primase subunit of the DNA polymerase, to recruit the replication complex to the viral replication origin (Masterson et al., 1998; Conger et al., 1999). E1 also interacts with multiple cyclins and is phosphorylated by cyclin/CDK complexes (Dalton et al., 1995; Cueille et al., 1998.). These interactions require the consensus RxL cyclin binding motif, present in the amino-terminal domain of the E1 protein. Moreover, mutation of RxL motif severely compromises replication of viral genome (Ma et al., 1999), suggesting that E1 is regulating HPV genome replication through interaction with cyclins and CDKs complexes (Deng et al., 2004).

The full-length E2 protein is a sequence-specific transcription factor that functions as an activator or repressor to tightly regulate the transcriptional activity of all HPV genes. This is achieved through four consensus E2-binding sites (E2-BSs), ACCGN4CGGT, whose locations within the upstream regulatory region (URR) are highly conserved among genital HPVs (Fig. 1; Hedge, 2002; Hou et al., 2002). It has also been seen that E2 participates in viral DNA replication via interaction with the protein E1 (Chiang et al., 1992). Hence, the versatile role of E2 protein functioning as a transcriptional repressor/activator and promoting genome DNA replication could be explained by E2-BSs occupancy in a context-dependent fashion. In this respect, E2 binding to E2-BS4 can specifically up-regulate viral early gene expression, including the expression of oncogenes E6 and E7. In contrast, E2 binding at the

promoter-proximal sites E2-BS1 and E2-BS2 leads to transcriptional repression of the early genes, including E6 and E7, whereas the E2-BS3 site is important for viral DNA replication (Steger & Corbach, 1997; Stubenrauch & Pfister, 1994; Stubenrauch et al., 1998). In this way, E2 contributes to the cell cycle control by regulating the expression of E6 and E7. Repression of HPV-early genes mediated by E2 appears to involve the displacement of cellular transcription factors from the viral promoter (Tan et al., 1994). Therefore, viral DNA replication and viral gene expression reflect the relative occupancy of different E2 binding sites, finely modulated by the concentration of the E2 protein (Figure 2A).



**Fig. 1. HPV viral cycle and cervical cancer development.** Human papillomavirus infect epithelial basal cells through mechanical microabrasions or by infecting the transformation zone, an abrupt transition from a columnar to a squamous epithelium (Phase 1). Infected cells actively express the early genes E1, E2, E4 and E5 (Phase 2). E6 and E7 are expressed in limited amounts due to transcriptional modulation exerted by E2, which permits to cells have a higher cell cycle rate. Infected basal cells migrate to the lumen as they differentiate expressing the late capsid genes L1 and L2 (Phase 3). Viral genome is replicated as an episome in sub-clinical infections or low grade intra-epithelial-lesions (LGSIL), and is encapsidated in the nucleus of the upper layer epithelium (Phase 4). Shed viral particles then can infect new zones of epithelium or be sexually transmitted. Only a limited number of infections progress to high grade intra-epithelial-lesions (HGSIL) and cervical carcinoma (CC). The progression of LGSIL to CC is associated with the integration of the HPV genome into the host genome and the loss of transcriptional repression exerted by E2.

However, E2 is not only a transcription factor, but can also induce apoptosis in absence of any other HPV open reading frame by its association to the DED motif of caspase-8. HPV-18 E2 protein induces caspase oligomerization through its amino-terminus motif containing a 27 amino-acid  $\alpha$ -helix (Demeret et al., 2003; Thierry & Demeret C., 2008). In this context, HPV-16 E2 protein induces apoptosis by means of the binding to p53; this interaction has important implications in the viral cycle. For example, it has been reported elsewhere that p53-E2 heterodimer can down-regulate HPV-16 DNA replication (Webster et al., 2000; Brown et al., 2008). It is known that E2 regulates the cell cycle progression through two main mechanisms: a) by means of apoptosis either p53-dependent or caspase induction pathway; and by b) balancing the expression/repression of oncoviral proteins E6 and E7. That balance executed by E2 is broken upon HPV integration onto the host genome.

HPV genome replicates as episome or extrachromosomal molecule in benign cervical precursor lesions. However, cancer tissues can contain both episomal and integrated HPV DNA that has been covalently incorporated into the host cell chromosomal DNA (Cullen et al., 1991; Hudelist et al., 2008). Because the HPV genome is a ring molecule, it requires to be open in order to be integrated; this process involves a breakage in the E1-E2 open reading frames region and deletion of E2 and adjacent regions E2-E4, E5, and L2, after integration. Hence, as we discussed above the fine tune on the expression levels of E6 and E7 exerted by E2 is lost and viral oncogenes E6 and E7 are actively expressed in CC tissue (Ueda et al., 2003). It has been suggested that common sites of viral integration are cellular genes that could contribute essentially to the enhanced progression risk of HPV-induced premalignant lesions to neoplastic lesions. Thus, it has been reported that frequent integration sites are near to *MYC*, *NR4A2*, *hTERT*, *APM-1*, *FANCC*, and *TNFAIP2* (reviewed by Wentzensen et al., 2004).

## 2.1 Effect of protein-protein interactions of E6 and E7 with nuclear proteins in the regulation of transcription

The active expression of E6 and E7 is required to increase the proliferation capacity of malignant cells and uncoupling differentiation through targeting prominent regulators of cell cycle control progression. E6 and E7 epithelial expression and its interactions with cellular proteins have been at the center of the HPV biomedical research scenario probably for the past 20 years. The central core of the classic E6/E7 model is the binding and inactivation of tumour suppressor proteins p53 and pRb, respectively; which was established between the late 1980's and the early 1990's (Dyson et al., 1989; Scheffner et al., 1990). Currently, it is well-known that E6 and E7 interact with a plethora of cellular proteins, in the nucleus and in the cytoplasm, that participate in molecular pathways involved in the activation and establishment of the malignant phenotype. We will not discuss about the cytoplasmic interactions between E6/E7 with cellular proteins but there are some available reviews previously published that are highly recommended, with extensive and comprehensive content (Moody & Laimins, 2010; Lavia et al., 2003).

It has been well described that E6 and E7 do not possess DNA-binding domains (Mallon et al., 1987; Grossman et al., 1988). However, in the nucleus these viral products interact with chromatin remodeling proteins, such as the histone acetyl transferase CREB-binding protein CBP/P300; with transcriptional coactivators, such as hADA3; with transcription factors, such as AP1, IRF3, E2F1, TBP, MPP2, SRC-1 and pCAF; DNA-methyl transferases, and the telomerase (Antinore et al., 1996; Phillips & Vousden, 1997; Ronco et al., 1998; Lüscher-Firzlaff et al., 1999; Huang & McCance, 2002; Hwang et al., 2002; Maldonado et al., 2002;

Baldwin et al., 2006; ; Burgers et al., 2007; Liu et al., 2009). These protein-protein interactions may conduct important changes in transcriptional regulation by the direct action of these viral oncoproteins upon specific genes. For instance, E6 and E7 recruit c-Jun, c-Fos and CBP/p300, and also inhibit the binding of the repressive histone deacetylase NCoR to the promoter of COX-2. This corepressor/coactivator exchange caused by E6 and E7 induce the expression of this target gene (Subbaramaiah and Dannenberg, 2007; Haertel-Wiesmann et al., 2000; Howe et al., 1999). In addition, the over-expression of COX-2 could accentuate the malignant phenotype induced by Wnt hyper-activation and correlates with the progression of cervical epithelial lesions and lymph node metastasis in cervical cancer patients (Liu et al., 2011; Balan et al., 2011). Likewise, the interaction between E6 and E7 with CBP/p300 has been described in the context of the promoters of *TP53* and the proinflammatory IL-8. This event inhibits the histone acetylation of *TP53* promoter region and prevents the interaction between CBP/p300 with NFkappa-B and SRC-1 in the promoter of IL-8, which results in the inhibition of the expression of p53 and IL-8 (Bernat et al., 2003). Altogether, this aberrant repression contributes to hinder apoptosis, induce malignant transformation and could compromise the immune response against HPV. Additionally, the E6-hADA3 interaction prevents the transactivation of *TP53* and the transcriptional induction mediated by the retinoic X receptor (Hu et al., 2009; Zeng et al., 2002; Kumar et al., 2002). Moreover, the binding between E7 and DNMT1 stimulate the methyltransferase activity of this enzyme, producing an aberrant hypermethylation state, which could lead to the silencing of tumour suppressor genes and cellular transformation (Burgers et al., 2007). Similarly, the promoter of the catalytic subunit of hTERT has E6 and cellular transcription factor Myc consensus sequences both actively participating in the induction of this gene (Sekaric et al., 2008). This effect is consistent with the increased hTERT activity observed in primary epithelial cells transfected with E6 and provides a molecular basis for the immortalization of these cells (Klingelutz et al., 1996). Furthermore, E6 can interact directly with hTERT, this interaction upregulates the activity of hTERT which could be determinant for cellular immortalization and progression to cancer (Liu et al., 2009).

As we have described above, E2 has a functional DNA-binding domain and regulates viral gene expression. In addition, E2 can also regulate the expression of relevant cellular genes. In this regard, E2 binds to and transactivates the promoter of the splicing factor SF2/ASF. SF2/ASF participates in the regulation of the alternative splicing, and its overexpression mediated by E2 could be related to the production of the viral alternative transcripts of L1 and L2 in the replicative cycle (Mole et al., 2009). E2 also interacts with the transcription factor Sp1 in the promoter of hTERT to repress its expression (Lee et al., 2002). This fact is consistent with the increased hTERT transcription observed in cells that have lost E2 as a result of viral genome integration, and is independent of E6 co-activation of the hTERT promoter (Lee et al., 2002; Sekaric et al., 2008). Additionally, E2 can interact with C/EBP to promote keratinocyte differentiation (Hadaschick et al., 2003). Altogether, these findings illustrate mechanisms that participate in differentiation, growth inhibition and senescence induction, which are associated to E2 function (Dowhanick et al., 1995).

These findings reveal that the interaction of E6, E7 and E2 with nuclear proteins could constitute a hallmark of transcriptional regulation. This direct trans regulation exerted by the presence *in situ* of these viral proteins in human promoters, in the form of co-regulatory complexes (E6 and E7) or by direct binding to DNA (E2), could occur at a global, genomic level. These events provide new molecular mechanisms of aberrant phenotype development, where E2 and E6/E7 have counteracting forces. Therefore, the new horizon in

the comprehension of the molecular pathology of HPV must address this complex nuclear scenario, in which cellular and viral proteins are partners in the promotion of the malignant phenotype (Figure 2B).

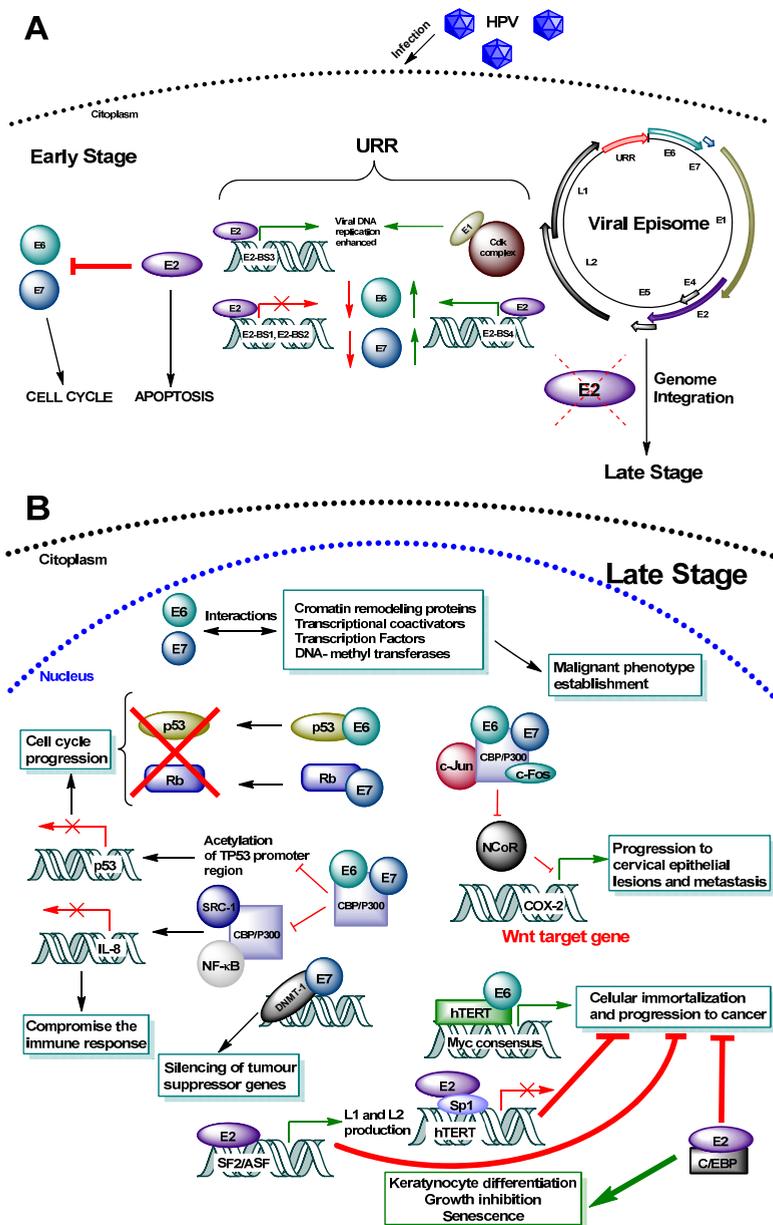


Fig 2. Molecular mechanisms induced by HPV early-expressed proteins. A. Meanwhile HPV is maintained as episome, E1 and E2 are actively expressed, which are

essential proteins for viral genome replication and viral cycle completion. E2 is a sequence-specific transcription factor which regulates the E6 and E7 rate of expression; depending on E2-B5s occupancy sites.

**B.** Human papilloma virus E6 and E7 interact with nuclear proteins such as transcription factors, chromatin remodelers, co-activators and DNA methyl-transferases to influence gene expression and cellular processes towards malignant phenotype and tumoral progression. In the late stage of the infection E2 expression is reduced with a concomitant increase in hTERT expression and reduction of differentiation, therefore the oncogenic activity of E6 and E7 are up-regulated (for details see text).

### 3. Wnt cell signalling pathway

One of the most relevant signaling pathways in tumourigenesis that has been proposed as a hallmark of CC initiation and progression is the Wnt/beta catenin pathway (Uren et al., 2005; Kloth, 2005; Pérez-Plasencia et al., 2007; Pérez-Plasencia et al., 2008). The proto-oncogenic effects of Wnt were discovered almost 30 years ago in C3H mice bearing mammary tumours induced by a viral agent, the mouse mammary tumour virus (MMTV), which genomic sequences were integrated in the host genome. After this seminal work it was clear that common sites of MMTV integration were *int-* called sequences, which were transcriptionally activated in C3H mice breast tumours and inactivated in their normal counterparts (Nusse et al., 1984). Since then, a wealth of evidence has put in the scene the significance of Wnt activation during neoplasm progression in a vast majority of tumour types. The activity of Wnt proteins encompass the regulation of three pathways: 1) the planar cell polarity (PCP) pathway, which controls the polarization and differentiation of cells within a plane of an epithelium and is essential in the neural tube closure and alignment of the neurosensory hair cells of the cochlea (Curtin et al., 2003; Quian et al., 2007.); 2) the Wnt/Ca<sup>2+</sup> pathway that regulates cell movement and adhesion (Kuhl et al., 2000); and 3) the Wnt/beta-catenin or “canonical” pathway, which we will further discuss in this chapter, and that is involved in the regulation of proliferation and that is considered as a hallmark of cancer as well (Ying & Tao, 2009; Hu & Li, 2010; Morris et al., 2010.).

Wnt glycoproteins are extracellular ligands found in many species, ranging from the ctenophore *Mnemiopsis leidyi* to humans (Pang et al., 2010). In mammals, Wnt signalling regulates the establishment of the anterior-posterior (A-P) axis, which was demonstrated by gene depletion in mouse (Zeng et al., 1997). In the adult body, Wnts are involved in the regulation of several biological processes, for instance, cell fate specification, proliferation, migration, cell adhesion, cell polarity, tissue architecture, organogenesis, and angiogenesis, among other. (Wodarz et al., 1988; Peifer et al., 2000; Ross et al., 2000; Gong et al., 2001; Goodwin et al., 2002.).

In tumours, the Wnt/beta-catenin pathway is activated and regulates the expression of genes involved in cell cycle progression, such as cyclin D1 (Tetsu & McCormick, 1999); transcription factors, such as cMyc, that enhance the shift to aberrant tumoural metabolism towards aerobic glycolysis (He et al., 1998); antiapoptotic proteins, such as survivin (Zhang et al., 2001a); proangiogenic factors including VEGF (Zhang et al., 2001b); and metalloproteinases related to tumour progression, invasion and metastasis (Brabletz et al., 1999; Crawford et al., 1999). The alterations in Wnt canonical pathway have been well characterized in colorectal cancer, in

which the via is activated in 80% of cases due to a high rate of mutations in the negative regulators, including axin, APC and GSKb1 and activating mutations on -catenin (Miyoshi et al., 1992; Bienz & Clevers, 2000; Segditsas & Tomlinson, 2009)(Figure 3).

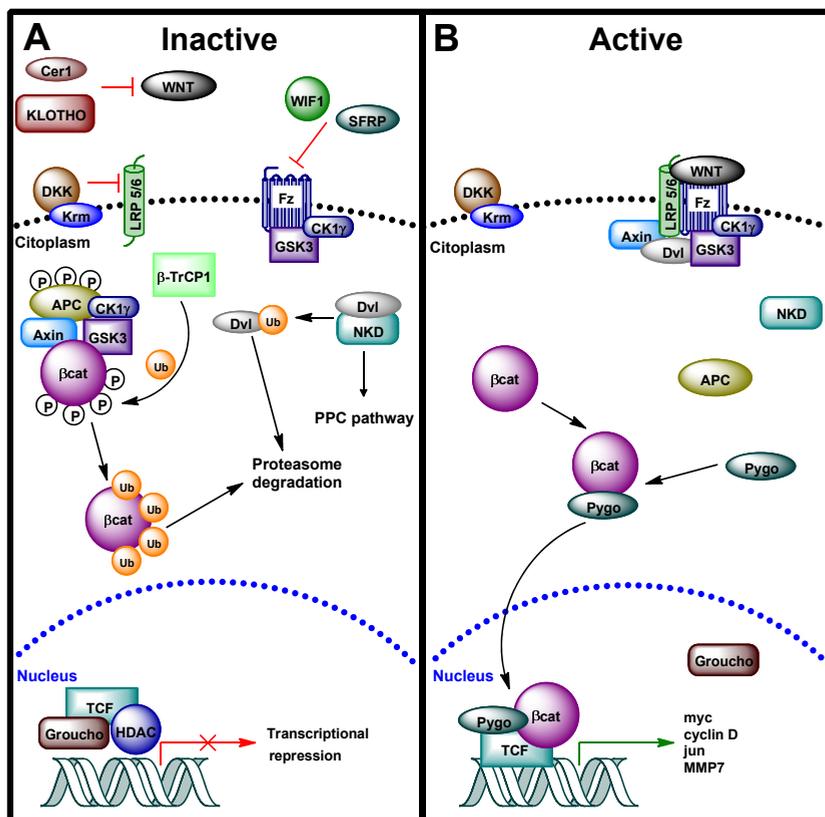


Fig. 3. Wnt canonical signaling pathway.

#### A. WNT Pathway not active.

When the wnt signaling pathway is not active,  $\beta$ -catenin binds to the degradation complex composed by APC, axin, and the serine/threonine kinases CK1 and GSK3. The main role of the degradation complex is to phosphorylate  $\beta$ -catenin leading to its degradation by means of the proteasome-ubiquitin pathway.  $\beta$ TrCP1 functions as an ubiquitinating protein. The pathway can be regulated by several proteins that operate at the receptor-ligand level; such as, Cer1, DKK, WIF1 and sFRP, whose function is to modulate positive signals induced by Wnts. Planar Cell Polarity pathway (PCP) is regulated by NKD genes which interacts to DVL and degrades it by ubiquitination.

#### B. WNT Pathway activated.

The contact of wnt with its receptors leads to the stabilization of  $\beta$ -catenin and its accumulation in cytoplasm and nucleus.  $\beta$ -catenin displaces the transcriptional repressor groucho from the LEF/TCF complex, leading to the activation of target genes, such as c-myc and cyclinD1, which are involved in cell proliferation and cell cycle progression.

Wnt binds to and activate the seven-transmembrane domain specific receptors denominated Frizzled (FZD). The secretion and post-translational modification of Wnt proteins are attained by accompanying molecules, such as porcupine and Wntless (WIs), a process needed to the optimal release and Wnt binding to their receptors and co-receptors (reviewed at Coudreuse & Korswagen, 2007). The broad range of cellular processes regulated by the Wnt pathway can be explained -at least in part- by the high diversity between Wnt proteins and FZD receptors: nineteen members of Wnt family and ten FZD genes have been identified in higher vertebrates (Wordaz & Nusse R, 1988). Besides, interactions between Wnt proteins and their receptors show an important rate of promiscuity (Bahnot et al., 1996). The interaction between Wnt and Fzd requires the cooperation of LRP-5/6 co-receptors, which are long single-pass transmembrane proteins (Wehrli et al., 2000). In this light, mutational studies have shown that both genes are involved in developmental processes; for instance, dorsal thalamic development, skeletal and neural tube abnormalities, decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization (Kokubu et al., 2004; Zhou et al., 2004; Lindvall, 2006.).

Another level of regulation operating on the interaction between LRP5/6, FZDs, and Wnts is achieved by secreted proteins acting as antagonists such as the secreted frizzled-related protein (sFRP), Dickkopf (Dkk), Cerberus1 (Cer1), and KLOTHO, which can inhibit Wnt signaling through direct binding to Wnt or co-receptor molecules. Dkk binds to LDL receptor-related protein (LRP) with other transmembrane proteins, the Kremens (Krm); thus promoting LRP internalization and inactivation (Mao et al., 2002; Pinson et al., 2000). It has been observed that the lack of expression of the sFRP1 is common in cervical, breast, ovary and kidney neoplasms, by mechanisms that include the loss of the *sFRP1* locus in chromosome 8p21 and promoter hipermethylation; moreover, sFRP1 downregulation is associated with tumour progression and invasion (Klopocki et al., 2004; Ko et al., 2002; Ugolini et al., 2001). Similarly, in primary colorectal carcinomas it has been detected a high frequency of hypermethylation on *sFRP1*, *sFRP2*, *sFRP4* and *sFRP5* promoters, which correlates to a reduced expression of these genes (Suzuki et al., 2002). In addition, *in vitro* experiments showed that Dkk-3 has reduced expression in immortalized and tumour cells and it is frequently downregulated in non-small cell lung cancer (Tsuji et al., 2000).

After Wnt binds to their receptor co-receptor complex, FZD recruits Disheveled (Dvl), which transduces the Wnt signal into the cell through interaction with several pathway components. Indeed, Dvl has a key role in the Wnt signal routing and amplification through pathway-specific effectors, by its interaction with axin, which performs a scaffolding function in the Wnt pathway by its association with key proteins for  $\beta$ -catenin phosphorylation and poly-ubiquitination, including GSK-3 $\beta$ , CK1, APC, and  $\beta$ -catenin itself (Zeng et al., 2005; Davidson et al., 2005; McDonald et al., 2009). The consequence of axin phosphorylation is inactivation of "degradation complex" and the subsequent activation of  $\beta$ -catenin (Xing et al., 2003). Degradation complex is a multi-protein assembly activated in the absence of Wnt signaling, whose main task is to add ubiquitins to  $\beta$ -catenin resulting in its inactivation by means of the ubiquitin-proteasome pathway (Peifer & Polakis, 2000). A key component of the degradation complex is APC. When  $\beta$ -catenin binds to APC, it displaces the bound to axin because the binding affinity of  $\beta$ -catenin increases dramatically upon phosphorylation and because the binding motifs of APC to axin and  $\beta$ -catenin overlap. Most colorectal tumours contain truncating mutations on APC, which leads to an inability to bind Axin or degrade  $\beta$ -catenin (Kinzler & Vogelstein, 1996; Xing et al., 2003).

Finally, stabilized non-phosphorylated  $\beta$ -catenin tends to accumulate in the cytoplasm leading to its nuclear translocation, where it is associated with lymphoid enhancer-binding factor 1/Tcell-specific transcription factor (LEF/TCF) and transcriptional activator Pygopus (Pygo). Pygo contains a PHD domain, which is shared by many nuclear proteins with a role in chromatin remodeling and transcriptional co-activation (Belenkaya et al., 2002). Several genes activated by the Wnt signaling pathway, which are involved in cell proliferation and differentiation processes have been identified (Daniels & Weis, 2005) (Figure 3).

The Naked cuticle (Nkd) protein family (NKD1,2), whose activity is required to restrict Wnt signaling during *Drosophila* embryonic segmentation, thus establishing a negative-feedback loop and ameliorating canonical Wnt signaling by binding and destabilizing Dsh/Dvl proteins (Rousset et al., 2001). NKD was the first Wnt antagonist found to be induced by the Wnt pathway (Zeng et al., 2000). Besides, Naked cuticle is proposed to function as a switch, acting to restrict classical Wnt signaling and to activate a second Wnt signaling pathway that controls planar cell polarity (PCP) during gastrulation movements in vertebrates (Wharton et al., 2001). Recently, it has been shown mutations in NKD1 in a subset of DNA mismatch repair-deficient colorectal tumours that are not known to harbor mutations in other Wnt-pathway genes. The mutant Nkd1 proteins were defective at inhibiting Wnt signaling; in addition, the mutant Nkd1 proteins stabilize  $\beta$ -catenin and promote cell proliferation, in part due to a reduced ability of each mutant Nkd1 protein to bind and destabilize Dvl proteins (Guo et al., 2009). Those results suggest that NKD1 is a negative regulator of Wnt and an important target of mutations during the carcinogenesis process.

### 3.1 Wnt pathway and Cervical Carcinoma

Wnt/ $\beta$ -catenin pathway activation is an established hallmark of cancer; hence, mutations in distinct components of this pathway have been studied and identified in nearly all human cancers. In contrast to what is observed in other tumours, Wnt canonical pathway activation caused by mutations is meaningless in CC. In this regard, cervical high grade lesions have an increased expression and nuclear localization of  $\beta$ -catenin with no mutations of CTNNB1 nor Axin (Shinohara et al., 2001; Pereira-Suárez et al., 2002; Su et al., 2003). Thus, in CC it is possible that activation of  $\beta$ -catenin occurs independently of activating mutations by an upstream level mechanism, which could be accomplished by the inactivation of negative regulators. It is well-known that during carcinogenesis aberrant CpG island methylation inactivates distinct tumour suppressor genes, a mechanism that could be explained by means of an increase in DNA methyltransferase (DNMT) activity (Robertson, 2001). In this context HPV16/E7 has the capacity to bind and increase the DNMT1 activity, (Burgers et al., 2007); thus, it is feasible that negative Wnt/ $\beta$  catenin-pathway regulators are inactivated by methylation. In this respect, sFRPs, axin, DICKKOPF (Dkk), KLOTHO and APC genes have enriched CpG islands in their promoters which can be found as hypermethylated in CC (Mikheev et al., 2004; Chung et al., 2009a; Chung et al., 2009b; Lee et al., 2009; Okino et al., 2003; Lee et al., 2010; Song et al., 2009). Therefore, it is probable that inactivation of these genes by promoter hypermethylation induce activation of Wnt canonical pathway during cervical carcinogenesis.

On the other hand, respecting the upstream activation of Wnt/ $\beta$ -catenin pathway, the over-expression of pathway activators such as Wnt ligands, frizzled receptors, and disheveled has been described. There is evidence showing over-expression of WNT10B, -14, FZD10, and

DVL-1 in cervical cell lines (Kirikoshi & Katoh, 2002; Kirikoshi et al., 2001; Koike et al., 1999; Okino et al., 2003); nonetheless, this has not been explored in pathological specimens.

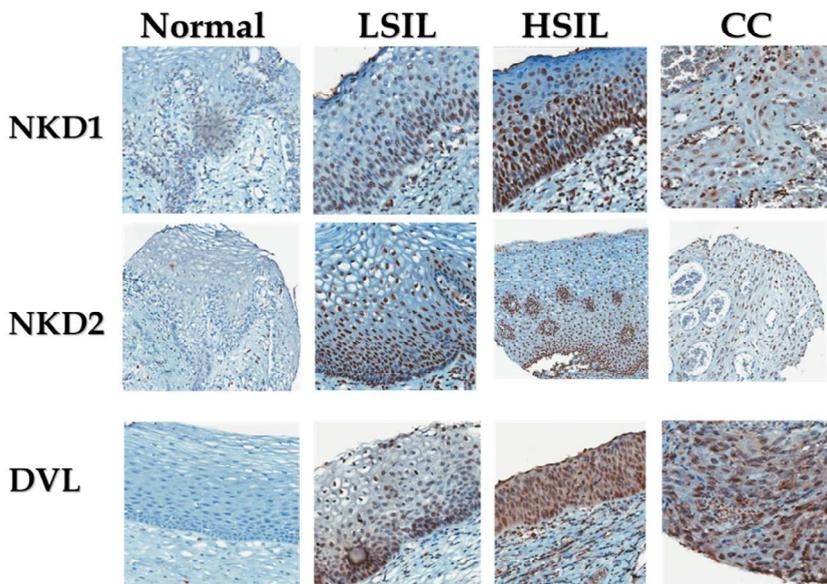


Fig. 4. NKD and DVL are over-expressed from early staged lesions.

Levels of Wnt regulators were assessed in cervical epithelial lesions by means of Immunohistochemistry. Unexpectedly NKD1 and 2 were over-expressed showing an aberrant nuclear staining. As expected, DVL is over-expressed in cervical lesions.

We have reported by genome-wide expression analysis in HPV16 CC tissues that one of the most altered pathways is Wnt/ $\beta$ -catenin. In our study, we observed a significant increment of Wnt4, -8a, Fzd2, GSK3 $\beta$ , and  $\beta$ -catenin in tumours. In addition, genes also belonging to this pathway are actively expressed in normal cervical epithelia, such as sFRP4, PPP2C, and FZD7 (Pérez-Plasencia et al., 2007). This evidence demonstrates two important facts: first, the deregulation in specific genes belonging to Wnt/ $\beta$ -catenin pathway could play an important role in cervical carcinogenesis, and second, the presence of some Wnt/ $\beta$ -catenin-related genes in normal tissues suggests that this pathway is involved in cervical epithelial differentiation. Interestingly, gene components of the planar cell polarity (PCP) pathway were actively expressed in normal cervixes, indicating that this branch of Wnt signaling is down regulated in CC. In vertebrates, PCP is considered as any process affecting cell polarity within an epithelial plane and involving one or more core PCP genes. PCP has shown to be an important developmental and adult tissue differentiation process (Wang & Nathans, 2007). To our knowledge, there are no previous reports showing active PCP genes in normal cervical epithelia. This result demonstrates that during the carcinogenesis process, infected cervical cells turn off the PCP pathway, activating the canonical pathway with a concurrent increase of genes participating in it, for instance, Wnt4, -8A, FZD2, CTNNB1, among others. The activation of the canonical pathway leads to the upregulation of target genes such as MYC, JUN, FOS, and RRAS, which are related to growth promotion (Pérez-

Plasencia et al., 2007). Notwithstanding, NKD1 and NKD2 expression in CC specimens show an increased expression and aberrant nuclear localization as an early event, occurring from low grade squamous intraepithelial lesions to carcinoma (Figure 4). Apparently, the aberrant localization is due to a lack of 300 bp in transcripts sequence (Pérez-Plasencia unpublished results), indicating a key role of NKD genes in Wnt pathway regulation on CC tumour progression.

Additionally, some experiments *in vitro* have shown that high-risk HPV16 E6 oncoprotein was capable of activate Wnt/beta-catenin pathway in an E6AP dependent fashion (Lichtig et al., 2010). Altogether, these data are in concordance with the fact that human genital keratinocytes immortalized with high-risk HPV need the activation of Wnt canonical pathway to be transformed and suggest that this event is essential in the cervical tumourigenesis (Uren et al., 2005).

#### 4. Conclusions

The extensive use of the Papanicolau smear and colposcopy examination have significantly decreased the CC mortality rates; however, this neoplasm still remains as the second cause of death in women worldwide. Concordantly, HPV presence has been found in more than 99% of CC, hence HPV infection is considered as the most important etiologic factor in cervical carcinogenesis. Even though HPV infection is very common among the young sexually active population, only a small fraction of infected individuals develop cervical carcinoma later in life. Thus, HPV is considered only as an initial hit in the multistep carcinogenesis that leads to the development of CC. The molecular pathways involved in the progression of HPV-infected cells to CC have not been accurately identified. Here, we reviewed the role of Wnt/ $\beta$ -catenin pathway over-activation and the inactivation of planar cell polarity pathway in CC cells as a second hit to develop CC; moreover, one key regulator of PCP, NKD, is aberrantly localized in nucleus and overexpressed in CC. In this regard, many reports have described that Wnt/ $\beta$ -catenin pathway is aberrantly active in CC, where common tumour-causing mutations on the genes of this pathway, such as APC, Axin and CTNNB1 have not been found. Thus Wnt/ $\beta$ -catenin pathway over-activation could be caused by dysregulation in upstream modulators, by means of negative regulators inactivation or over-expression of activators. On the other hand, an additional branch in Wnt signaling pathway, that could be determinant during CC pathogenesis, is the planar cell polarity (PCP) pathway, which is involved in cellular differentiation. PCP is a key via in differentiation and the morphogenetic process involved in development of epithelia. In normal cervical epithelia, cells are polarized and migrate from basal to the luminal space as they differentiate. Interestingly, PCP component genes are repressed in CC, indicating that this pathway could be abated prior the establishment of the neoplasia. From the diagnostic point of view, this fact could be of great importance because the possibility to reveal PCP downregulation as an early tumourigenic process could provide for potential methods of early molecular markers detection in patients who have HPV and will develop CC.

#### 5. Acknowledgments

This work was supported in part by UNAM (Grant IACOD-TB200111, Grant PAPCA-2010-2011 No.21) and by CONACyT (Grant SALUD-2009-01-113948).

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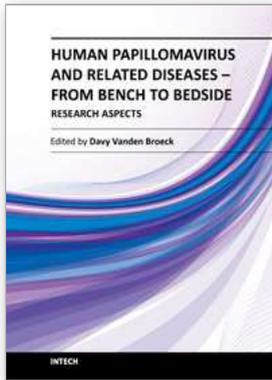
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## **Human Papillomavirus and Related Diseases - From Bench to Bedside - Research aspects**

Edited by Dr. Davy Vanden Broeck

ISBN 978-953-307-855-7

Hard cover, 406 pages

**Publisher** InTech

**Published online** 25, January, 2012

**Published in print edition** January, 2012

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

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