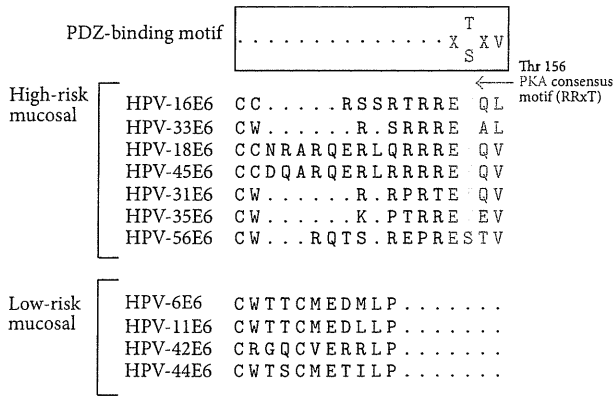

FIGURE 1: The E6 proteins of high-risk or oncogenic HPVs uniquely contain class I -PBM (sequence x-S/T-x-V/I/L-COOH) located at the C-terminus. Representative alignment of the C-terminal ends of the E6 proteins from the most frequently found. HPV types associated with cervical carcinoma (HPV 16, 33, 18, 45, 31, 35, 56) and compared with low risk types (HPV 6, 11, 42, 44). PKA consensus motif (RRxT) marked in purple.

mutant viruses defective in PDZ binding lead to reduced growth rates, loss of viral episomes, and increased numbers of unstable viral genomes that are either lost or become integrated into the host chromosomes [23]. Considering the fact that modulation takes place in the earlier stages of HPV life cycle, that is, in the viral proliferation and maintenance phase rather than later in malignant progression, it is reasonable to speculate that E6 PBM-PDZ interactions occur in a variety of cellular contexts and at different stages along the time axis of malignant progression.

In the discussion below, we focus on what effect these varieties of HPV E6-PDZ interactions potentially have in relation to HPV-induced malignancy, depending upon "where" (different localization) and "when" (different stages in viral life cycle or in cancer progression) they take place and upon various posttranslational modifications to which they are subject.

4. HPV-PDZ Interplay during the Course of HPV Life Cycle

4.1. A Model for Progression from HPV Infection towards Malignancy. The existing model for the progression from HPV infection towards malignancy is shown in Figure 2 [24, 25]. Initial HPV infection occurs when microtraumas secondary to sexual intercourse allow HPVs to enter the mucosal basal cell layer of genital tract epithelium. Initially, HPVs maintain their genome at low copy number, around 10–200 copies per cell, as episomes in the basal cells of the epithelium, and thereby are capable of establishing long-term latent infections. For viral replication, viruses depend on the terminal differentiation of stratified epithelium and are known to alter the host's differentiation program to allow it to reenter cell cycle through the coordinate expression of viral gene products including E6 and E7. This process, known as the proliferative phase, is subsequently followed by genome amplification, virus synthesis, and shedding of new viral particles within a short period of 2-3 weeks postinfection. As previously mentioned, when considering high prevalence of HPV infections in the young sexually active population, the number of lesions that ultimately progress to cancer is fairly low that is, the majority of infections are transient and cleared by the immune system. However, when HPVs succeed in evading innate immune recognition and elimination, continued persistence of the viruses can cause dysplasia, the precancerous but reversible changes that may slowly progress to cancer.

4.2. Stabilization of HPV16 E6 Protein by PDZ Proteins. Studies on NIKS cells, a spontaneously immortalized human keratinocyte cell line that can support the entire life cycle of HPV-16 [26], have shown that the presence of hScrib significantly increased the levels of E6 protein, revealing that the interactions between E6 PBM and its PDZ-containing targets may increase the stability of E6 during early stages of the viral life cycle [27]. Furthermore, mutant HPV-16 genomes lacking E6 PBM cannot persist in an episomal state, and this instability leads either to their being rapidly degraded by the proteasome or being integrated into the host cellular DNA. In addition to hScrib, other PDZ domain-containing proteins, including hDlg1 and MAGI-1, have also been suggested to stabilize E6 expression in a PBM-dependent manner.

This is consistent with the previous study on HFK cells transfected with HPV-31, where mutation of PBM resulted in significant retardation in their growth rates and reduction in their viral copy number, together with the unusual morphological differentiation of cells [23].

It is somewhat unexpected, but appears to be a curious paradox, that the same series of proteins which E6 targets for degradation during malignant progression fulfill an important role for the stabilization of E6 and the episomal maintenance in earlier stages of the HPV life cycle, suggesting the possibility of the multiple functions for PDZ-PBM interactions, depending upon different cellular contexts.

5. HPV-PDZ Interplay during Malignant Progression

5.1. Perturbation of the Functions of PDZ Proteins Results in Major Characteristics of Various Cancers. As definitively discussed in previous studies, both hDlg and hScrib are intimately linked to homeostatic maintenance of several epithelial tissues, including regulation of cell polarity and proliferation. Deregulation of these proteins has been shown in a number of human epithelial cancers: mislocalized and deregulated hScrib expression is seen in colorectal [28], breast [29], prostate [30], and endometrial cancers [31], providing further powerful evidence supporting a role for these proteins in tumor suppression. As for cervical cancer, hDlg and hScrib are degraded to varying degrees by high-risk HPV E6 oncoproteins, both in cells derived from cervical tumors and in a variety of experimental settings, and their expression levels are low in late-stage cancer [32]. Thus, loss of these

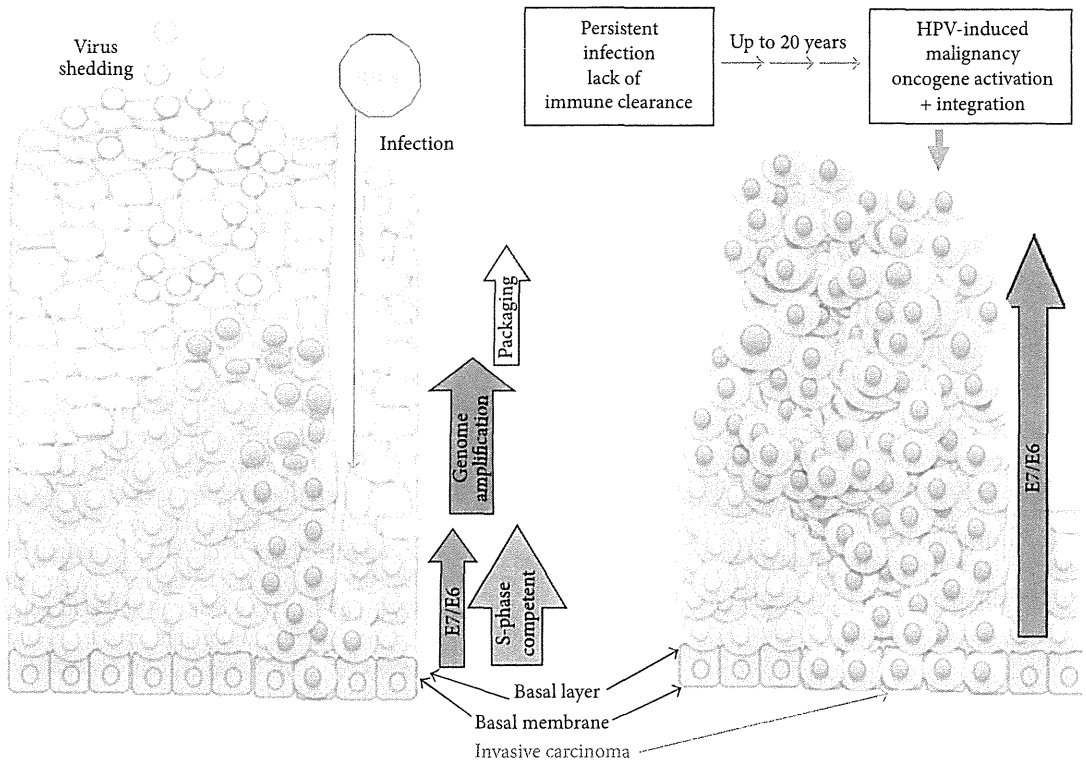

FIGURE 2: The existing model for the progression from HPV infection towards malignancy. HPV infection occurs when microtraumas allow HPVs to enter the mucosal basal cell layer of genital tract epithelium. Initially, HPVs maintain the genome at low copy number as episomes in the basal cells of epithelium and thereby are capable of establishing long-term latent infections. However, when HPVs succeed in evading innate immune recognition and elimination, continued persistence of the viruses can cause dysplasia, the precancerous but reversible change, which may slowly progress to cancer.

regulatory functions appears to be a major feature in diverse malignancies.

5.2. Spatiotemporal Regulation of PDZ Proteins—Their Loss Might Be a Late-Stage Marker in Cervical Cancer. Interestingly, changes in the levels of expression and localization of Dlg and Scrib occur dependently upon different stages of malignant progression to cervical cancer. For Dlg, analysis by immunohistochemistry showed that the expression of Dlg in cervical intraepithelial precursor lesions displays a distribution pattern different from what is seen in normal epithelium, that is, an unusual increase in the cytoplasm and loss at the sites of cell-cell contact (normal localization). It was only in the invasive form of cervical cancers that a severe decrease in the amount was observed [33], suggesting that loss of Dlg is a late-stage marker in cervical malignancy but that alterations in its normal expression and localization may also contribute to the progression from precancerous lesions to cervical cancer. Also, it has been demonstrated that for proteasome-mediated degradation, E6 preferentially targets nuclear pools of hDlg rather than membrane-bound forms, which shows an example of a localization-dependent difference in susceptibility of PDZ-containing protein to E6 [34].

In addition, the analysis of Scrib showed redistribution during cervical carcinogenesis, from cell contact sites in the squamous cells towards the cytoplasm in dysplastic cells, with a gradual reduction in the Scrib expression levels along with the tumor progression [35].

5.3. HPV-PDZ Interaction Could Have Oncogenic Potential in Certain Context. A recent study, showing that SGEF (Src homology 3 domain-containing guanine nucleotide exchange factor) is a strong binding partner of Dlg and that the interaction of the two induces activation of RhoG both *in vitro* and *in vivo*, has revealed that specific forms of Dlg, which remain bound by E6 but not degraded, may actually have oncogenic potential [36], which is another novel aspect of E6 and its PDZ-containing interaction.

Thus, PDZ domain-containing proteins are implicated in diverse aspects of tumor growth, development, and metastasis.

6. Changes in Cell Signaling by Posttranslational Modifications Also Greatly Influence the Interplay between E6 and Its PDZ-Containing Targets

In addition to the two distinct aspects of HPV E6-PDZ interactions at different stages along the time axis of HPV-induced

malignant progression, as mentioned above, recent studies have shown that these PDZ domain interactions can be modulated by a multitude of posttranslational modifications, the most important of which is phosphorylation. Indeed, specific phosphorylation states have been shown to greatly affect the localization and the susceptibility of each PDZ-containing protein to proteasome-mediated degradation by E6 (Figure 3).

6.1. Phosphorylation of hDlg by JNK, CDK1, and CDK2. For instance, hyperphosphorylation of hDlg by Jun N-terminal kinase (JNK), which occurs in response to osmotic shock, results in the rapid accumulation of Dlg at the sites of cell contact and renders it more susceptible to degradation induced by HPV-18 E6 [37]. Another example of the phosphorylation-dependent regulation of hDlg is mediated by two major cell cycle regulatory kinases, the cyclin-dependent kinases 1 and 2 (CDK1 and CDK2). When hyperphosphorylated on the specific phosphoacceptor sites of Ser 158 and Ser 442 by these two kinases, Dlg shows a preferential nuclear accumulation and thereby enhanced susceptibility to E6-induced degradation, implicating its nuclear function as a tumor suppressor in the late-stage progression of cervical cancer [38]. It is also important to note that these phosphoacceptor sites can also be phosphorylated by mitogen-activated protein kinase (MAPK) in response to the environmental change of osmotic stress, shown by the previous study [39].

6.2. hScrib and ERK/MAPK Cascade. The ERK/MAPK cascade, another notable cell signaling pathway, has been reported to be activated in cervical cancers [40], and hScrib is also a substrate of ERK [41]. In human skin keratinocytes, the interaction between hScrib and ERK, through hScrib's recruitment of a protein phosphatase, PP1gamma, for the dephosphorylation of ERK, subsequently inactivates ERK and inhibits its nuclear translocation, ultimately resulting in the downregulation of the MAPK signaling cascade [42]. Whether this interaction between hScrib and ERK actually happens in the progression of cervical cancer deserves further investigation, but it is highly likely that the downregulation of ERK/MAPK cascade is one of hScrib's multifunctional activities as tumor suppressor (Figure 3).

6.3. Regulation of HPV-PDZ Interactions through PKA-Dependent Phosphorylation. Interestingly, not only are the PDZ-containing proteins regulated through phosphorylation, but so also is HPV E6. Embedded within the HPV E6 PBM there is a consensus recognition site for protein kinase A (PKA) phosphorylation on the Thr/Ser residue at the −3 position (Figure 1). Previous studies showed that both PKA- and PKC-dependent phosphorylation can lead to the inhibition of many ligand-PDZ interactions ([43, 44] and more), and this would also seem to be true for HPV-18 E6 *in vivo*. Thus, the E6-PBM phosphorylation by PKA greatly inhibits E6-Dlg recognition and subsequent degradation, thereby maintaining high Dlg protein levels under conditions of high PKA activity [45]. A striking feature of this PKA recognition motif is its strict conservation among the high-risk HPV E6 proteins and its absence in the low-risk HPV E6 proteins, suggesting that PKA-regulated degradation of hDlg is an essential function in the process of malignancy [46]. However, the PKA recognition motif within the E6-PBM not only has a role in regulating PDZ-binding activity, but a recent study shows that the phosphorylation of E6 by PKA or AKT is also significant for the interaction between certain types of HPV E6 and 14-3-3ζ and that this E6/14-3-3ζ interaction is essential to maintain E6 levels [46]. Thus, this finding suggests that, in addition to the endogenous PKA activity, the expression level of 14-3-3ζ in high-risk HPV positive cervical tissues could be considered as a potential biomarker for predicting the development of cervical cancer.

At present, no information is available about at which stage, either during malignancy or during the course of viral life cycle, and to what degree, this E6 phosphorylation by PKA or AKT takes place, but this feature assumes great importance for the prospective designs for both progression-risk prediction and treatment for cervical cancer, considering the correspondent fact shown by many previous studies that mutations that disrupt normal PKA activity are frequently found in many forms of malignancy.

Thus, modifications by kinases and changes in cellular signaling pathways can profoundly influence, and sometimes dramatically alter, the efficiency of E6-PDZ interplay.

7. Other Possible Functions of PDZ-Containing Proteins in relation to the Development of Cervical Cancer

7.1. Immune Response. There are some fascinating recent studies that indicate PDZ targeting by E6 might be related to viral evasion of the host innate immune response. The important virulence factor of the Influenza A virus, NS 1 (nonstructural protein 1), has no oncogenic potential but does have a PBM at the carboxyl terminus and binds to PDZ-containing proteins as HPV E6 does [47]. The importance of the PBM for Influenza virus virulence has been associated with impairment of interferon activity via the downregulation of JAK/STAT signaling pathway [48]. Thus a possible analogy is that E6, through targeting certain PDZ substrates, can weaken the innate immune response. Indeed, previous studies have shown that E6 can impair JAK/STAT signaling [49], although whether E6-PDZ domain-containing substrates play a role in this activity remains to be determined.

7.2. Chronic Inflammation. The relationship between cancer progression and chronic inflammation has long been reported, and a transcription factor, nuclear factor-kappa B (NFκB), is well known as a driving force for generating chronic inflammation in the pathogenesis of many cancers, in addition to being implicated in a variety of other processes including proliferation, migration, angiogenesis, and prevention of apoptosis. Toll-like receptors (TLR) are possible signal initiators for NFκB activation, due to inflammation-inducing carcinogenesis, and are also known to be the forefront receptors in response to microbial infection. Therefore,

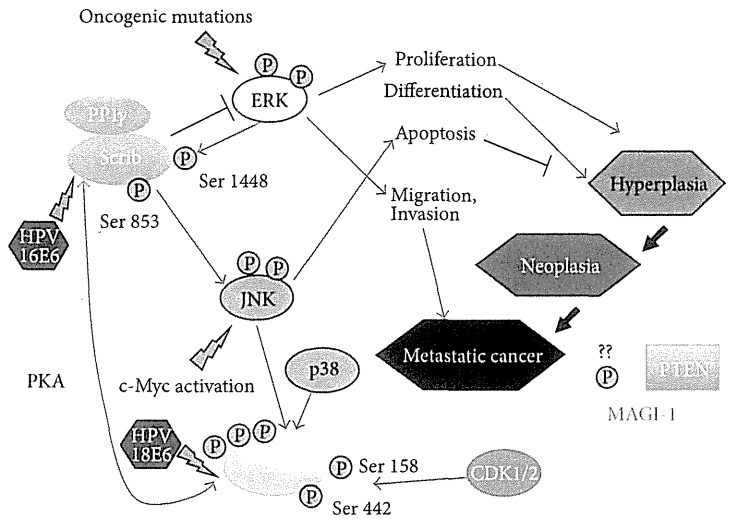

FIGURE 3: Multifunctional phosphorylated PDZ proteins integrate signaling pathways to control hyperplasia, neoplasia, and cell metastasis. Modified forms of PDZ proteins are potential molecular markers predicting the aggressiveness of malignancy. Candidates include Dlg phosphorylated by PKA, JNK, CDK1, or CDK2; Scrib interaction with ERK; Scrib recruitment of PP1gamma for downregulation of the MAPK pathway. No studies that could offer an explanation of the phosphorylation of MAGI-1 and PTEN by MAPK are currently available.

activation of NFκB through stimulated TLR in local chronic inflammation may serve as an initiator of carcinogenesis [50]. Previous studies have reported the activation and subsequent overexpression of NFκB in HPV-related cancers, and again the PBM of E6 appears to be required for this process [51].

7.3. Epithelial-Mesenchymal Transition. Furthermore, it was shown that in keratinocytes the PBM is also important for both HPV-16 and HPV-18 E6 to promote morphological changes that eventually lead to epithelial-mesenchymal transition (EMT), another crucial hallmark of cancer progression, especially in the progression of metastasis [52]. Therefore it is natural to infer that loss of PDZ-containing substrates of E6, such as Dlg1, Scrib, and MAGI-1, might contribute to the promotion of EMT, thereby enhancing the metastatic behavior of the virally transformed cells. A recent intensive study in human keratinocytes shows that an important consequence of Scrib-depletion in these cells is a significant increase in the invasive potential; in contrast, Dlg1-depletion in the same cells has distinct and opposite effects [53]. Also, the observations demonstrate that loss of Scrib can enhance cell invasiveness without the presence of extra oncogenes, such as *myc* and *ras*. In contrast, loss of Dlg-1 can result in the increased resistance of the cells to anoikis. Although the mechanism by which invading tumor cells survive the anoikis process remains largely unknown, we now know that the situation is rather more complex and that loss of Scrib and Dlg-1, which is often seen in malignant tumors, cooperatively contributes to the malignant state. Thus, the study demonstrating the opposing function of these polarity proteins may shed a light on their critical roles in regulating the promotion of EMT. Obviously, future studies are warranted to clarify how these epithelial cell polarity proteins may contribute to tumor suppression.

8. What Can We Learn from These HPV-PDZ Interactions for Cervical Cancer Prevention and Treatment?

Whilst previous studies have revealed much of the requirement for the oncoproteins of E6 and E7 in the development and maintenance of cervical cancer, much still merits further investigation to precisely understand the functions of these two viral proteins. In this review, we have attempted to feature one particular aspect of E6 function that may help to provide some clues to the molecular mechanisms that underlie HPV-induced development of cancer. Now the subject we must focus on is the prospective application of PBM-PDZ interactions for both the prevention and treatment of cervical cancer.

8.1. Possible Candidates for Molecular Markers Predicting Both Malignant Progression and Its Aggressiveness. Focusing on diagnostic tools, there is an urgent demand for the development of new biomarkers that can discriminate lesions with a high risk of progression towards cancer from those that will spontaneously regress. As noted above, most infections even with the high-risk HPVs occur without any clinical symptoms, are eliminated by the host immune system within the short period, and do not progress to cancer. High-grade cervical intraepithelial neoplasia (CIN) is normally the subject of therapeutic intervention, involving either removal or destruction of the lesions, to prevent malignant progression, and while this intervention certainly reduces the cancer development rates, the number of patients subject to overmedication increases at the same time. It is fairly reasonable to suggest that a more direct CIN predictor for malignant progression would be desirable.

Among the PDZ-containing proteins, MAGI-1, hDlg, and hScrib are degraded to varying degrees by high-risk HPV E6 oncoproteins, both in cells derived from cervical tumors and in a variety of experimental settings. However, although Dlg is targeted by E6 for degradation and is lost during the later stages of cervical cancer progression, only certain pools of the Dlg are degraded; therefore substantial amounts of Dlg protein, though often with unusual distribution pattern or phosphorylated forms, are found in HPV-positive tumor cells [34]. As mentioned above, several modified forms of PDZ proteins, for example, specific phosphorylated forms of Dlg (by PKA, JNK, CDK1, and CDK2), direct interaction between Scrib and ERK, or expression levels of PP1gamma recruited by Scrib for the downregulation of MAPK pathway could be candidates both for molecular markers predicting the aggressiveness of malignancy (high growth rate, invasiveness, and metastatic capacity) and for therapeutic targets.

Moreover, one of the hallmarks of cervical cancer progression is the frequent integration of the viral DNA into the host genome, and although there is considerable debate as to the role of this in disease development, the presence of integrated DNA sequences is likely to be of concern due to the concomitant high levels of E6 and E7 gene expression [54]. Therefore one possibility is that loss of PDZ-binding capacity through PKA phosphorylation of E6 might create an environment that is conducive to the loss of viral episomes and to consequent viral integration into the host genome. Whilst this remains speculative, it does nonetheless suggest that the levels of PKA activity within the infected cervical epithelium might be one marker that could be assessed as a means of determining whether viral DNA integration was more or less likely, and studies are currently underway to investigate these aspects further.

8.2. Drug Designs of PDZ-Domain Inhibitors; Possibilities for Novel Potent Therapeutic Intervention. Whether any of these PDZ targets of E6 might have therapeutic potential is also an intriguing possibility. In general, protein-protein interactions provide attractive possibilities for therapeutic intervention, and disruption of specific protein interactions with high affinity is a key concept for producing a molecularly targeted drug. However, targeting protein interactions are clearly more difficult than those targets that naturally bind molecules [55, 56]. Thanks to the advances in technology, we can now identify small molecules that can block specific proteins by screening an extensive small molecule database *in silico*, but the problem is that a large proportion of candidates identified by *in silico* screening prove to be false positive results [57]. Additional methods such as NMR spectroscopy are needed to evaluate these screening results, and indeed some small molecule inhibitors of the PDZ domain have been identified using this novel approach of NMR-assisted virtual screening, and they are shown to potently block specific cell signaling pathways [57, 58]. Thus, this novel *in silico* approach to drug design could be a valuable tool in identifying novel inhibitors of the E6-PDZ interactions, potentially leading to significant advances in the treatment of cervical cancer.

9. Conclusions

Although studies are still in their infancy, the HPV-PDZ interactions could have vast potential as both predictive and therapeutic targets in HPV-induced malignancies. And as these proteins appear to be multifunctional, sometimes exerting completely opposite influences on cancer progression depending on contexts, a comprehensive analysis of the PDZ protein expression at different stages along the time axis of malignant progression, and in various posttranslationally modified states, is essential to precisely elucidate the functions of HPV-PDZ interactions, before novel molecular biomarkers or drugs targeting these interactions come into practical use.

Conflict of Interests

The authors have no conflict of interest related to this article.

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A Novel Interaction between hScrib and PP1γ Downregulates ERK Signaling and Suppresses Oncogene-Induced Cell Transformation

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Abstract

Previous studies have shown that the cell polarity regulator hScrib interacts with, and consequently controls, the ERK signaling pathway. This interaction occurs through two well-conserved Kinase Interacting Motifs, which allow hScrib to bind ERK1 directly, resulting in a reduction in the levels of phospho-ERK. This suggests that hScrib might recruit a phosphatase to regulate this signaling pathway. Using a proteomic approach we now show that Protein Phosphatase 1γ (PP1γ) is a major interacting partner of hScrib. This interaction is direct and occurs through a conserved PP1γ interaction motif on the hScrib protein, and this interaction appears to be required for hScrib's ability to downregulate ERK phosphorylation. In addition, hScrib also controls the pattern of PP1γ localization, where loss of hScrib enhances the nuclear translocation of PP1γ. Furthermore, we also show that the ability of hScrib to interact with PP1γ is important for the ability of hScrib to suppress oncogene-induced transformation of primary rodent cells. Taken together, these results demonstrate that hScrib acts as a scaffold to integrate the control of the PP1γ and ERK signaling pathways and explains how disruption of hScrib localisation can contribute towards the development of human malignancy.

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Introduction

The control of cell polarity and the maintenance of tissue architecture are intimately related and are, in part, controlled by a tri-partite macromolecular signaling complex consisting of the Scrib complex, the Par complex and the Crumbs complex [1,2]. Through a series of antagonistic interactions the components of these three complexes control a variety of downstream signaling pathways that, in turn, directly contribute to the regulation of cell polarity and cell proliferation [3]. It is now clear that the loss of control of these pathways is a common event during the development of diverse human malignancies [1,4–7]. These defects are particularly evident at the later stages of malignant progression, and a variety of studies in both Drosophila and transgenic mice have provided additional supporting evidence of tumour suppressor activity for the various components of these signaling complexes [8–11].

The hScrib complex consists of three proteins, hScrib, hDlg1 and Hugl-1/2. In *Drosophila*, loss of either Scrib or Dlg produces imaginal disc overgrowth with invasive characteristics [8] [12], phenotypes that can be functionally complemented by the mammalian equivalents [13–15]. More recently Scrib has been implicated in the control of the JNK and ERK signaling cascades, and loss of hScrib appears to enhance the effects of the Ras and Myc oncogenes, and can contribute to mammary tumour development [16–21]. Recent studies have also demonstrated that hScrib can interact directly with ERK, and control both ERK activation and its nuclear translocation [19]. However, the physical interaction between ERK and hScrib is not sufficient to explain the inactivation of ERK, since high levels of hScrib appear capable of directly reducing the levels of ERK phosphorylation [19]. Since hScrib has no known phosphatase activity itself, it therefore seemed possible that a protein phosphatase might be recruited by hScrib to fully inactivate the ERK signaling pathway.

Control of ERK activation reflects an exquisite balance between the activities of the activating kinases and the de-activating protein phosphatases. Activated ERK can translocate to the nucleus, where it activates several transcription factors and also phosphorylates cytoplasmic and nuclear kinases [22–24]. Since phosphorylation of both the threonine and tyrosine residues of ERK is required for its activation, dephosphorylation of either is sufficient for its inactivation [25]. There are several reports demonstrating that dephosphorylation of active ERK can be achieved by tyrosine-specific phosphatases, by serine/threonine-specific phos-

phatases or by dual specificity (threonine/tyrosine) protein phosphatases [26–29]. One of the important negative regulators of the ERK signaling pathway is PP2A, a member of the PPP family of protein serine/threonine phosphatases which also includes PP1 [30,31]. However, PP2A is thought to exert its activity mainly upon other activating kinases within the cascade, rather than upon ERK itself [32–34]. In addition, recent studies have also shown that hScrib can directly regulate the Akt signaling cascade by recruitment of the protein phosphatase PHLPP1 to the plasma membrane, thereby resulting in de-phosphorylation of Akt [35]. Here, we have used a proteomic approach to extend our investigations into the regulation of the ERK signaling cascade by hScrib. We now show that hScrib interacts with PP1γ, and that this association correlates with the ability of hScrib to downregulate ERK activation. We also provide compelling evidence that hScrib directly contributes to the regulation of PP1γ function by controlling its translocation between the cytoplasm and the nucleus. Thus, loss of hScrib expression results in both ERK activation and aberrant nuclear translocation of PP1γ.

Materials and Methods

Cells and treatments

HEK293 (human embryonic kidney cells) and HaCaT (Human keratinocytes) were obtained from ATCC [36,37]. HEK293, HaCaT and Baby Rat Kidney (BRK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 U/mL) and glutamine (300 μg/mL) in a humidified 5%CO_2 incubator. Transfection was carried out using calcium phosphate precipitation as described previously [37] or using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The depleted Scribble cell lines were generated as described previously [19]. Cell transformation assays were done using BRK cells obtained from 9 day old Wistar rats with a combination of HPV-16 E7 and EJ-ras, plus the appropriate hScrib and PP1γ expression plasmids. Cells were placed under G418 selection for three weeks, and then fixed and stained.

Plasmids

The wild type pCDNA3-HA-PP1γ was the kind gift of Dr. Wilhelm Krek (Swiss Federal Institute of Technology (ETH) Zurich). The wild type HA-tagged pcDNA hScrib expression plasmid and the truncated mutant pGEX hScrib PDZ1-C, PDZ1-4, S1445A, S1445D, and CT expression plasmids have been described previously [19]. The L1266Y1268→AA mutation (KADA) to doubly change the Leucine (L) and Tyrosine (Y) residues to Alanine (A) in hScrib was done using the QuikChange site-directed mutagenesis kit from Stratagene Cloning Systems (Celbio) according to the manufacturer's instruction. The mutants were confirmed by DNA sequencing. See Figure S1 for a detailed description of the location of the different hScrib mutations.

Antibodies

The following commercial antibodies were used at the dilution indicated: anti-hScrib goat polyclonal antibody (Santa Cruz, WB 1:1000), anti-PP1γ goat polyclonal antibody (Santa Cruz, WB 1:1000), anti-PP1γ sheep polyclonal antibody (Abcam, WB 1:1000), anti-p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technology, WB 1:1000), anti-phospho p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell Signaling Technology, WB 1:1000), anti-HA monoclonal antibody 12CA5 (Roche, WB 1:500), anti-γ-tubulin monoclonal antibody (Sigma, WB 1:5000), anti-p84 mouse monoclonal antibody (Abcam, WB 1:1000), anti-E-Cadherin rabbit polyclonal antibody (Santa Cruz, WB 1:500), anti-α-tubulin mouse monoclonal antibody (Abcam, WB 1:1000).

Immunofluorescence and Microscopy

For immunofluorescence cells were grown on glass coverslips and fixed in 3.7% paraformaldehyde in PBS for 20 mins at room temperature. After washing in PBS the cells were permeabilised in PBS/0.1% Triton for 5 mins, washed extensively in PBS and then incubated with primary antibody diluted in PBS for 1 hour followed by the appropriately conjugated secondary antibodies. Secondary antibodies conjugated to Alexa Fluor 488 or 548 were obtained from Invitrogen. The cells were then washed several times in water and mounted on glass slides. Cells were visualized by using a Zeiss Axiovert 100 M microscope attached to a LSM 510 confocal unit.

siRNA transfection

HEK293 cells were seeded on 6 cm dishes and transfected using Lipofectamine 2000 (Invitrogen) with control siRNA against Luciferase (siLuc), or siRNA against hScrib and PP1γ sequences (Dharmancon). 48 hours post-transfection cells were harvested and total cells extracts or cell fractionated extracts were then analysed by western blotting.

Fusion protein purification and in vitro binding assays

GST-tagged fusion proteins were expressed and purified as described previously [19]. Proteins were translated in vitro using the Promega TNT kit and radiolabelled with (^{35}S) cysteine or (^{35}S) methionine (Perkin Elmer). Equal amounts of in vitro-translated proteins were added to GST fusion proteins bound to glutathione agarose (Sigma) and incubated for 1 hour at 4°C. After extensive washing with PBS containing 0.25% NP-40, or as otherwise indicated, the bound proteins were analysed by SDS-PAGE and autoradiography.

In vitro phosphorylation

Purified GST fusion proteins were incubated with commercially purified ERK1 (Cell Signaling Technology) or PKA (Promega) for 20 mins at 30°C in phosphorylation buffer (0.25 M Tris pH7.5, 1 M $MgCl_2$, 3 M NaCl, 0.3 mM aprotinin, 1 mM Pepstatin) or using the kinase buffer supplied by New England Biolabs supplemented with 56 nM (^{32}P) γ-ATP (Perkin Elmer) and 10 mM ATP following the manufacturer's instruction. After extensive washing, the phosphorylated proteins were monitored by SDS-PAGE and autoradiography.

Mass spectrometry analysis

HEK293 cells were transfected with HA-tagged Scrib and after 24 hours the cells were extracted in mass spectrometry lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 0.25% NP40) and extracts incubated with anti-HA beads (Sigma) for 2–3 hours on a rotating wheel at 4°C. The beads were then extensively washed with PBS, dried and the immunoprecipitated proteins were subjected to proteomic analysis as described previously [38].

Subcellular Fractionation assays

Differential extraction of HEK 293 cells to obtain cytoplasmic, membrane, cytoskeleton, and nuclear fractions was performed using the Calbiochem ProteoExtract Fractionation Kit according to the manufacturer's instructions. To inhibit phosphatase activity during the preparation of cell lysates, phosphatase inhibitors

A

log (e)+	#	total	HA-Scribble
-1189.3	91	200	SCRIB, scribble
-327.5	29	37	VIM, vimentin
-73.2	6	10	NPM1P21, nucleophosmin
-60.5	6	6	PPP1CC, protein phosphatase 1, catalytic subunit, gamma isoform
-59.6	6	6	NCL, nucleolin

[redacted] [15]llev
[redacted] [96]gkqs
[redacted] [122]gnhe
[redacted] [122]gnhe
[redacted] [260]rqlv
[redacted] [314]gmit

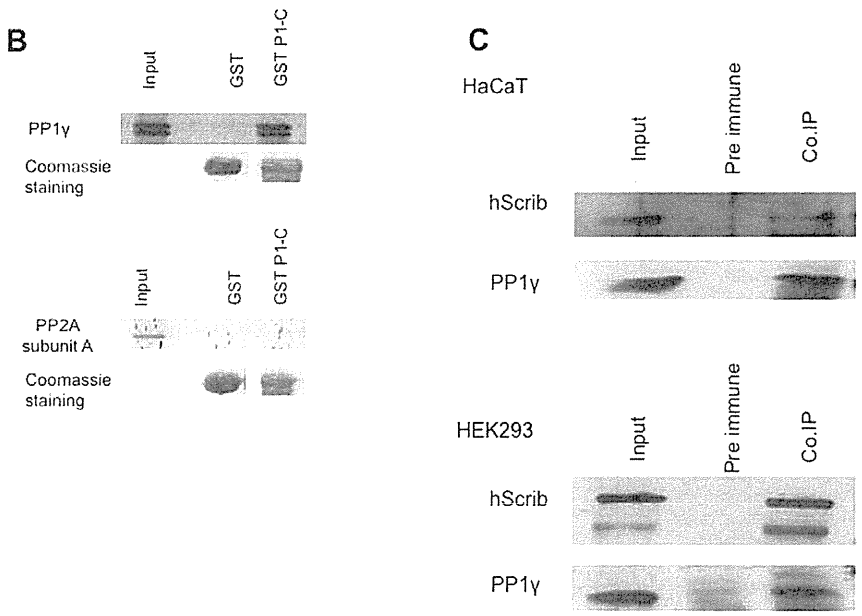

Figure 1. Interaction between hScrib and PP1γ in vivo. A) Results from the mass spectroscopy analysis of hScrib containing immunoprecipitates identified 6 peptides (indicated) corresponding to PP1γ. B) In vitro translated PP1γ (upper panels) and PP2A subunit A (lower panels) were incubated for 1 hour at 4°C with purified GST-hScribP1-C or GST alone immobilized on Glutathione agarose. After extensive washing, the bound proteins were analysed by SDS–PAGE and autoradiography which are shown in each of the upper panels. The gels were rehydrated and stained with Coomassie to show equal levels of GST loading in the respective lower panels. C) Endogenous PP1γ was immunoprecipitated from HaCaT (upper panels) and HEK 293 cells (lower panels), with pre-immune antibody used as control. The immunoprecipitated proteins were then analysed by western blotting using anti-hScrib and anti-PP1γ antibodies.
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(1 mM Na_3VO_4, 1 mM β-Glycerophosphate, 2.5 mM Sodium Pyrophosphate, 1 mM Sodium Fluoride) were also included.

Immunoprecipitation and Western blotting

Total cellular extracts were prepared by directly lysing cells from dishes in SDS lysis buffer. Alternatively cells were lysed in either E1A buffer (25 mM HEPES pH 7.0, 0.1% NP-40, 150 mM NaCl, plus protease inhibitor cocktail; Calbiochem) or RIPA buffer (50 mM Tris HCl pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, plus protease inhibitor cocktail; Calbiochem) and the cell extracts were analysed by SDS-PAGE and western blotting. For immunoprecipitations, total cell lysates were transferred into a tube of equilibrated EZview Red Anti-HA Affinity Gel beads (Sigma), and incubated for 2 hours at 4°C. Immunoprecipitates were extensively washed four times in lysis buffer and solubilised in SDS-PAGE sample buffer. For western blotting, 0.45 μm nitrocellulose membrane (Schleicher and Schuell) was used and membranes were blocked for 1 hour at 37°C in 10% milk/PBS followed by incubation with the appropriate primary antibody diluted in 10% milk/0.5% Tween 20 for 1 hour. After

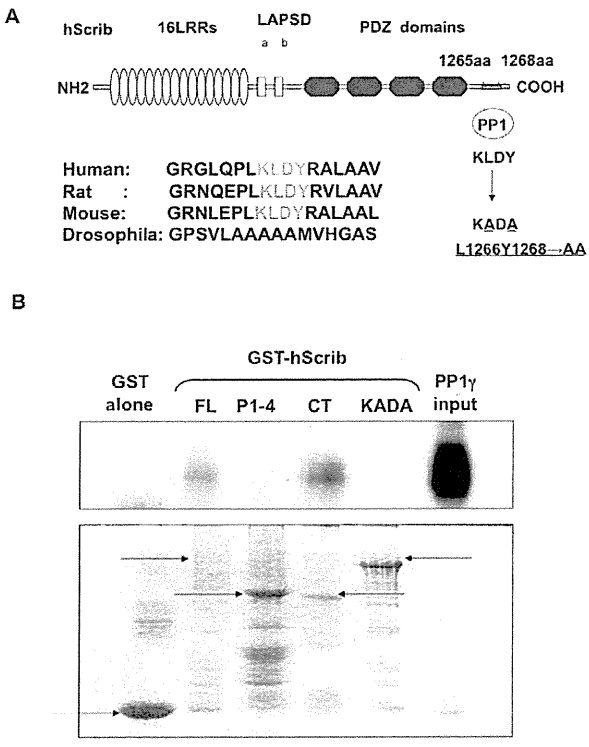

Figure 2. hScrib contains a consensus PP1-binding motif. A) The schematic shows the arrangement of the functional domains on the hScrib protein, highlighting the LRR, LAPSD and PDZ domains. The putative PP1-binding site, the RVXF (the consensus sequence is K/R/H/N/S V/I/L X F/W/Y) motif is also shown, where X is any amino acid. The hScrib mutant in which the PP1-binding site KLDY was mutated to KADA in order to disrupt the interaction with PP1 is shown. A comparison sequence alignment of the region of hScrib containing the PP1-binding motif indicating its absence in Drosophila also shown. B) In vitro translated and radiolabeled PP1γ was incubated with purified full length GST-hScrib fusion protein (FL), GST-hScrib PDZ1-4 (P1-4), GST-hScrib CT (CT), GST-hScrib L1266Y1268→AA (KADA) and GST alone as a control. After extensive washing the bound PP1γ was ascertained by SDS PAGE and autoradiography. The upper panel shows the autoradiograph, with the input of PP1γ also shown for comparison. The lower panel shows the Coomassie stain of the gel showing the levels of GST fusion protein loading, with the arrows indicating the relevant full length fusion proteins.
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several washings with PBS 0.5% Tween 20, secondary antibodies conjugated with HRP (DAKO) in 10% milk/0.5% Tween 20 were incubated for 1 hour. Blots were developed using Amersham ECL reagents according to the manufacturer's instructions.

Results

PP1γ is a direct binding partner of hScrib

Based on our previous studies we reasoned that down-regulation of ERK phosphorylation by hScrib might involve the recruitment of a protein phosphatase [19]. To investigate this possibility we performed proteomic analyses to identify additional interacting partners of hScrib. HEK293 cells were transfected with an HA-tagged hScrib expression plasmid and after 24 hours the cells were extracted, and hScrib–bound protein complexes were immunoprecipitated with anti-HA agarose beads and then subjected to mass spectroscopy analysis. Several previously reported interacting partners were identified, including vimentin. However, of the novel interacting partners, the most prominent phosphatase identified was the catalytic subunit of PP1γ (Figure 1A), a major eukaryotic serine/threonine protein phosphatase. To investigate whether hScrib can interact with PP1γ, an in vitro pull-down assay was performed using purified GST-hScrib P1-C fusion protein and in vitro translated radiolabeled PP1γ. For comparison a similar assay was also done using in vitro translated radiolabeled protein phosphatase 2A (PP2A). After extensive washing the bound PP1γ and PP2A were detected by SDS PAGE and autoradiography, and the results in Figure 1B demonstrate strong interaction between hScrib and PP1γ. In contrast, no interaction was observed between hScrib and PP2A, confirming the specificity of the association between hScrib and PP1γ. To determine whether endogenous hScrib and PP1γ could exist in a complex in vivo, immunoprecipitations were performed on cell extracts from HEK293 and HaCaT epithelial cells using anti-PP1γ antibody. Co-immunoprecipitated hScrib was then detected by western blotting, and the results in Figure 1C show a significant degree of co-immunoprecipitation of hScrib with PP1γ in both cell lines. Taken together, these results demonstrate that hScrib and PP1γ can exist as a complex in vivo.

hScrib interacts with PP1γ through a conserved RVxF motif

The PP1 holoenzyme is composed of a catalytic subunit and several regulatory subunits, which target the catalytic subunit to specific subcellular locations. The RVxF motif is a short conserved PP1-binding motif initially identified in previous studies showing that these residues can block the interaction of regulatory subunits with the PP1 catalytic subunit [39]. As shown in Figure 2A, analysis of the hScrib sequence reveals the presence of a putative PP1 binding motif, KLDY (the consensus sequence is: {K/R/H/N}.{S/V/I/L}.X.{ F/W/Y}) [40,41] spanning residues 1265–1268. This sequence is also highly conserved in mammalian Scrib proteins, but is absent in Drosphila. Based on previous studies, mutation of the L and Y residues would be expected to severely perturb the interaction with PP1 [39–42]. To investigate whether this KLDY motif is responsible for the capacity of hScrib to bind to PP1γ, a panel of GST-hScrib fusion proteins consisting of the full length (FL), two truncated proteins encompassing PDZ domains 1–4 (P1-4) and the carboxy terminal third of hScrib (CT), plus a full length hScrib with the KLDY/KADA mutation, were used in pull-down assays with in vitro translated radiolabeled PP1γ. The levels of bound PP1γ were then assessed by SDS PAGE and autoradiography and, as can be seen from Figure 2B, PP1γ binds to the carboxy terminal region of hScrib which contains the predicted PP1 binding motif. Furthermore the KLDY/KADA mutation significantly decreases the capacity of PP1γ to interact with hScrib, confirming that the major site of interaction is through the KLDY consensus motif.

hScrib and ERK are substrates of PP1γ

We have previously shown that hScrib is a substrate for both PKA and ERK. Furthermore, hScrib can downregulate ERK activation through a direct protein-protein interaction [19], although the precise mechanism by which hScrib can achieve this is still unknown. We therefore wanted to determine whether phosphorylation of hScrib by either PKA or ERK1 could influence the ability of hScrib to interact with PP1γ and, furthermore, whether hScrib itself was a substrate of PP1γ. To do this, purified GST-hScrib fusion protein was subject to phosphorylation by either PKA or ERK1 in the presence of non-radiolabeled ATP, and after extensive washing binding assays

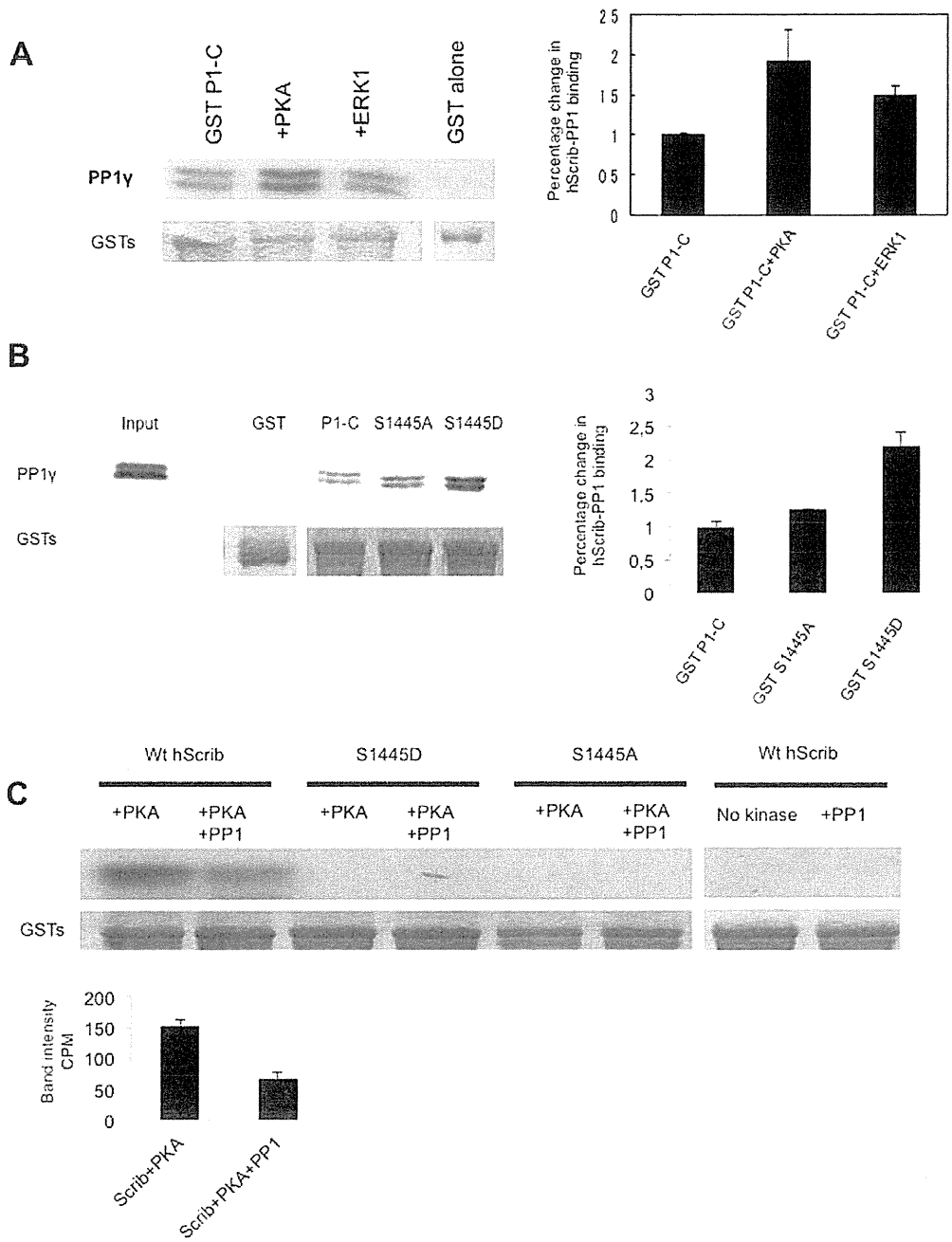

Figure 3. hScrib is a substrate of PP1γ. A) Purified GST-hScrib fusion protein was in vitro phosphorylated with purified PKA or ERK1 as described previously (19) and then incubated with PP1γ for 20 mins at 30°C. Bound PP1γwas detected by western blotting with anti PP1γ antibody. The lower panel shows the ponceau stain of the nitrocellulose, and the upper right panel shows the quantitations from three independent experiments. Note that hScrib phosphorylated by PKA exhibits increased association with PP1γ. B) Purified PP1γ was incubated with purified full length wild type GST-hScrib fusion protein (P1-C), the mutants S1445A, S1445D or GST alone as a control. After extensive washing the bound PP1γ was ascertained by western blotting. The upper panel shows the result of the western blot, with the 20% input of PP1γ also shown for comparison. The lower panel shows the ponceau stain of the nitrocellulose. The histogram shows the quantitation from three independent experiments. C) Purified GST-hScrib wild type and PKA phospho-site mutants of hScrib were in vitro phosphorylated with purified PKA in the presence of radiolabeled ATP as described previously (19) and incubated with PP1γ for 20 mins at 30°C. The remaining level of phosphorylated hScrib was then determined following SDS PAGE and autoradiography. The two right-hand lanes show lack of phosphorylation of hScrib in the absence of PKA, whilst the lower panels show the Coomassie stain of the gel demonstrating equal levels of the GST-hScrib fusion protein throughout. The quantitation of hScrib phosphorylation from three independent experiments is also shown.
doi:10.1371/journal.pone.0053752.g003

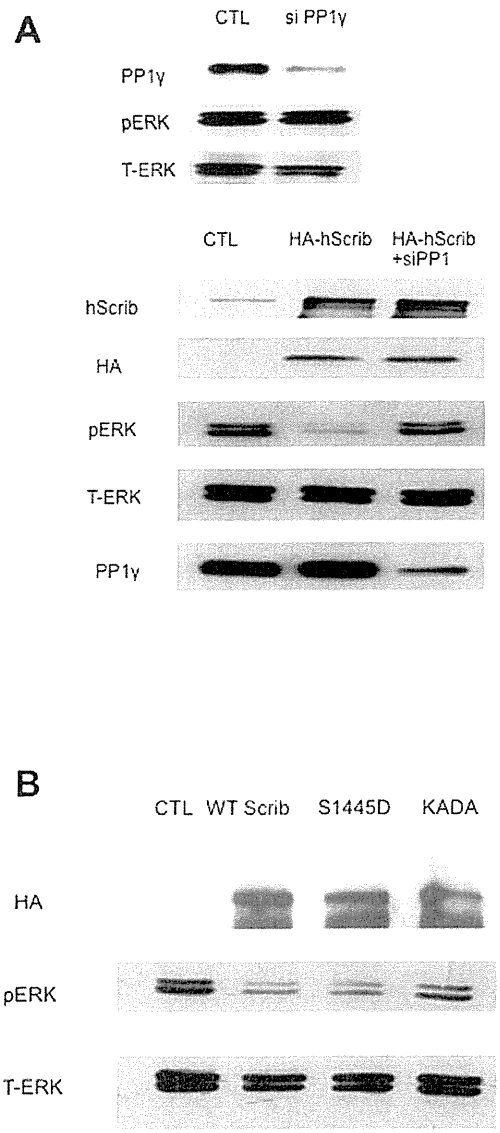

Figure 4. PP1γ is required for hScrib-induced de-phosphorylation of ERK. A) HEK 293 cells were transfected with PP1γ si RNA or si Luc RNA as control (CTL) and after 24 hours were then transfected with a plasmid expressing HA-tagged hScrib. After a further 24 hours the cells were extracted and levels of phospho and total ERK determined by western blot analysis. The upper three panels shows the changes in the ERK profiles when cells were transfected with siRNA PP1γ alone, whilst the lower set of five panels show the effects in the presence of ectopically expressed hScrib. B) HEK 293 cells were transfected with HA-tagged wild type hScrib, or the S1445D and KADA mutants. Total cell extracts were then made after 48 hours and the hScrib, phospho-ERK and total ERK were detected by western blotting.
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were performed using commercially purified PP1γ. The bound protein was then detected by western blotting using anti-PP1γ antibodies. The results in Figure 3A demonstrate a number of important features. In the absence of phosphorylation there is a strong interaction between hScrib and the purified PP1γ, demonstrating that the interaction between hScrib and PP1γ is indeed direct. However, there is also a clear increase in the association between hScrib and PP1γ when hScrib is phosphorylated by PKA, but not when it is phosphorylated by ERK1. We had previously shown that the major PKA phosphorylation site on hScrib was S1445 [19]. Therefore, to further confirm that phosphorylation of hScrib by PKA at S1445 can influence its capacity to interact with PP1γ, we repeated the pull down assays using the phospho-mimic mutation of hScrib, S1445D. As can be seen from Figure 3B, the S1445D mutant exhibits a significantly increased capacity to interact with PP1γ, which is similar to that seen following phosphorylation by PKA. These results demonstrate that phosphorylation of hScrib by PKA at S1445 can indeed increase the ability of hScrib to directly interact with PP1γ.

We then analysed whether hScrib was a potential substrate of PP1γ. Purified GST-hScrib fusion protein was subjected to in vitro phosphorylation with purified PKA and radiolabeled ATP. After extensive washing the radiolabeled hScrib fusion protein was incubated with purified PP1γ, and the amount of phosphorylated protein determined by SDS PAGE and autoradiography. The results obtained in Figure 3C demonstrate that the level of phosphorylated hScrib is decreased following incubation with PP1γ, demonstrating that hScrib is a potential substrate of the phosphatase and, furthermore, that hScrib can directly recruit active PP1γ. Also shown are the non-phosphorylatable mutants of hScrib, confirming the specificity of the phosphorylation reaction.

We then proceeded to determine whether the interaction of hScrib with PP1γ might be involved in the capacity of hScrib to downregulate ERK activation. Cells were transfected with control siRNA against luciferase or against PP1γ, and after 24 hours the cells were then transfected with an hScrib expression plasmid. After a further 24 hours the cells were extracted and the levels of activated phospho-ERK analysed by western blotting. The results obtained are shown in Figure 4A. As can be seen, in the absence of hScrib, siRNA PP1γ has minimal effect on the levels of phospho-ERK (Figure 4A upper three panels). In contrast, overexpression of hScrib significantly reduces the levels of phospho-ERK (Figure 4A lower five panels), and this is in agreement with previous studies [19]. However, the ability of hScrib to down-regulate the levels of phospho-ERK is largely abolished following treatment with siRNA PP1γ, suggesting that this activity of hScrib is in part PP1γ-dependent. To further investigate this, we repeated the assay using the PKA phospho-mimic mutant (S1445D) and the non-PP1γ binding mutant (KADA) of hScrib. After 24 hours the levels of phospho-ERK were analysed by western blotting and the results obtained are shown in Figure 4B. As can be seen the wild type and S1445D mutant of hScrib both strongly inhibit the levels of phospho-ERK, whilst the non-PP1γ binding mutant of hScrib is decreased in this activity. Taken together these results demonstrate that the ability of hScrib to interact with PP1γ correlates with its ability to down-regulate the levels of phospho-ERK.

Loss of hScrib enhances PP1γ nuclear localization

Having found that PP1γ plays a role in hScrib regulation of ERK signaling, we were next interested in determining whether hScrib could also potentially affect PP1γ localisation. Therefore, we first analysed the pattern of PP1γ expression in human keratinocytes after stably silencing hScrib expression in these cells. The distribution of PP1γ in control and shScrib HaCaT cells were analysed by immunofluorescence. The results in Figure 5A and Figure 5B, show that most of the PP1γ localises in the nucleus, although some also co-localises with hScrib at the plasma membrane and within the cytoplasm. More importantly, however, upon loss of hScrib expression there is a significant increase in the amount of nuclear PP1γ, with a corresponding decrease in the cytoplasmic pool. In order to verify these results we also performed

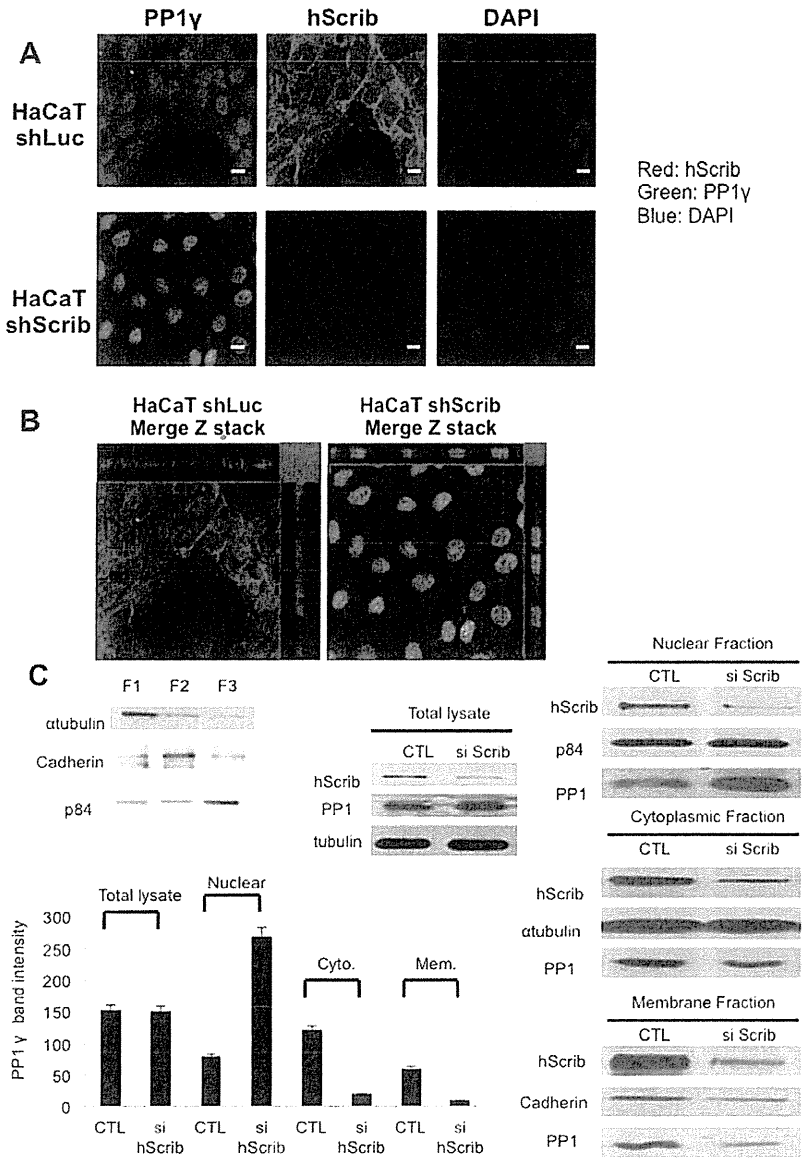

Figure 5. hScrib regulates PP1γ nuclear localization. A) Immunofluorescence analysis of hScrib and PP1γ expression in sh-Luc control HaCaT cells and sh-hScrib knockdown cells. The cells were grown on coverslips and then fixed and double-stained with the anti-hScrib antibody and the anti-PP1γ antibody. Note the significant increase in the levels of nuclear PP1γ in the absence of hScrib expression. B) Z-reconstruction (x-z direction) of a z-stack (15 planes, z-distance 0.2 μm), showing sh-hScrib knockdown cells have enhanced PP1γ localisation into the nucleus. C) HEK 293 cells were transfected with hScrib siRNA and si Luc RNA as control. Cells were either extracted in SDS PAGE sample buffer (Total lysate) or were fractionated into cytoplasmic (F1), membrane (F2) and nuclear (F3) pools (the example shows the integrity of a typical extraction procedure) and then PP1γ was detected by western blotting. p84 was used as a loading control for the nuclear fraction, cadherin was used as a loading control for the membrane fraction and α-tubulin was used as the loading control for the cytoplasmic fraction and total cell extracts. Note the relative increase in nuclear PP1γ following hScrib knockdown but no overall change in total PP1γ levels.
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a series of transient siRNA experiments, where hScrib levels were ablated in 293 cells, and the levels of PP1γ, both in total cell extracts or in the respective cellular fractions (Fig. 5C), were analysed by western blotting. As can be seen, loss of hScrib resulted in decreases in the cytoplasmic and membrane pools of PP1γ, but a corresponding increase in the amounts of the nuclear form of the protein.

To investigate the pattern of pERK expression following hScrib depletion we repeated the immunofluorescence assays staining for hScrib, PP1γ and pERK. The results obtained are shown in Figure 6. As can be seen, under conditions of hScrib depletion there is a marked increase in the levels of both nuclear and cytoplasmic pERK, consistent with previous observations [19]. This is also accompanied by an increase in the levels of nuclear PP1γ.

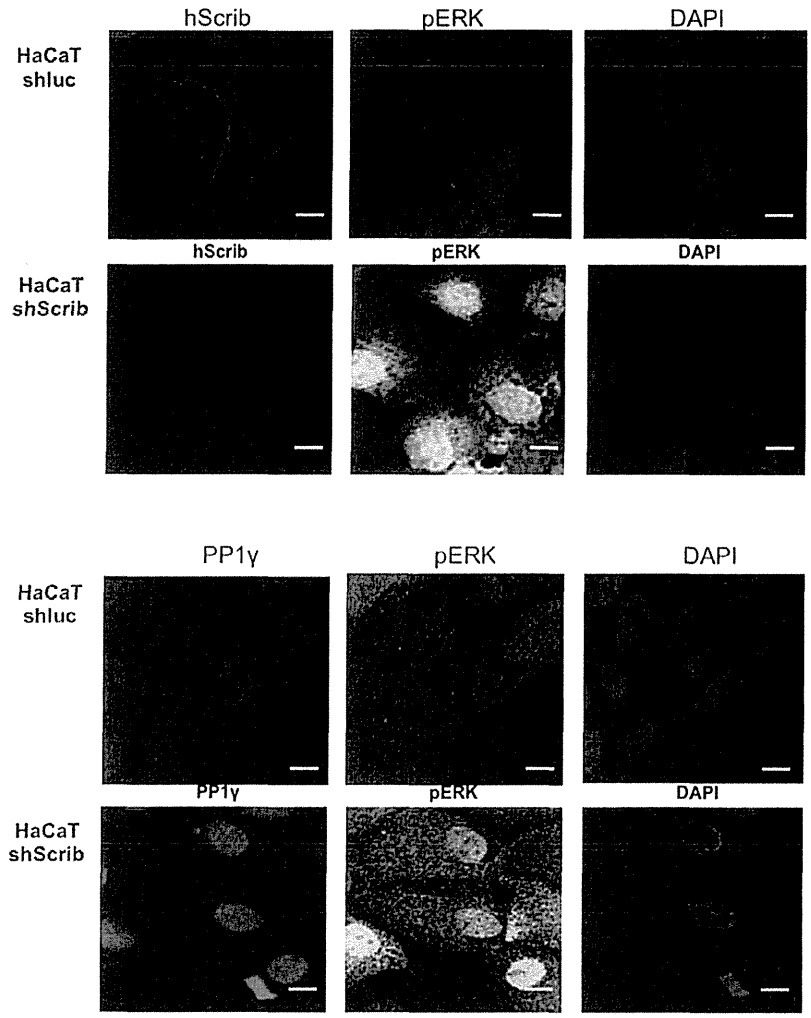

Figure 6. Loss of hScrib results in enhanced nuclear accumulation of both PP1γ and pERK. Control and shScrib HaCaT cells were stained for hScrib, phospho-ERK and PP1γ as indicated.
doi:10.1371/journal.pone.0053752.g006

hScrib tumour suppressor activity requires an intact PP1γ binding motif

We have previously shown that hScrib can suppress cell transformation induced by EJ-ras and Human Papillomavirus (HPV)-16 E7 [19]. To determine whether the interaction between hScrib and PP1γ was physiologically relevant in this context, primary BRK cells were transfected with HPV-16 E7 plus EJ-ras in the presence or absence of the hScrib wild type and KLDY/KADA mutant hScrib expressing plasmids, with or without the PP1γ expression plasmid. After 3 weeks the cells were fixed and stained and the numbers of colonies counted. As can be seen from Figure 7, co-expression of wild type hScrib and PP1γ strongly inhibits the oncogene cooperation between E7 and EJ-ras, whilst the KADA mutant of hScrib is compromised in this activity. These results demonstrate that the hScrib-PP1γ interaction is functionally relevant in an assay of oncogene cooperation.

Discussion

We have shown previously that hScrib can regulate ERK signaling in two ways. The first involves a direct protein interaction, which is mediated via two KIM binding sites located within hScrib. The second appears to involve the recruitment of a protein phosphatase [19]. In this study we provide evidence that a candidate phosphatase is PP1γ. We have also found that hScrib can control PP1γ sub-cellular localisation, with a loss of hScrib promoting PP1γ nuclear translocation.

Regulation of the ERK signaling cascade can occur at multiple levels and can involve Raf dephosphorylation, MEK1,2 phosphorylation, and also MEK1,2 dephosphorylation [24,43–44]. Furthermore, it has been reported that whilst the kinases in the pathway control signal amplitude, the phosphatase PP2A mediates both signal amplitude and signal duration [32–33]. Previous studies have also implicated PP1 in regulating ERK signaling through its ability to dephosphorylate Raf-1 at Ser 259 [45]. Since we have consistently observed that overexpressed hScrib results in a decrease in ERK phosphorylation, we initiated a series of studies to identify the potential phosphatases with which hScrib might interact. Using a proteomic approach we identified PP1γ as a direct interacting partner of hScrib, an interaction that we could confirm both in vitro and in vivo. Analysis of the hScrib amino

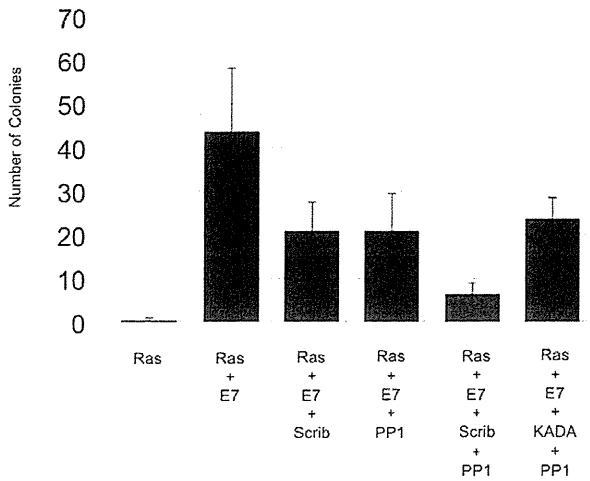

Figure 7. hScrib suppresses HPV-16 E7 and EJ-ras induced transformation in cooperation with PP1γ in a RVxF motif-dependent manner. BRK cells were transfected with EJ-ras alone, HPV-16 E7 plus EJ-ras, HPV-16 E7 plus EJ-ras and wild type hScrib, HPV-16 E7 plus EJ-ras and PP1γ, and HPV-16 E7 plus EJ-ras and wild type hScrib with PP1γ, and HPV-16 E7 plus EJ-ras and PP1γ plus the KADA non-PP1γ binding mutant of hScrib. After three weeks the dishes were fixed and stained and the colonies counted. Results represent the mean number of colonies from 3 independent assays and standard deviations are shown.
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acid sequence identified a potential site of interaction, KLDY, mutation of which abolished the ability of hScrib to bind PP1γ. Furthermore, this consensus PP1 recognition motif is conserved in mammalian forms of Scrib, but is absent in Drosophila.

We also analysed the effects of PP1γ ablation upon hScrib control of ERK phosphorylation, and found that loss of PP1γ greatly diminished the ability of hScrib to downregulate the levels of phospho-ERK in vivo. Furthermore, we also found that this activity of hScrib was in part dependent upon an intact PP1γ binding site motif. Interestingly, we also noted that the interaction between PP1γ and hScrib was increased following PKA phosphorylation of hScrib, one potential consequence of which is PP1γ-mediated de-phosphorylation of hScrib. Whether this has an important role with respect to other functions of hScrib remains to be determined and is worthy of further study. Taken together these studies demonstrate that hScrib can interact with PP1γ, an activity which appears to play a role in the ability of hScrib to downregulate the ERK signaling pathway. Interestingly, this regulation of ERK by hScrib has many parallels with a recent study showing that hScrib could also regulate Akt signaling [35]. This required hScrib interaction with the phosphatase, PHLPP1, resulting in the de-phosphorylation of Akt. In this case the interaction between hScrib and PHLPP1 requires sequences in the LRR region of hScrib. Thus hScrib could potentially interact simultaneously with multiple protein phosphatases to control diverse signaling pathways. It should also be emphasized that hScrib is a multifunctional protein, and loss of hScrib also results in increased levels of MEK activity, suggesting multiple mechanisms by which hScrib can control ERK signaling [46].

To investigate whether the capacity of hScrib to interact with PP1γ had any physiological relevance, we made use of an oncogene cooperation assay in primary rodent cells. Previous studies had shown that hScrib could suppress cell transformation induced by HPV-16 E7 and EJ-ras in these cells, and that this activity was dependent in part upon the ability of hScrib to interact with ERK [19]. We reasoned that this activity of hScrib might also be influenced by the ability of hScrib to interact with PP1γ. Indeed, both hScrib and PP1γ, either alone or in combination, could dramatically decrease the levels of HPV-16 E7 and EJ-ras induced cell transformation. However, the additive effects upon the levels of cell transformation, seen with the combination of hScrib and PP1γ, was abolished if a mutant hScrib defective in its ability to interact with PP1γ was included in the assay. This demonstrates that, in the context of an oncogene cooperation assay, the ability of hScrib to interact with PP1γ does play a role in the ability of hScrib to suppress cell transformation.

PP1γ has been linked to the regulation of a variety of different cellular processes, including the DNA damage response, nuclear function and diverse aspects of the cell cycle [47–52]. One of the important aspects of PP1γ regulation is believed to be related to the control of its nuclear expression, which can be mediated by proteins possessing the consensus RVxF PP1 binding motifs, and which can thereby control the correct cellular localization of PP1 [42,52]. We therefore investigated whether hScrib might have a similar potential regulatory function with respect to the pattern of PP1γ localization within the cell. This was indeed found to be the case; in two different assay systems we observed that loss of hScrib resulted in an increased nuclear accumulation of PP1γ, with a concomitant decrease in the levels found in membrane and cytoplasmic fractions. Thus hScrib would appear to contribute directly to the regulation of PP1γ expression patterns. Whether this is related to some of hScrib's previously reported pleiotropic effects upon cell proliferation and cell survival remains to be determined. Taken together, these studies have defined PP1γ as a novel interacting partner of hScrib, an interaction which correlates with hScrib downregulation of ERK signaling and suppression of oncogene-induced cell transformation.

Supporting Information

Figure S1 Schematic diagram showing the different hScrib expression constructs. The schematic shows the arrangement of the functional domains on the hScrib protein, highlighting the LRR, and PDZ domains. The putative PP1-binding site, KLDY is also shown in the carboxy terminal third of hScrib. Also summarized are the results on the interaction assays with PP1γ.
(TIF)

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Author Contributions

Conceived and designed the experiments: KN LB. Performed the experiments: KN TS AY VKS CK PM MT. Analyzed the data: KN LB. Contributed reagents/materials/analysis tools: KN KK SN TY YT TF SK. Wrote the paper: KN LB.

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子宮頸がん

新たなHPVワクチンの開発

川名 敬

● 子宮頸がんの予防効果を高めるための次世代のHPVワクチンの開発が進んでいる.
● HPVを標的にした細胞性免疫を誘導する免疫療法が期待される.
● CIN2～3に対する治療薬としてE7標的がんワクチンを開発中である.

はじめに

ヒトパピローマウイルス（human papillomavirus：HPV）の感染を予防する“HPVワクチン”が世界中で普及し定着してきた．国内においては，2012年度からは学童女子を接種対象とし公費助成がすべての自治体で始まり，2013年度からはHPVワクチンは定期接種化された．ここまで急激にHPVワクチンが普及している理由は，子宮頸がんの予防につながること，予防効果が世界中で再現性を以て証明されていること，の2点によるところが大きい．しかし現行HPVワクチンにはいくつかの問題点があり，これだけでは子宮頸がんの根絶が可能であるとは考えられない．子宮頸がんの根絶をめざした次世代HPVワクチン開発も同時に進んでいる．本稿では，現在開発が進んでいる次世代HPVワクチンを紹介する．

HPVワクチン開発の経緯

ハイリスクHPVは，ほぼすべての子宮頸がんにとって必要条件である．ハイリスクHPVには約13～15種類の遺伝子型（タイプ）が存在し，それらの抗原性は基本的に

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はタイプ特異的ということが数多くの基礎研究，臨床研究から判明している．すなわち，13タイプのハイリスクHPVの感染を予防するためには，13タイプのワクチン抗原を用意しないといけないわけである．さもなければ，13タイプに共通の抗原を見つけ出してワクチン抗原として利用できるかを検証する必要がある．HPVはDNAウイルスであり，遺伝子がダイナミックに変異することがないのでワクチンの有効性は半永久的に保障されているが，その反面，タイプが多く存在することがHPVワクチンの最大のネックである．

このような基礎的・臨床的根拠に基づき，HPVワクチン開発においては，各HPVタイプに合わせたワクチン抗原（L1-VLPという）をそれぞれ用意して混ぜあわせたカクテルワクチンを用いている．ハイリスクHPV13タイプの抗原をカクテルにすることはコスト的にも免疫学的にも現実的ではないため，ハイリスクHPVのうち最もインパクトの大きいHPV16，18型をまずターゲットにする戦略となった．HPV16，18型はHPV感染から子宮頸がんに至るまでの期間が短いことが知られており，4～5年追跡によってプラセボ群と比べて有意にHPV関連疾患の発生率に差があることが証明された．そして，世界中で承認され，実用化に至ったのである．しかも，HPV16，18型はがんの発生年齢が他のHPVと比べて若く，若年の子宮頸がんの80%超がこの2つのHPVによるともいわれていることから，その予防の必要性は特に大きい．

Merck（MSD）社とGlaxo Smith Kline（GSK）社が開発したHPVワクチンが現在世界中で普及している．これらのワクチンは，HPVの粒子を形成するL1蛋白質で構成されるウイルス様粒子virus-like particles（L1-VLP）であり，MSD社のHPVワクチンは6，11，16，18型の4価ワクチン（ガーダシル®），GSK社は16，18型の2価ワクチン（サーバリックス®）である．HPVタイプに未感染の成人女性を対象とした大規模な臨床試験では，HPV16，18型による子宮頸がん前癌病変（CIN2～3）はほぼ100%予防されており，未感染者に対する予防効果が実証された[1～4]．

現行HPVワクチンの限界

先に述べたように現行HPVワクチンの最大の問題点は，接種したL1-VLPと同じタイプのHPV感染しか阻止できないという点である．わが国をはじめとする東アジアに多いHPV52，58型感染に対する予防効果は期待できない．HPVワクチンの疾患予防効果は，HPV31型の約50%を最高としておおむね5～30%程度である[4～6]．HPV16，18型以外のタイプへの感染予防効果には交差性があるという情報もこれまで目にしてきたが，長期的な追跡をしないと確かなことはいえないと考えられる．HPV16，18型用に誘導された抗体では，ほかのタイプへの予防効果は，HPV16，18型と同じように長期間は持続しないと考えられる．やはり現行のHPVワクチンによって予防できるのはわが国の子宮頸がんのうち6～7割であろう[7]．

HPVワクチンのもう1つの限界は，すでに感染している女性やHPV関連病変を有する患者に対する有効性は期待できないことである．HPVワクチンの大規模臨床試験では，ASC-US以上の細胞診異常がある女性では予防効果はほとんどなかった．