Abstract

Cervical carcinoma, the second leading cause of cancer deaths in women worldwide, is associated with human papillomavirus (HPV). HPV-infected individuals are at high risk for developing cervical carcinoma; however, the molecular mechanisms that lead to the progression of cervical carcinoma have not been established. We hypothesized that in a multistep carcinogenesis model, HPV provides the initial hit and activation of canonical Wnt pathway may serve as the second hit. To test this hypothesis, we evaluated the canonical Wnt pathway as a promoting factor of HPV-induced human keratinocyte transformation. In this in vitro experimental cervical carcinoma model, primary human keratinocytes immortalized by HPV were transformed by SV40 small-t (smt) antigen. We show that smt-transformed cells have high cytoplasmic β-catenin levels, a hallmark of activated canonical Wnt pathway, and that activation of this pathway by smt is mediated through its interaction with protein phosphatase-2A. Furthermore, inhibition of downstream signaling from β-catenin inhibited the smt-induced transformed phenotype. Wnt pathway activation transformed HPV-immortalized primary human keratinocytes even in the absence of smt. However, activation of the Wnt pathway in the absence of HPV was not sufficient to induce transformation. We also detected increased cytoplasmic and nuclear staining of β-catenin in invasive cervical carcinoma samples from 48 patients. We detected weak cytoplasmic and no nuclear staining of β-catenin in 18 cases of cervical dysplasia. Our results suggest that the transformation of HPV expressing human keratinocytes requires activation of the Wnt pathway and that this activation may serve as a screening tool in HPV-human keratinocytes requires activation of the Wnt pathway. Therefore, tumor formation following HPV infection most likely is a result of multistep carcinogenesis.

In vitro models of cervical carcinoma have supported this hypothesis. Human genital keratinocytes can be maintained in cell culture for ~10 passages. In the presence of serum and calcium, they undergo terminal differentiation. Either the entire HPV genome or the isolated E6/E7 genes can immortalize primary keratinocytes and inhibit their ability to differentiate. These immortalized keratinocytes are anchorage dependent and do not proliferate in soft agar. However, SV40 small-t antigen (smt) can transform these cells to an anchorage-independent state. Transfection of HPV-expressing cells with smt therefore mimics the progression to malignancy.

Wnt proteins comprise a large family of secreted growth factors that control cell fate, proliferation, migration, tissue architecture, and organogenesis during embryonic development (6, 7). In adult organisms, Wnts regulate the homeostasis of hematopoiesis, osteogenesis, angiogenesis, and adipogenesis (8–11). Recent findings provide evidence for a major role of Wnt signaling in various pathologic conditions including cancer (12–14). Wnt binding to its receptors activates a canonical pathway, which is characterized by accumulation of β-catenin in the cytoplasm and nucleus. Cytoplasmic β-catenin protein levels are under strict control by phosphorylation-induced ubiquitination and degradation (15). In the absence of Wnt signaling, GSK-3β is active and phosphorylates β-catenin resulting in its degradation. Activation of Wnt pathway inhibits GSK-3β and causes increased cytoplasmic β-catenin. Excess β-catenin then goes to the nucleus to form an active transcriptional complex with T-cell factor (TCF), which activates transcription of target genes including c-myc and cyclin D1 (16–18). Degradation of β-catenin requires the presence of adenomatous polyposis coli (APC gene product) and axin to allow GSK-3β to phosphorylate β-catenin (19). A protein phosphatase, PP2A, is also involved in the degradation complex of β-catenin. PP2A inhibits Wnt signaling through its direct interactions with APC and axin (20–22).

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In this study, we link the prior evidence that transformation of HPV-immortalized human keratinocytes requires a second hit with activation of the canonical Wnt pathway. Activation of this canonical Wnt pathway is necessary and sufficient to induce the transformation of HPV-immortalized cells in our experimental system. We also provide evidence that Wnt pathway activation occurs in human cervical carcinoma samples.

Materials and Methods

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CO₂. HFK cells were grown in keratinocyte medium with 25 mg of bovine pituitary extract and 2.5 µg human recombinant epidermal growth factor added (Invitrogen Life Technologies, Grand Island, NY). HFK-HPV, HFK-HPV-smt, HFK-HPV-smt-43/45, and HFK-HPV-smt-97 cells were grown in 1 volume DMEM + 10% fetal bovine serum (FBS) and 3 volumes keratinocyte medium with added supplements. Pathologic specimens were obtained from five archives of Pathology departments in Georgetown University and Hacettepe University. Category 4b exemption for this study was obtained from Georgetown University Oncology Institutional Review Board. Immunoprecipitations and Western blotting were done as described elsewhere (23).

Transfection of cDNA by electroporation. Standard 1-mL electroporation cuvettes and a Cell-Porator (Invitrogen Life Technologies) were used for these experiments. Cells were trypsinized and collected into their appropriate medium with 10% FBS to inhibit trypsin activity. Cells were then washed twice with serum-free medium before electroporation; 3 x 10⁶ cells/mL were placed into each electroporation cuvette along with plasmid DNA (10 µg) and mixed gently. Cells were electroporated at 350 V and 1,600 μF and transferred to 60-mm tissue culture dishes with prewarmed serum-containing medium.

TOPFLASH/FOPFLASH reporter assay. HFK, HFK-HPV, HFK-HPV-smt cells, and HFKHPV-smt-DN-TCF cells were electroporated as described above with TOPFLASH or FOPFLASH reporter construct; 20 ng of Renilla plasmid with a truncated cytomegalovirus promoter (kindly provided by Dr. Stephen Byers, Departments of Oncology and Cell Biology; Georgetown University, Washington, DC) were also added as a control. Cells were then plated in 6-well tissue culture plates and grown for 24 hours. Cells were lysed in 100 µL of 1× passive lysis buffer (Promega, Madison, WI). Ten microliters of each lysate was plated in triplicate on a 96-well white polystyrene assay plate. The Dual-Luciferase Reporter assay (Promega) was done following manufacturer’s protocol. Cytoplasmic β-catenin protein levels were measured by glutathione S-transferase (GST) pulldown as described earlier (24, 25).

Soft agar colony formation. A 6% SeaPlaque GTG agarose was prepared in PBS as a stock solution and kept in a 70°C water bath. From the stock solution, 0.6% and 0.4% agaroses were prepared in the appropriate cell culture medium. To form the supporting bottom part of the soft agar, 1.5 mL of the 0.6% agarose were added to each well of a 24-well culture cell and was set aside to solidify at room temperature. Cells were washed twice with medium and resuspended in the 0.4% agarose at 5,000 cells/mL. One milliliter of this cell suspension was added in triplicate on top of the bottom agar. The plate was kept at room temperature until the top solidified and was incubated at 37°C in a cell culture incubator for 10 to 14 days. Pictures of wells were taken by a Nikon SMZ-1500 Stereozoom and quantitation was done on digital images by Kodak 1D image analysis software (Eastman Kodak Co., Rochester, NY). Experiment was repeated thrice.

Wnt-3a conditioned medium. Wnt-3a-transfected and control L cells were purchased from American Type Culture Collection (Manassas, VA). Wnt-3a-conditioned medium was collected from L cells as described earlier except that serum-free medium was used in the present study (26). Cells were grown on TripleFlask (Fisher Scientific, Newark, DE) with 500 cm² culture area until they were 80% confluent, then medium was switched to 100 mL of RPMI. Two successive 72-hour harvests of conditioned medium were collected and pooled.

Immunohistochemistry. Staining was done on 5-mm sections of formalin-fixed, paraffin-embedded tissue from patients with diagnosed cervical carcinoma. Antigen retrieval occurred by steam heat for 20 minutes with 0.01 M sodium citrate buffer, pH 6.0. Antigen retrieval was then performed in a 3N HCl solution, 0.6% and 0.4% agaroses were prepared in the appropriate cell culture medium. To form the supporting bottom part of the soft agar, 1.5 mL of the 0.6% agarose were added to each well of a 24-well culture cell and was set aside to solidify at room temperature. Cells were washed twice with medium and resuspended in the 0.4% agarose at 5,000 cells/mL. One milliliter of this cell suspension was added in triplicate on top of the bottom agar. The plate was kept at room temperature until the top solidified and was incubated at 37°C in a cell culture incubator for 10 to 14 days. Pictures of wells were taken by a Nikon SMZ-1500 Stereozoom and quantitation was done on digital images by Kodak 1D image analysis software (Eastman Kodak Co., Rochester, NY). Experiment was repeated thrice.

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Immunohistochemistry. Staining was done on 5-mm sections of formalin-fixed, paraffin-embedded tissue from patients with diagnosed cervical carcinoma. Antigen retrieval occurred by steam heat for 20 minutes followed by a standard avidin-biotin peroxidase complex technique and automated immunostaining (Ventana Medical System, Tucson, AZ). The primary antibody was mouse anti-β-catenin diluted 1:100 (BD Biosciences, San Jose, CA) and the secondary antibody was biotinylated-mouse anti-mouse antibody (Ventana Medical System).

Results

Transformation of human papillomavirus–immortalized keratinocytes by small-t correlates with increased cytoplasmic β-catenin levels. We studied cervical cancerogenesis in vitro, using a human genital keratinocyte model system. This model uses primary human foreskin keratinocytes (HFK) obtained from human neonatal foreskin that undergoes senescence after 10 passages in cell culture (27). HPV immortalizes these keratinocytes (HPF-HPV) and induces resistance to serum and calcium-induced differentiation. Although HFK-HPV cells are immortalized, they are not transformed. Transfection with smt induces transformation of HFK-HPV (HFK-HPV-smt; ref. 5). Hence, in this model system, smt transforms HPV-immortalized keratinocytes, which mimics the biology of cervical cancer progression. We analyzed cytoplasmic β-catenin protein levels in the presence or absence of Wnt-3a conditioned medium to assess the state of Wnt signaling in our in vitro experimental model of cervical carcinogenesis (Fig. 1A).

Due to the abundance of membrane-bound β-catenin, Western blot analysis of total cell lysates is not sensitive enough to detect the changes in the cytoplasmic pool of β-catenin. Therefore, cytoplasmic fractions of β-catenin were isolated by GST pulldown using a GST/E-cadherin construct that contains the β-catenin-binding domain of E-cadherin (24, 28). Because β-catenin on the membrane is already bound to endogenous E-cadherin, only the cytoplasmic β-catenin is pulled down with GST/E-cadherin. We measured the cytoplasmic β-catenin levels in HFK, HFK-HPV, and HFK-HPV-smt cells in their steady-state growth phase and tested the effect of Wnt-3a treatment on these cells. Untreated HFK and HFK-HPV cells showed low levels of cytoplasmic β-catenin (Fig. 1A, lanes 1 and 3). Both HFK and HFK-HPV cells responded to Wnt-3a treatment with a significant increase in cytoplasmic β-catenin protein levels in 3 hours. Total β-catenin levels did not change in any condition (Fig. 1A, bottom). Interestingly, HFK-HPV-smt cells had high steady-state levels of cytoplasmic β-catenin before Wnt stimulation. This suggests constitutive activation of Wnt signaling (Fig. 1A, lanes 5 and 6). Conditioned medium from untransfected L cells was used as a negative control for Wnt-3a treatments. HFK, HFK-HPV, and HFK-HPV-smt cells treated with L cell–conditioned medium did not show any changes in their cytoplasmic β-catenin protein levels (data not shown).

Because we detected elevated cytoplasmic β-catenin in transformed cells, we investigated whether this elevated β-catenin led to increased nuclear function. Using a TCF-responsive reporter construct (TOPFLASH), we measured TOPFLASH activity in HFK, HFK-HPV, and HFK-HPV-smt cells (Fig. 1B). As a negative control, we used FOPFLASH, which is the same reporter construct as TOPFLASH except with mutated TCF-binding sites. We found that HFK-HPV-smt cells had 35-fold increased TOPFLASH activity compared with that of HFK and HFK-HPV cells. This finding was consistent with the high cytoplasmic β-catenin levels in HFK-HPV-smt cells. Because HFK-HPV-smt cells previously showed a transformed phenotype, we hypothesized that β-catenin might be a final common pathway of transformation.

Activation of canonical Wnt pathway by smt is mediated through PP2A. We investigated a possible mechanism that can induce β-catenin accumulation in HFK-HPV-smt cells. Previous studies have shown that smt can directly interact with PP2A and inhibit its cellular functions (29–32). Because PP2A also interacts with APC and axin, components of β-catenin degradation machinery, we tested if smt-induced β-catenin stabilization is PP2A dependent. We used three smt mutants (smt-97, smt-103, and smt-43/45; refs. 33, 34); smt-97 and smt-103 were mutated in the PP2A dependent. We used three smt mutants (smt-97, smt-103, and smt-43/45; refs. 33, 34); smt-97 and smt-103 were mutated in their PP2A-binding sites but retained their transcriptional activities. On the other hand, smt-43/45 was mutated on its transcriptional activation site but retained its PP2A-binding
domain. HFK-HPV cells were transfected with wild-type (WT) and mutant smts and individual clones were selected. All clones were tested for their β-catenin levels (Fig. 2). As expected, HFK and HFK-HPV cells showed low β-catenin levels compared with those for HFK-HPV-smt cells. Transcriptionally inactive mutant smt-43/45 was still able to induce β-catenin stabilization in HFK-HPV cells, suggesting that the smt-induced increase in cytoplasmic β-catenin protein levels does not require the transcriptional activity of smt. In contrast, PP2A-binding mutants of smt (i.e., smt-97 and smt-103) failed to induce β-catenin stabilization, suggesting that smt-PP2A interaction is critical for this function. Therefore, it is likely that PP2A inhibition by smt is the molecular mechanism for increased β-catenin levels in HFK-HPV-smt cells. Moreover, transcriptionally inactive mutant smt-43/45 can still induce colony formation in soft agar, whereas PP2A-binding mutants of smt (i.e., smt-97 and smt-103) lack this phenotype (data not shown).

Inhibition of β-catenin signaling attenuates smt-induced malignant transformation of HFK-HPV cells. Our results suggested a strong correlation among cytoplasmic β-catenin accumulation, activated Wnt signaling, and smt-induced transformation of HFK-HPV cells (Figs. 1 and 2). We then investigated whether the activation of β-catenin signaling is necessary for smt-induced malignant phenotype. We transfected HFK-HPV-smt cells with a dominant-negative TCF construct, which can inhibit the canonical Wnt pathway downstream of β-catenin. We tested the smt-induced malignant phenotype using the soft agar assay, an anchorage-independent growth assay (Fig. 3A). As reported earlier, HFK-HPV cells did not form colonies in soft agar and HFK-HPV-smt cells formed colonies. Inhibition of β-catenin signaling by dominant-negative TCF significantly reduced the number of colonies. Two identical experiments were done in triplicates and colonies were counted, showing consistent reduction in the dominant-negative TCF group (Fig. 3B). The reduced activity of β-catenin signaling in these cells was confirmed by a TOPFLASH reporter assay (Fig. 3C). Consistent with soft agar results, HFK-HPV-smt cells with dominant-negative TCF showed significantly reduced TOPFLASH activity when compared with untransfected cells or empty vector–transfected cells. To rule out the possibility that the reduction in malignant phenotype may be due to decreased smt expression in HFK-HPV-smt cells with dominant-negative TCF, we did Western blotting (Fig. 3D). HFK-HPV-smt cells with dominant-negative TCF showed comparable levels of smt expression with untransfected cells and empty vector–transfected cells.
Activation of β-catenin signaling via Wnt pathway induces transformation of HFK-HPV cells. Transformation of HFK-HPV cells by smt was found dependent on activation of canonical Wnt signaling (Fig. 3). Therefore, we investigated whether activation of canonical Wnt pathway is sufficient to induce transformation of HFK-HPV cells in the absence of smt. We generated two HFK-HPV cell lines with an activated Wnt pathway, using an HA-tagged mouse Wnt-1 in one line and using Flag-tagged constitutively active h-catenin (S37A-h-catenin) in the other line. S37A-h-catenin has a serine-to-alanine substitution on position 37. The mutation prevents phosphorylation at Ser 37 and consequently prevents degradation of h-catenin. Hence, h-catenin accumulates in the cytoplasm and nucleus, mimicking an upstream Wnt signal. Both cell lines formed colonies in soft agar, suggesting that the activation of Wnt pathway at different stages is sufficient to induce transformation in the absence of smt (Fig. 4A). Furthermore, neither Wnt-1 nor S37A-β-catenin-transfected HFK cells formed colonies in soft agar, suggesting a requirement for HPV initiation. To show reproducible results, the experiment was done thrice in duplicates (Fig. 4B). Expression of Wnt-1 and S37A-β-catenin was confirmed by Western blotting (Fig. 4C and D). We obtained similar results when we activated the Wnt pathway at different levels by WT β-catenin, disheveled-2, Wnt-3a, or a GSK-3β inhibiting peptide, GID-5-6 (data not shown). GID-5-6 is a 25-amino-acid peptide corresponding to the GSK-3β-binding domain of axin, which specifically binds to GSK-3β and prevents phosphorylation of β-catenin. Expressing these constructs induced colony formation in soft agar, with HFK-HPV cells but not with HFK cells.

β-Catenin signaling is activated in the cervical carcinoma samples. As previously described, a hallmark of canonical Wnt signaling is the accumulation of β-catenin in the cytoplasm and nucleus as opposed to its normal plasma membrane localization. Because our in vitro experimental model of cervical carcinoma showed a critical role of Wnt signaling in human genital keratinocyte transformation, we evaluated β-catenin localization in human cervical carcinoma specimens by immunohistochemistry (Fig. 5). Forty-three of 48 tumors showed β-catenin staining in the cytoplasm and/or nucleus (Fig. 5E and F, arrows). Five tumors had matched normal cervical epithelium on the same slide. Normal epithelium showed staining of β-catenin only on the membrane (Fig. 5A and B). There was cytoplasmic and nuclear staining only on the basal proliferating layer of the normal stratified squamous epithelium (Fig. 5A, arrowhead). Furthermore, staining of 18 cases with cervical dysplasia, a precancerous lesion, showed a β-catenin
staining pattern that falls between normal epithelium and invasive cancers (Fig. 5C and D). These results suggest that only cervical carcinoma cells but not normal cervical epithelial cells show signs of activated β-catenin signaling. Because β-catenin is the downstream target of Wnt signaling, this finding supports our hypothesis that activation of Wnt signaling at some stage may contribute to cervical carcinoma development.

Discussion

Our data indicate that smt transformation of HPV-immortalized cells correlates with increased cytoplasmic β-catenin levels and higher TCF-responsive reporter construct activity. Furthermore, in our model, this effect seems dependent on smt interaction with PP2A, because mutants of smt that cannot bind PP2A did not induce β-catenin stabilization. These findings were also complemented by the immunohistochemistry data showing activation of Wnt signaling in cervical carcinoma specimens. Our results suggest that Wnt signaling may contribute in the pathogenesis of cervical cancer.

Foreskin genital keratinocytes, like cervical epithelial cells, are an in vivo target for the oncogenic mucosal HPVs. Thus, HPV-16 induces dysplastic cells in the cervix as well as foreskin epithelium. In addition to these clinical correlations, the HPV genome induces cancerous epithelial alteration in foreskin cells and foreskin cultures that closely mimic cervical dysplasia. Finally, the HPV E6 and E7 genes induce chromosomal changes in foreskin cells that parallel those observed in cervical neoplasia. Whereas there are anticipated differences in estrogen signaling in these different cell types, the basic cell growth controls in the foreskin and cervical cells seem very similar and, due to their much greater bioavailability, foreskin cells have become the accepted "standard" cell type for assaying HPV oncogene activities (35).

Widespread use of the Papanicolaou smear in developed countries has significantly reduced the mortality rates from cervical carcinoma by early detection and removal of premalignant lesions. However, cervical carcinoma remains the number two killer among women worldwide. More than 99% of all cervical cancers are associated with HPV and HPV infection is the most important etiologic factor in cervical carcinogenesis. Although HPV infection is very common among the young sexually active population, only a small fraction develops cervical carcinoma later in life. Hence, HPV provides an initial hit in a multistep carcinogenesis. The molecular mechanisms of how HPV-infected cells progress to cervical carcinoma are unclear. Understanding these mechanisms will allow screening of the HPV-infected population and will allow preventive intervention before cancer develops. Our results suggest that activation of canonical Wnt pathway may play a significant role in malignant transformation of HPV positive cervical epithelial cells. An earlier publication supports this hypothesis as well. When invasive cervical squamous cell carcinoma specimens were analyzed by immunohistochemistry, 73% showed increased cytoplasmic and nuclear staining (36). Interestingly, 80% of these samples had no mutations in the β-catenin gene, which suggests an activation of Wnt signaling at an upstream level. A similar outcome was observed with
In summary, there is evidence for increased β-catenin levels in cervical carcinoma without any β-catenin mutations. However, the relationship between this activated β-catenin signaling and HPV has not been established.

Activation of canonical Wnt signaling without frequent β-catenin mutations is an intriguing finding. One possible mechanism for this activation could be up-regulated expression of Wnts. Another possible mechanism for this activation could be modulation of gene expression by DNA methylation in promoter regions. Because multiple gene promoters are hypermethylated in cervical carcinoma, it is possible that APC and sFRP promoters could also be methylated and be responsible for high cytoplasmic β-catenin protein levels. Furthermore, Ko et al. detected diminished sFRP-1 expression in the cervical carcinoma cases compared with three normal cervical tissues by Northern blot analysis, which could potentially result in Wnt signaling activation. Additionally, genetic mutations of APC and AXIN2 occur in 24% and 69% of sporadic colon cancers, respectively. Hence, there may be yet to be discovered genetic mutations in cervical carcinoma that result in activated canonical Wnt signaling.

Results of our immunohistochemistry analysis of 48 tumor samples showed an activated β-catenin signaling. Activation of the Wnt pathway at some level is the most common mechanism for β-catenin stabilization and is most likely the case in cervical carcinoma. However, an increased cytoplasmic β-catenin level can be achieved by other oncogenes including nonreceptor tyrosine kinase v-src and mutants of RON and MET receptor tyrosine kinases. Therefore, future studies aiming to find the underlying cause of β-catenin stabilization in cervical carcinoma should include these alternative signaling pathways too.

Our in vitro experimental model of cervical carcinogenesis reflects the multistep tumor formation in clinical cases. HFK cells have been widely used in studying HPV-induced immortalization and changes in differentiation and viral replication. HFK cells take the role of normal cervical epithelium, which can be immortalized by HPV. HFK-HPV cells represent a large population that is HPV positive and at high risk to develop cervical cancer. HFK-HPV cells can be transformed by many oncogenes, one of which is smt. In this model of smt-induced malignant transformation of HFK-HPV cells, we have discovered that activation of canonical Wnt signaling plays a central role. Our findings suggest that the activation of Wnt signaling by smt is through inactivation of a phosphatase, PP2A.

PP2A is a family of multimeric serine/threonine phosphatases that regulates a wide range of cellular activities, including cell cycle, apoptosis, and signal transduction. Many functions of PP2A support its role as a tumor suppressor protein like PTEN; however, definitive proof remains to be discovered. PP2A has three subunits: catalytic, regulatory A, and regulatory B. There are two catalytic, two A, and 15 B subunits, which makes it a very diverse and strictly controlled phosphatase. PP2A modulation of Wnt signaling comes through its binding to axin and APC in the β-catenin degradation complex. Phosphorylation of β-catenin by GSK-3β mediates its degradation. GSK-3β is inhibited by phosphorylation at residue Ser\(^{\alpha}\). Hence, removal of this inhibition by PP2A might activate GSK-3β and inhibit Wnt.
signaling (50, 51). Therefore, active PP2A will function as a Wnt antagonist and reduce cytoplasmic β-catenin levels (21). Inhibition of PP2A by ΔNp63, a p53 homologue, results in increased levels of β-catenin (52). A similar conclusion can be reached based on the expression pattern of β-catenin and one of the B subunits of PP2A in lung development (53). Smt binds to PP2A and inhibits its function (29–32). Therefore, it is possible that smt might stimulate Wnt signaling by inhibiting PP2A, which leads to inhibition of GSK-3β and accumulation of β-catenin.

Hence, transformation of HPV-immortalized keratinocytes by smt (5) could be through activation of the Wnt/β-catenin pathway. Conversely, there have been reports of Wnt signaling activation by PP2A. The catalytic subunit of PP2A augments Disheveled-induced secondary axis formation in Xenopus laevis embryos (54). Wnt induces dephosphorylation of axin, which may be mediated by PP2A (55). Dephosphorylated axin may then fail to function in the β-catenin-degrading complex thereby giving rise to increased β-catenin levels. These seemingly paradoxical results are most likely due to experiments done in different systems with different PP2A subunits.

In summary, our results suggest that activation of Wnt signaling may have a role in the pathogenesis of cervical cancer and this observation may be useful for development of molecular diagnostics and therapies.

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References


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