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Therapeutic Human Papillomavirus (HPV) Vaccines: A Novel Approach

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Abstract: Cervical cancer is the second largest cause of cancer-related death in women worldwide, and it occurs following persistent infection, sometimes for decades, with a specific subset of human papillomavirus (HPV) types; the approximately 13 oncogenic subtypes. Prophylactic vaccines against HPV infections hold promise for cost-effective reductions in the incidence of cervical cancer, but this may not be enough. Two prophylactic HPV vaccines are presently available and both contain L1 virus-like particles (VLPs) derived from the HPV subtypes most frequently associated with cervical cancer, HPV-16 and -18. Since the L1-VLP vaccines can only effectively prevent infection by the specific HPV subtype against which the vaccine was developed, cervical cancers caused by high-risk HPV subtypes other than HPV-16 and -18 may still occur in recipients of the current HPV vaccines. Furthermore, HPV vaccination coverage for adolescents is insufficient in most countries and therefore even HPV-16 and -18 infections are unlikely to be fully eradicated using the existing strategies. The development of HPV therapeutic vaccines remains essential. Many therapeutic vaccines aimed at clearing HPV-related cervical lesions have been developed and tested in patients with HPV16-positive cervical intraepithelial lesions (CIN) or cervical cancers. To date, definitive clinical efficacy and appropriate immunological responses have never been demonstrated for cervical neoplasia although promising results have been reported in patients with vulvar intraepithelial neoplasia. Here we discuss shortcomings of previous HPV therapeutic vaccine candidates and propose a novel vaccination strategy that leverages newly gained knowledge about mucosal immunity and the induction of mucosal immune responses.

Keywords: HPV therapeutic vaccine, mucosal vaccination, cervical mucosal immune system, E7-expressing lactobacillus-bases vaccine.

EPIDEMIOLOGY OF HPV INFECTION

At present, there are about 100 identified genotypes (types) of human papillomavirus (HPV) of which about 40 are genital HPV types that invade genital organs such as the uterine cervix, vaginal wall, vulva, and penis. Genital HPV types are classified into high-risk types commonly associated with cervical cancer and low-risk types known to cause condyloma acuminatum. This classification varies among researchers, but, in general, types 16/18/31/33/35/39/45/51/52/56/58/66/68 are classified as high-risk and 6/11/40/42/43/44/54/61/72 as low-risk [1]. Interestingly, the HPV type distribution varies depending on the stage of cervical neoplasia (Fig. **1**).

The HPV DNA detection rate in the genital organs of healthy adult females varies between advanced and developing countries but is approximately 20-40% collectively [2, 3]. In Japan, the HPV-positive rate in pregnant females aged 20-29 years has been reported to be 20-30%, which is similar to or higher than that among similarly aged females in the U.S [4]. The World Health Organization (WHO) has estimated an annual increase of 3 hundred million in the number of HPV carriers in the world [5, 6]. Overall HPV prevalence with normal cervical cytology was estimated to be 10.4 % [6]. Epidemiological data show HPV infection at least once during their lifespan in approximately 75 % of U.S. women [3]. Thus, HPV infection is common and can affect any female. Frequent sexual activity has been reported to increase the risk of HPV infection but this is not always the case [7].

NATURAL HISTORY OF CERVICAL INTRAEPITHELIAL NEOPLASIA

Natural history studies of CIN show that most infections and CIN lesions resolve spontaneously but some persist and progress to cervical cancer. The incidence of cervical intraepithelial neoplasia (corresponding to squamous intraepithelial lesion: SIL) is about 1 per 10 females with HPV infection [8]. The incidence of high grade SIL (corresponding to cervical intraepithelial neoplasia 2 and 3: CIN2-3) is about 3 per 10 females with low grade SIL, and that of CIN3 is about 1-2 per 10 females with low grade SIL [9]. Without treatment, the incidence of the progression of CIN3 to cervical cancer is about 30% [10]. Therefore, the incidence of the spontaneous development of cervical cancer is about 1 per 200-300 females with HPV infection. Factors associated with progression to cervical cancer in females with HPV infection have been extensively studied [1]. Many prospective studies have identified persistent HPV infection as the most important risk factor. They have also shown that persistent infection tends to occur in women with high risk HPV subtypes.

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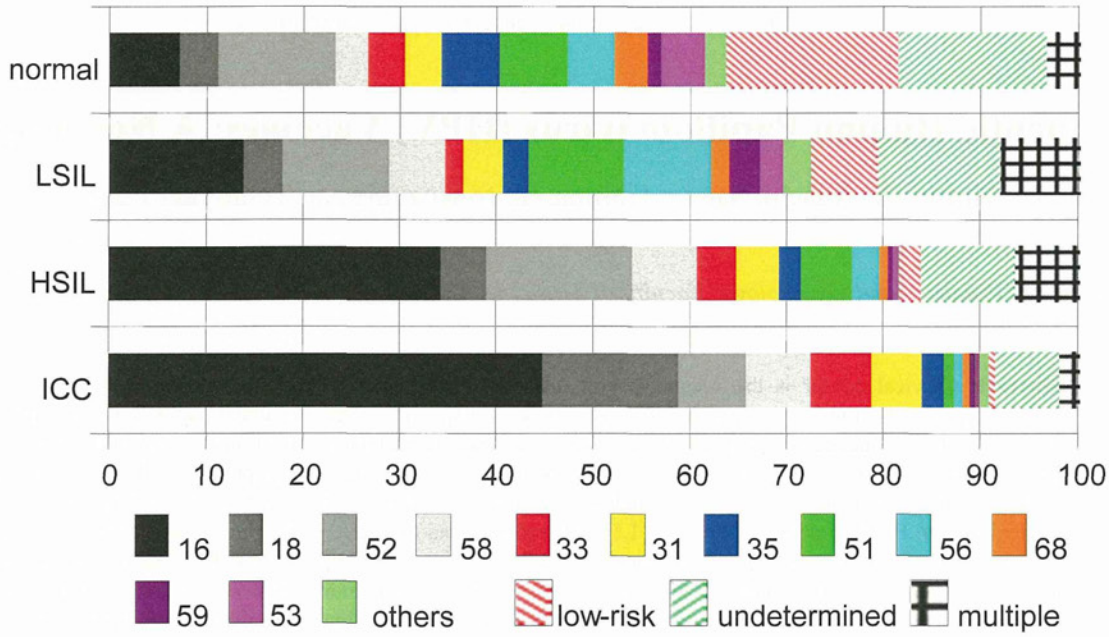

Fig. (1). HPV subtype distribution in cervical neoplastic lesions in Japan [18]. HPV16 and 18 are the most common subtypes found in invasive cervical cancer (ICC) but more than 40% of invasive lesions are associated with other oncogenic subtypes in Japan. HPV52 is the most common HPV subtype present among Japanese women with with normal cervical cytology [19].

Chronic virus proliferation induces the active proliferation/differentiation of infected epithelial cells, and some infected cells incidentally immortalize, which is the first step of carcinogenesis [1]. In contrast, transient infection involves short-term virus proliferation followed by the long-term latent presence of low copies of the viral genome in the basal cells of the genital epithelium [11]. Studies showing that HIV-infected women and patients who are under treatment with immunosuppressive agents have an increased incidence of CIN lesions [12, 13] suggest that cell-mediated immune response against HPV antigens is important in the control of HPV infection and progression to CIN. More controversial are the relative roles of systemic and local mucosal immune responses in HPV pathogenesis [14]. Trimble *et al.* reported that naturally occurring systemic immune responses to HPV antigens do not predict regression of CIN 2/3 lesions [15] but Nakagawa *et al.* demonstrated a positive association between systemic cell-mediated immune responses to HPV E6 and the regression of HPV/CIN [16].

SHORTCOMINGS OF THE CURRENT L1-VLP VACCINES

Theoretically, if HPV infection could be completely eradicated, HPV-associated cancers could be prevented. With this in mind, HPV vaccines began to be studied nearly 10 years ago. In 2002, Koutsky *et al.* were the first to show the clinical prophylactic effects of an HPV vaccine [17]. Soon thereafter, Merck in the United States and Glaxo Smith Kline (GSK) in Europe launched full-scale development of prophylactic vaccines against HPV. These products were approved and became commercially available just a few years ago. The vaccine antigens used by the two companies are virus-like particles (VLP) produced by overexpressing HPV16 L1 protein in yeast or insect cells. These particles have a 3-dimensional external structure similar to that of virus particles, but having no internal contents, they are not infective. The vaccine first reported by Koutsky *et al.* also used HPV16L1-VLP as an antigen.

One integral drawback of L1-VLP based vaccines is their negligible prophylactic effect on many HPV subtypes not specifically targeted by the vaccine [18]. For this reason, GSK and Merck developed cocktail vaccines composed of L1-VLPs corresponding to several HPV subtypes. The vaccine developed by Merck is a quadrivalent vaccine against HPV types 6, 11, 16, and 18 (Gardasil®) [19] and that developed by GSK is a bivalent vaccine against types 16 and 18 (Cervarix®) [20]. Unfortunately these L1-VLP vaccines are very specific and may not protect for long time against HPV types that exhibit very close genetic similarities to HPV-16 or -18, such as HPV-58 or -45 respectively. Ultimately, the most effective L1-VLP-based vaccines vaccines would be multivalent for the 13 described oncogenic HPV types. Such prophylactic vaccines would likely be much more expensive than their current counterparts.

HPV-16 or -18-related cervical cancers, which constitute less than 60% of all invasive cervical cancer cases in Japan [21], could be prevented if the appropriate subtype cocktail vaccine were available (Fig. **1**). However, the HPV subtype distribution in cervical cancer varies (60-70%) by worldwide location [22] and current vaccines are unable to address all oncogenic subtypes in even a single population. While current HPV vaccines are distributed without cost to the patient due to government subsidies or full coverage by insurance [23] these facile approaches will ultimately fail to eradicate the disease. Further, even with broad vaccination coverage, deficiencies in vaccine design mandate that even vaccinated females must continue cervical cancer screening.

The commercially available GSK and Merck HPV vaccines are indicated for uninfected females to prevent

HPV infection/spread. Due to the high prevalence of HPV infection, effective mass prophylactic vaccination strategies for uninfected females should include girls age 10 and above to predate the onset of sexual activity. Ph-III clinical studies in which females approximately 20 years of age were randomly inoculated with Gardasil® or Cervarix® revealed protective efficacy on the development of CIN2-3 associated with HPV-16 or -18 in 93-98% of vaccine-type naïve females who completed the vaccination protocol [24, 25]. However, intention-to-treat analysis revealed protective efficacy was only 19-30% for non-vaccine HPV subtypes [24, 25].

DEVELOPMENT OF HPV THERAPEUTIC VACCINES

The limitations of current prophylactic HPV vaccines demonstrate a pressing need for novel approaches to the eradication of HPV-related neoplasia and suggest that the development of therapeutic vaccines for the treatment of HPV-associated lesions will remain an important goal even if worldwide prophylactic vaccine programs are successfully implemented [26]. The past two decades has seen several inroads into the development of therapeutic HPV vaccines. The combined actions of the high-risk E6 and E7 oncoproteins are essential for the maintenance of the neoplastic phenotype and the evasion of apoptosis. Several functions have been described for E6 and E7. Initial observations revealed that E6 interacts with p53 and E7 interacts with Rb to block the activity of these tumour suppressors [1]. There are only two possible antigenic targets, E6 and E7, since these are the only viral proteins that will be expressed in all cancers and precursor lesions [1]. The approach of deliberate immunization with E6 and/or E7 of HPV 16 and 18 predominantly, and the generation of antigen-specific CTL as an immunotherapy for HPVassociated cancer has been tested with a wide array of potential vaccine delivery systems. Here we will summarize the results of the therapeutic vaccine clinical trials reported to (Table **1**) [14].

1. SGN-00101 (s.c.) is a fusion protein consisting of a heat shock protein (Hsp) from *Mycobacterium bovis* and HPV16 E7. The Ph-II study looking at the effects of SGN-00101 in women with CIN3 revealed histological regression to CIN1or less (complete remission: CR) in 13 (22.5%) of 58 cases, although immunological responses were not studied [27]. Another Ph-II study of the same agent administered to women with CIN showed the induction of cytotoxic T lymphocyte (CTL) against HPV16E7 in peripheral monocytes in 5 of 7 patients which obtained CR [28].
2. L1VLP-E7 (s.c.) is a vaccine using chimeric particles composed of HPV16 L1-VLP and E7. In the Ph-I/II study of women with CIN2-3, histological regression to CIN2 (partial remission; PR) was shown in 39% of vaccine recipients compared with 25 % of placebo recipients. This was not significant significant [29]. Clinical response was coupled with detectable cellular immune responses in some cases.
3. TA-HPV (i.m.) is a recombinant vaccinia virus expressing E6 and E7 of HPV-16 and -18. The Ph-II study of TA-HPV in women with vulvar intraepithelial neoplasia (VIN) revealed PR of lesions in 8 of 13 cases and responders also had an increase in lesion-infiltrating CD4 and CD8 positive cells [30].
4. TA-CIN (i.m.) is a fusion protein consisting of E6, E7 and L2 from HPV-16 and -18. The Ph-II study in women with VIN revealed CR or PR in only 6 of 29 cases. CTL against E6/E7 were induced in 4 of 29 cases [31]. Correlations between clinical efficacy and cellular immune responses to the vaccine remain unclear.
5. MVA-E2 (TGA4001) (intrauterine) is also a recombinant vaccinia virus expressing bovine papilloma virus (BPV) E2. A Ph-II study in subjects with CIN2-3 confirmed the down grade of CIN in some cases (19/34 cases) [32].
6. ZYC-101a (i.m.) is a DNA vaccine synthesized from proteins containing CTL epitopes against E6 and E7 of HPV-16 and -18. A Ph-III study was performed in subjects with CIN2-3. CR or PR was observed in 41% of vaccinated women and 27% of those receiving placebo. This was not a significant difference. Subset-analysis limited to those subjects aged 25 years or less revealed a statistically significant increase in the percentage of women with CR or PR in the vaccination group (72%) when compared to placebo controls (23%). However, no correlation was shown between CTL induction against E6/E7 and clinical effect [33].

Table 1. Clinical Trials of Therapeutic Vaccine for HPV-Associated Cervical Lesion

Trial Phase	Target Proteins	Vaccine Vectors	Inoculation	Target Types
Ph-I/II [27]	L1, E7	Chimera-VLP	S.C.	16
Ph-II [26]	E7	Hsp (SGN-00101]	S.C.	16
Ph-II [28]	E6, E7	Vaccinia virus (TA-HPV)	I.M.	16, 18
Ph-II [29]	L2, E6, E7	Fusion protein L2E6E7 (TA-CIN)	I.M.	16, 18
Ph-II [30]	BPV E2	Vaccinia virus (MVA-E2]	intrauteral	all
Ph-III [31]	E6, E7	plasmid vaccine (ZYC101a)	I.M.	16, 18
Ph-II [32]	E6, E7	Cocktailed Synthetic peptide	S.C.	16

S.C.: subcutaneous injection, I.M.: intramuscular injection, BPV: bovine papillomavirus.

7. Synthetic long-peptide vaccine (s.c.) is a peptide vaccine comprised of nine HPV16 E6 peptides and four HPV16 E7 peptides solubilized in incomplete Freund's adjuvant. A Ph-II study was performed in patients with VIN3. 5 of 20 patients demonstrated complete regression of their lesions [34].

In summary, no therapeutic HPV vaccines are presently available that exert significant clinical efficacy against CIN. Some of the tested therapeutic vaccines elicited systemic cellular immunity after intramuscular or subcutaneous injection, but none of the trials have assessed local cellular immune responses to vaccine antigen in the cervix.. The outcomes of vaccination strategies involving intramuscular or subcutaneous injection of E6/E7-based antigens for the treatment of VIN have been more promising [30, 31, 34]. We hypothesize that these findings are the direct result of the predicted poor response of cervical mucosal lesions to systemic cellular immune responses when compared to the effects of systemic immunity on epidermal lesions including those of VIN.

THE CERVICAL MUCOSAL IMMUNE SYSTEM AND HPV THERAPEUTIC VACCINES

Induction of adaptive cellular immune responses to HPV in the cervical mucosa is indispensable for treating cervical mucosal lesions such as CIN. Since precancerous lesion of the cervix develops essentially exclusively in the mucosal epithelium it would be predicted that intraepithelial lymphocytes (IELs) should be central to the elimination of CIN. To this poin, there are substantial differences between cellular and humoral immune responses in the female reproductive tract mucosa. It is well-known that intramuscular injection of L1-VLP based vaccines leads to systemic humoral immune responses characterized by the induction of anti-L1 IgG neutralizing antibody which leaks from the serum to protect the reproductive tract mucosa from HPV infection. However, the requirements for induction of mucosal cellular immune responses against microbial infected lesions differ from and are independent of those for systemic cellular immunity. Therefore, systemic intramuscular or subcutaneous vaccination strategies may be unsuitable for the induction of mucosal cellular immunity, at least in the reproductive tract mucosa.

In the uninduced state, the specific lymphocytes involved in mucosal immunity reside in the inductive sites of organized mucosa-associated lymphoid tissues (MALT); these are present in a variety of effector sites, including the mucosa of the intestine, respiratory tract and genital tract [35]. Efficient homing of lymphocytes to the gut is dependent on the homing receptors integrin $\alpha4\beta7$ and C-C chemokine receptor type 9 (CCR9). Lymphocyte-expressed integrin $\alpha4\beta7$ and CCR9 bind to their natural ligands, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and CCL25 (TECK), respectively, which are expressed on the cell surface of endothelial cells in submucosal post-capillary venules. In the intestine, mucosal dendritic cells (DCs) in gut-associated lymphoid tissues (GALT) regulate the expression of integrin $\alpha4\beta7$ on activated effector and regulatory lymphocytes in a retinoic acid-dependent manner [36]. Integrin $\alpha4\beta7$+ T cells reside the lamina propria in submucosa as lamina propria lymphocytes (LPL) and can differentiate into integrin $\alpha E\beta7$+ T cells upon exposure to TGF-β and expression of integrin $\alpha E\beta7$ facilitates retention of lymphocytes in the epithelium *via* interactions with E-cadherin [37] (Fig. **2**). Integrin $\alpha E\beta7$ is a specific marker of IELs residing in mucosal epithelia and those cells expressing this antigen on their surface were initially educated in the gut.

Several studies have demonstrated that human genital tract mucosa expresses MAdCAM-1 endogenously [38] and that GALT-derived integrin $\alpha4/E\beta7$+ T cells home to the genital mucosa [39-41]. This T cell homing and the expression of integrin αE increase in the presence of cervicitis and vaginitis [39, 40]. Although integrin $\beta7$+ mucosal T cells have been found in the cervical mucosa, a local inductive site (i.e., MALT) has never been demonstrated histologically [39, 40]. Taken together, GALT is thought to act as the inductive site for cervical IELs. GALT and the cervical mucosal connect through mucosa-specific T cells which express the homing receptors, integrin $\beta7$ and/or CCR9. Using flow cytometry, we have demonstrated that 25-30% of CD3-positive mucosal cervical lymphocytes are positive for the homing receptors integrin $\beta7$ and CCR9 and are thereby educated in GALT [41]. Approximately half of the integrin $\beta7$-positive T cells are CD45RO memory T cells while the other half are CD45RA effector T cells. Accumulation of integrin $\alpha E\beta7$+ IEL in CIN lesions varies markedly among patients and higher IEL numbers are associated with spontaneous regression of CIN [41]. These and related investigations have dramatically improved our understanding of cervical mucosal immunity which should hasten the development of a therapeutic HPV vaccine.

ORAL ADMINISTRATION OF HPV THERAPEUTIC VACCINES: A NOVEL APPROACH

Mucosal vaccination *via* oral administration of vaccine antigen is an effective method for the induction of mucosal immunity. Bermudez-Humaran *et al.* have evaluated the induction of CTL activity and the prevention/reduction of tumor formation following nasal or oral administration of live lactobacillus engineered to produce lactic acid-expressing HPV16E7 and IL-12, in tumor challenged murine models [42]. They found more marked induction of mucosal responses after nasal *vs* oral administration and a more effective induction of immunity when using *Lactobacillus plantarum vs Lactococcus lactis* [43]. Poo *et al.* have shown that oral immunization of C57BL/6 mice with *Lactobacillus casei* expressing HPV16 E7 reduces tumor formation induced by TC-1 cell administration. Immunization in these experiments elicited type 1 T cell immune responses to E7 in lymphocytes isolated from the spleen and from anogenital regional lymph nodes [44]. Although both studies used transmucosal immunization with Lactobacillus-based vaccines, they examined E7-specific systemic cellular immune response and regression of subcutaneous TC-1-induced tumors. These investigations provide no insight into mucosal cellular immune responses after immunization nor into the antigen specificity of mucosal lymphocytes. We have observed a marked induction of mucosal T cells possessing HPV16 E7-specific cellular immune recognition (E7-CMI) within intestinal mucosa after oral administration of *Lactobaccilus casei* expressing HPV16 E7 in mice [45].

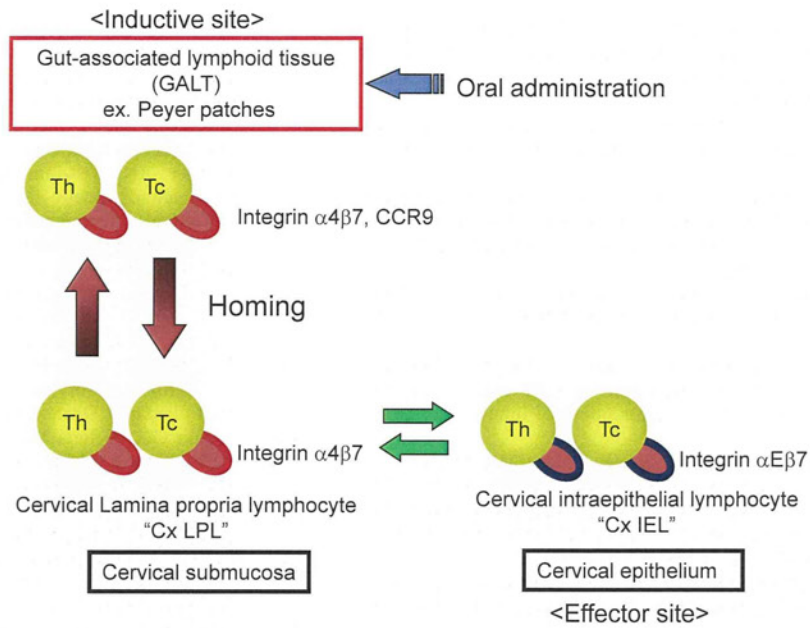

Fig. (2). Mucosal immune system in cervix. GALT is thought to act as the inductive site for cervical IELs. GALT and cervical mucosal connect through mucosa-specific T cells which express homing receptors, integrin α4β7 and/or CCR9. Integrin α4β7+ T cells can differentiate into αEβ7+ T cells upon exposure to TGF-β and expression of integrin αEβ7 facilitates retention of lymphocytes in the epithelium *via* interactions with E-cadherin. Integrin αEβ7 is a specific marker of IELs residing in mucosal epithelia and those cells expressing this antigen on their surface were initially educated in the gut. Oral administration of the therapeutic vaccine can stimulate directly to the inductive site. LPL: lamina propria lymphocytes.

In these studies, full-length mutated E7 was transduced into the *Lactobacillus casei* common to many lactic acid containing foods, and the bacterial cells were attenuated to the destroy exogenous plasmid gene. We compared mucosal vaccination *via* oral administration of the agent (GLBL101c) to systemic vaccination *via* intramuscular or subcutaneous injection of HPV16 E7 protein. Intramuscular and subcutaneous antigen administration induced small numbers of mucosal E7-CMI, but oral administration doubled these levels [45]. This implies that oral vaccination may surmount some of the deficiencies seen with systemic immunization that have been documented in previous clinical trials. Our preclinical data encouraged us to embark on a clinical trial using GLBL101c, which has now been advanced to the Ph-I/IIa stage. Patients with CIN3 who are positive for only for HPV16 alone are presently being enrolled in dose escalation study of the effects of orally administer GLBL101c on the progression or remission of their neoplastic lesions (unpublished data).

SUMMARY

The utility of the commercially-available HPV vaccines is great but incomplete. These vaccines are a valuable step toward the control of cervical cancer and should be advanced for worldwide distribution. However, cervical cancer and its precursor lesions cannot be eradicated extant vaccination strategies costly cervical cytology screening will remain essential until new, more broadly protective HPV vaccines are developed and vaccination coverage approaches 100 % among adolescents worldwide. Until then, strategies for the development of the next generation of HPV vaccines must include both prevenative and therapeutic products.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ORIGINAL ARTICLE

The Prevalence of Cervical Regulatory T Cells in HPV-Related Cervical Intraepithelial Neoplasia (CIN) Correlates Inversely with Spontaneous Regression of CIN

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Keywords
CD4+CD25+Foxp3+ regulatory T cells, cervical intraepithelial neoplasia, cervical lymphocytes, programmed cell death-1

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Problem
Local adaptive cervical regulatory T cells (Tregs) are the most likely direct suppressors of the immune eradication of cervical intraepithelial lesion (CIN). PD-1 expression on T cells induces Tregs. No studies have quantitatively analyzed the Tregs and PD-1+ cells residing in CIN lesions.

Method of study
Cervical lymphocytes were collected using cytobrushes from CIN patients and analyzed by FACS analysis. Comparisons were made between populations of cervical Tregs and PD-1+ CD4+ T cells in CIN regressors and non-regressors.

Results
A median of 11% of cervical CD4+ T cells were Tregs, while a median of 30% were PD-1+ cells. The proportions of cervical CD4+ T cells that were Tregs and/or PD-1+ cells were significantly lower in CIN regressors when compared with non-regressors.

Conclusions
The prevalence of cervical tolerogenic T cells correlates inversely with spontaneous regression of CIN. Cervical Tregs may play an important role in HPV-related neoplastic immunoevasion.

Introduction

HPV infection is a major cause of cervical cancer and its precursor lesion, cervical intraepithelial neoplasia (CIN). Natural history studies of CIN[1,2] show that most infections and most CIN lesions resolve spontaneously; only a minority persists and progress to cervical cancer. Studies showing that HIV-infected women and patients who are under treatment with immunosuppressive agents have an increased incidence of CIN lesions[3,4] suggest that cell-mediated immune response against HPV viral protein is important in the control of HPV infection and progression to CIN. We have previously reported that the presence of gut-derived effector lymphocytes within the cervix plays an important role in local cell-mediated

immune responses and correlates with CIN regression.[5] The presence of robust local tolerogenic cervical T-cell responses to HPV-related neoplastic lesions would be predicted to attenuate the effects of these local effector responses. We hypothesized that the proportion of tolerogenic lymphocytes among the CD4+ T cells in the cervix would decrease among women experiencing CIN regression, thereby allowing full effect of the changes previously seen among local effector cells.

It has been reported that CD4+CD25+Foxp3+ regulatory T cells (Tregs) play an important role in tumor-associated immunoevasion in cancers (ovarian, uterine cervical, endometrial, lung, breast, pancreas, renal cell, and thyroid cancers) as well as in other proliferative disorders such as melanoma and hepatoma.[6–15] Mechanisms underlying Treg suppressive functions have been abundantly reported. The high expression of CD25 (IL-2R) on Tregs has been thought to result in cytokine deprivation-induced apoptosis of effector T cells.[16] IL-10, TGF-β, and IL-35 are also important mediators of Treg suppressive function.[16] Tregs have been reported to suppress T effectors by ligating T-effector-expressed CD80, thereby inhibiting T-cell proliferation and cytokine production. Tregs kill effector T cells, other antigen-presenting cells, and NK cells in a manner dependent on granzyme and perforin.[16]

Natural Treg cells (nTregs) differentiate in the thymus and migrate to peripheral tissues while adaptive/induced Treg cells (iTregs) differentiate in secondary lymphoid organs and tissues including mucosa-associated lymphoid tissues (MALT).[17] iTregs play essential roles in mucosal tolerance, in the control of severe chronic allergic inflammation, in the prevention of parasite and other microorganism clearance, and in the obstruction of tumor immunosurveillance while nTregs have roles in preventing autoimmunity and preventing exaggerated immune responses. iTregs appear in the mesenteric lymph nodes during induction of oral tolerance, differentiate in the lamina propria of the gut in response to microbial signals, and are generated in chronically inflamed tissues. At a minimum, Foxp3+ iTreg development requires TCR stimulation and the cytokines TGF-β and IL-2. Integrin αEβ7+ dendritic cells (DCs) residing in the MALT produce both TGF-β and retinoic acid (RA), which mediate the differentiation of naïve T cells into Foxp3+ iTregs.[17]

The programmed cell death-1 (PD-1) and PD-ligand (PD-L) pathway is also critical in the suppression of immune responses. PD-1 is a molecule inducibly expressed on peripheral CD4+ and CD8+ T cells, NKT cells, B cells, monocytes, and some DC subsets when these cells are activated by antigen receptor signaling and cytokines.[16] nTregs and iTregs can express PD-1 and PD-L1, and the expression of ligand and receptor on the same cell conveys interesting implications. Engagement of PD-1 by its ligands during T-cell receptor (TCR) signaling results in two possible T-cell responses: 1) a diminution in T-effector responses and 2) an augmentation in differentiation of naïve T cells into Foxp3+ iTreg in a TGF-β-dependent manner.[16] There are synergistic effects between the PD-1/PD-L1 pathway and TGF-β in promoting Treg development. PD-L1 is expressed on a wide variety of tumors, and high levels of PD-L1 expression strongly correlate with unfavorable prognosis in a number of cancers.[18] To this point, ligation of PD-1 may induce and maintain iTregs within the tumor microenvironment, enhance the suppression of anti-tumor T-cell responses, and thereby allow tumor progression.

Several previous studies have shown that the prevalence of Tregs among PBMCs increases in CIN patients when compared with healthy controls.[19,20] These studies assess populations of circulating Tregs using flow cytometry. Characterization of the local lymphocytes residing in cervical lesions should better reflect local immune responses to pathogen. While Nakamura et al.[21] used Foxp3 immunostaining of human CIN lesions to report the number of local Foxp3+ cells residing in the CIN lesions by immunostaining of the tissues for Foxp3 and report that the number of Foxp3-immunoreactive cells is higher in CIN3 lesions than normal or CIN1-2 lesions, no studies have quantitatively assessed populations of local Tregs, likely iTregs, in the CIN lesions using flow cytometry. Possible associations between iTregs and the natural course of CIN have also never been studied.

We have previously characterized cervical lymphocytes collected from CIN lesions using a cytobrush and have demonstrated that the majority of cervical lymphocytes in these lesions are CD3+ T cells (median 74%) and that half of the cervical CD3 + T cells are CD4+ (median 54%).[5] In the present investigations, we have analyzed the relative proportions of two tolerogenic T-cell subsets, CD25+Foxp3+ Tregs and PD-1+ T cells, among cervical CD4+ T cells collected from CIN lesions. To determine whether there was a correlation between the frequency of cervical tolerogenic T cell and the natural course of

CIN, comparisons were made between tolerogenic T-cell subsets in the lesions of CIN regressors and non-regressors.

Materials and methods

Study Population

Cervical cell samples were collected using a cytobrush from 24 patients under observation after being diagnosed with CIN by colposcopically directed biopsy. All women gave written informed consent, and the Research Ethics Committee of the University of Tokyo approved all aspects of the study. Patients with known, symptomatic or macroscopically visible vaginal inflammation, or sexually transmitted infections were excluded from our study. To study the association between cervical tolerogenic lymphocytes and CIN progression, CIN patients with regression of cervical cytology (cases) were matched with control patients who did not exhibit cytologic regression over the same time period (measured from initial detection of abnormal cytology). In this study, cytological regression was defined as normal cytology at two or more consecutive evaluations conducted at 3–4 months intervals. For the comparison of CD4+CD25+Foxp3 Tregs and PD1+CD4+ cells, 12 patients were enrolled in the regression group, and the median follow-up duration was 16.5 (8–33) months. Twelve pairs of follow-up time-matched patients with persistent cytological abnormalities were enrolled in the non-regression group, and the median follow-up time was 19 (9–34) months. Patients were interviewed about their smoking history and their last menstrual period.

Collection and Processing of Cervical Lymphocytes

Cervical cells were collected using a Digene cytobrush as described previously.[5] The cytobrush was inserted into the cervical os and rotated several times. The cytobrush was immediately placed in a 15-mL tube containing R10 media (RPMI-1640 medium, supplemented with 10% fetal calf serum, 100 mg/mL streptomycin, and 2.5 μg/mL amphotericin B) and an anticoagulant (0.1 IU/mL of heparin and 8 nM EDTA). After incubating the sample with 5 mM DL-dithiothreitol at 37 °C for 15 min with shaking, the cytobrush was removed. The tube was then centrifuged at 330 *g* for 4 min. The resulting pellet was resuspended in 10 mL of 40% Percoll. This mixture was layered onto 70% Percoll and centrifuged at 480 *g* for 18 min. The mononuclear cells at the Percoll interface were removed and washed with PBS. Cell viability was greater than 95%, as confirmed by trypan blue exclusion, and fresh samples were immediately used for further analyses.

Immunolabeling and Flow Cytometry

Cervical immune cell preparations were immunolabeled with fluorochrome-conjugated mouse monoclonal antibodies specific for the following human leukocyte surface antigens: a programmed death-1 marker (FITC–anti-PD-1), a phycoerythrin cyanine 5.5 (PC5.5)-conjugated helper T-cell marker (PC5.5–anti-CD4), and an allophycocyanin (APC)-conjugated IL-2 receptor marker (APC–anti-CD25). After exposure to primary surface-labeling antibodies, cells were washed twice with FACS buffer (10% fetal calf serum, 1 mM EDTA, 10 mM NaN3), permeabilized with Foxp3 Fixation/Permeabilization working solution (eBioscience, San Diego, CA, USA), and immunolabeled with the anti-intracellular antigen antibody, phycoerythrin (PE)-conjugated anti-Foxp3 marker (PE–anti-Foxp3). Cells were then washed twice with Flow Cytometry Staining Buffer (eBioscience) and resuspended in Flow Cytometry Staining Buffer. Additional aliquots of the cell preparations were labeled in parallel with appropriate isotype control antibodies. Antibodies were purchased from eBioscience and BD (Franklin Lakes, NJ, USA). Data were acquired using four-color flow cytometry on FACSCalibar (Becton-Dickinson, Texarkana, TX, USA). A minimum of 5000 CD4+ T cells was analyzed per sample. The position of CD4+ T cells was determined by CD4 vs SSC gating. We used KALUZA® Flow Analysis Software (Becman Coulter, Brea, CA, USA) for data analysis.

HPV Genotyping

DNA was extracted from cervical smear samples using the DNeasy Blood Mini Kit (Qiagen, Crawley, UK). HPV genotyping was performed using the PGMY-CHUV assay method.[22] Briefly, standard PCR was conducted using the PGMY09/11 L1 consensus primer set and human leukocyte antigen-DQ (HLA-DQ) primer sets. Reverse blotting hybridization was performed. Heat-denatured PCR amplicons were hybridized to specific probes for 32 HPV genotypes

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and HLA-DQ reference samples. The virological background (HPV genotyping) of 24 patients in our study is shown in Table I. HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73, and 82 were defined as high-risk HPVs according to an International Agency for Research on Cancer (IARC) multicenter study.[23]

Statistical Analysis

Statistical analyses, including calculation of medians and interquartile ranges (IQRs), were performed using the commercial statistical software package JMP® (SAS, Cary, NC, USA). Wilcoxon rank sum tests or Fisher's exact tests were applied for matched pair comparisons. P-values ≤ 0.05 were considered significant.

Results

Isolation of Cervical Tolerogenic T-cell Subsets in CIN Lesions

To assess cervical tolerogenic T cells, cervical samples were collected from CIN lesions positive for any HPV genotype and fractionated over a discontinuous Percoll density gradient to remove cervical epithelial cells. Cervical lymphocytes were then isolated from the interphase between Percoll and culture medium.[5] Cervical CD4+ T cells were identified among the isolated lymphocytes using CD4 vs SSC gating. The percentages of CD4+ cervical T cells that were CD25+Foxp3+ Tregs or that were PD-1+ were determined by flow cytometry. Two representative cases are displayed in Fig. 1(a,b), respectively. The proportion of cervical CD4+ T cells that were CD25+Foxp3+ was 14.2% whereas the proportion of CD4+ T cells that displayed PD-1 was 33.6% (bold lines). Among all CIN patients, a median of 11.7% (IQR: 7.3–14.6, $n = 24$) of CD4+ cervical T cells were CD25+Foxp3+ Tregs, while a median of 30.7% (20.2–38.5, $n = 24$) of CD4+ cells expressed PD-1. The proportions of tolerogenic T-cell subsets found in cervical preparations were markedly higher than those reported in circulating peripheral blood where approximately 5% of PBMCs are CD25+Foxp3+ Tregs[24] and 5% of peripheral CD4+ T cells are PD-1+.[25] These data indicate that the cervical mucosal T cells separation technique used for these investigations isolated a population of T cells with characteristics that suggest little to no contamination by peripheral blood. Further, should small amounts of contamination occur during isolation the effect on overall results would be predicted to be minimal.

Table I Patients infected with multiple HPV types were included.

HPV type	Total numbers (%)
16	5 (16.6)
18	2 (6.6)
31	1 (3.3)
45	1 (3.3)
51	1 (3.3)
52	3 (10)
53	3 (10)
55	3 (10)
56	4 (13.3)
58	5 (16.6)
70	2 (6.6)
Total	30 (100)

Of 24 patients, 4 (16.6%) were infected with multiple types. HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73, and 82 were defined as high-risk HPVs.

Correlation of Cervical Tregs and PD-1+ CD4+ cells in CIN Lesions with Menstrual Phase, HPV Types, Smoking History, and CIN Course

Many factors, including HPV genotypes, smoking, and other microbial infections, have been reported to associate with spontaneous regression or progression of CIN.[26] In this study, we obtained cervical Tregs from histologically diagnosed CIN patients and sought correlations between cervical Tregs and potential clinical factors, which may associate with the natural course of CIN. Patients with known, symptomatic or macroscopically visible vaginal inflammation, or sexually transmitted infections other than HPV were excluded from our study. All patients were diagnosed with CIN1-2 at the time of enrollment and followed with colposcopy and cervical cytology smears every 4 months.

To account for possible confounding factors, samples from our 24 CIN patients were reanalyzed after segregation by each of the following characteristics: menstrual phase (proliferative vs secretory), HPV genotype (high risk vs low risk), and smoking history (smoking vs non-smoking). The prevalence of CD25+Foxp3+ Tregs and of PD-1+ T cells among cervical CD4+ cells was compared between each of the

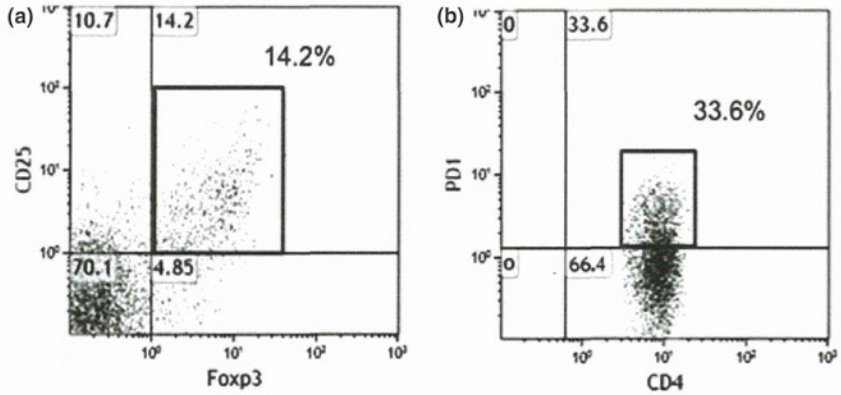

Fig. 1 Representatives of flow cytometric analysis of immune cells isolated from cervical intraepithelial neoplasia lesions. Bold lines delimit cervical CD4+CD25+Foxp3+ Tregs (a) and PD1+ CD4+ T cells (b). The indicated percentages represent percentage of total CD4+ T cells.

two groups using Wilcoxon rank sum testing (Table II). None of these possible confounders correlated with CD25+Foxp3+ Tregs and PD-1+ T cells results in CIN lesions, indicating that the tolerogenic T cells residing in the cervical mucosa were not influenced by smoking, hormonal status, or infecting HPV subtypes.

Next, we compared populations of CD25+Foxp3+ Tregs and PD-1+ T cells residing in the CIN lesions of regressors ($n = 12$) and non-regressors ($n = 12$) to determine whether there was an association between the frequency of cervical tolerogenic T-cell subsets and spontaneous regression of CIN. Twelve patients had spontaneous regression of their CIN lesions, and these women had a median follow-up duration of 16.5 (8–33) months. The non-regression group consisted of twelve women with persistent cytological abnormalities who were matched to the spontaneous regressor cohort by follow-up time. No significant differences were seen in the detection rates of high-risk HPV (58.3% vs 83.3%, $P = 0.37$), percent of CIN 2 at the enrollment (33.3% vs 58.3%, $P = 0.4$), and the median ages (33 years old vs 36, $P = 0.44$) of patients in the regression and non-regression groups. Among regressors, cervical CD25+Foxp3+ Tregs comprised a median of 7.3% (IQR: 6.3–11.4) of cervical CD4+ cells; the rate among non-regressors was 13.9% (IQR: 11.6–16.9). The frequency of cervical CD25+Foxp3+ Tregs in regressors was significantly lower than that in non-regressors ($P = 0.0012$) (Table II and Fig. 2). Similarly, cervical PD1+ CD4+ cells comprised a median of 20.8% (IQR: 15.8–31.9) of cervical CD4+ cells among regressors whereas a median of 35.1% (IQR:

Table II Correlation of the proportions of cervical Treg and PD-1+ cells among cervical CD4+ T-cell populations with clinical characteristics

		Percentage of total cervical CD4+ T cells			
Factors	Groups	CD25+Foxp3+ Tregs		PD-1+ cells	
Menstrual phase	Proliferative	10.26 (7.04–15.4)	$P = 0.94$	29.8 (22.7–39.5)	$P = 0.72$
	Secretory	12.0 (7.1–14.2)		28.1 (18.9–36.7)	
HPV genotype	High risk	11.8 (7.8–14.2)	$P = 0.67$	29.8 (20.3–38.2)	$P = 0.82$
	Low risk	7.4 (6.7–15.7)		33.5 (18.5–45.4)	
Smoking	Smoking	10.2 (7.3–14.7)	$P = 0.73$	29.8 (19.5–39.5)	$P = 0.80$
	Non-smoking	10.8 (5.0–15.9)		24.6 (19.6–40.9)	
CIN course	Regression	7.3 (6.3–11.4)	$P = 0.0012$	20.8 (15.8–31.9)	$P = 0.018$
	Non-regression	13.9 (11.6–16.9)		35.1 (30.2–42.6)	

Association of cervical CD4+CD25+Foxp3+ Tregs and PD1+CD4+ cells with menstrual cycle, HPV genotype, smoking, and cervical intraepithelial neoplasia (CIN) course were shown.

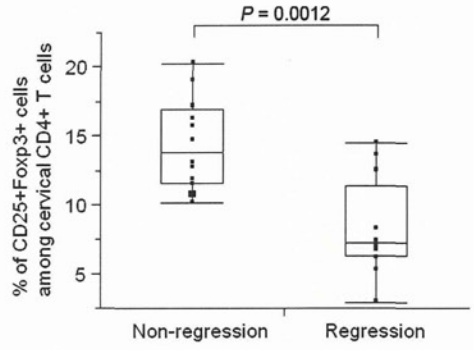

Fig. 2 Association of cervical Tregs with the natural course of cervical intraepithelial neoplasia. Among regressors, cervical Tregs comprised a median of 7.33% [Interquartile ranges (IQR): 6.38–11.4, $n = 12$] of CD4+ cervical T cells; the rate among non-regressors was 13.9% (IQR: 11.6–16.9, $n = 12$); $P = 0.0012$.

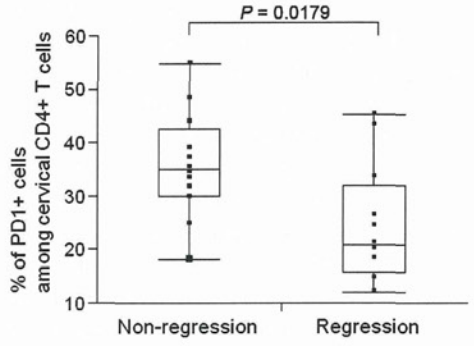

Fig. 3 Association of cervical PD-1+ CD4+ T cells with the natural course of cervical intraepithelial neoplasia. Among regressors, cervical PD1+ cells comprised a median of 20.8% [Interquartile ranges (IQR): 15.8–31.9, $n = 12$] of CD4+ cervical T cells; the rate among non-regressors was 35.1% (IQR: 30.2–42.6, $n = 12$); $P = 0.0179$.

30.2–42.6) among non-regressors. Again, the frequency of cervical PD-1+ CD4+ cells in regressors was significantly lower than that in non-regressors ($P = 0.017$) (Table II and Fig. 3).

Discussion

Although many studies have been reported about the positive association between tolerogenic lymphocytes and poor prognosis in many cancers, there are limited data on similar associations in women with HPV-related cervical precursor lesions. Our results show that the prevalence of CD25+ Foxp3+ Tregs and of PD1+ CD4+ T cells residing in cervical precursor lesions inversely correlates with spontaneous regression of CIN.

The peripheral population of Foxp3+ Tregs includes nTregs and iTregs. iTregs play essential roles in mucosal tolerance, in the control of severe chronic allergic inflammation, and in the prevention of organism clearance and tumor immunosurveillance, while nTregs have roles in preventing autoimmunity and exaggerated immune responses.[17] We would predict that the majority of cervical CD25+Foxp3+ Tregs assessed in this study are iTregs although definitive isolation of iTregs is hampered by the lack of suitable surface markers that distinguish iTreg and nTreg cell populations.

In this study, cervical Treg prevalence negatively correlated with regression of CIN (Fig. 2) but did not correlate with CIN grade (data not shown). Supporting our data, several previous studies have shown a positive correlation between Treg prevalence in peripheral blood and high grade of CIN.[19,20] Of course, cervical iTregs and circulating Tregs may differ in their TCR repertoire. iTregs are known to differentiate from mature naïve CD4+ cells through the effects of TGF-β and RA secreted by mucosa-associated DCs.[17] In our data, the proportion of CD25+Foxp3+ Tregs among total cervical CD4+ cells (a median of 11%) was twofold higher than previously reported peripheral blood levels (approximately 5%). This suggests that iTregs may be generated continuously, probably in an antigen-depending manner, and accumulate in chronically HPV-infected tissues and CIN lesions. Others have reported that Foxp3 mRNA levels in cervical samples that included exfoliated epithelial cells and cervical lymphocytes are higher among high-grade squamous intraepithelial lesion (HSIL) patients when compared with low-grade squamous intraepithelial lesion (LSIL) patients.[27] However, it is unknown whether Foxp3 mRNA levels in these cervical samples parallel the number of Tregs because cervical lymphocytes were not specifically isolated in this study.

Although the persistence of HPV infection was not followed in the present study, Molling et al.[20] reported that CD4+CD25hi Treg frequency correlates with persistence of HPV type 16. Tregs may inhibit the HPV clearance by immune cells such as invariant natural killer T cells.

TGF-β is critical to the induction and maintenance of Foxp3+ Tregs, with particular importance in the induction of iTregs from naive T cells and in the conversion of effector T cells to iTregs. Several studies have demonstrated that the expression of TGF-β and RA receptors in cervical specimens is lower in

CIN lesions when compared with normal epithelium.[28,29] In these studies, there was no correlation between TGF-β mRNA levels and either CIN grade or CIN natural course. TGF-β-induced iTreg frequency may be a more direct predictor of CIN progression than TGF-β. In fact, measurement of tolerogenic T-cell frequency in CIN lesions has the potential to prove useful in determining individualized screening and treatment paradigms.

Whether sex hormones modulate the prevalence and function of Tregs remains controversial. Arruvito et al. reported that the proportion of Foxp3+ cells within the peripheral blood CD4+ T-cell population increases during the late follicular phase when compared with the luteal phase.[29] The expansion of Tregs during the follicular phase was highly correlated with serum estradiol (E2) levels.[30] In contrast, Weinberg et al. reported recently that there are no significant correlations between changes in serum E2 levels and the prevalence of any circulating Treg subtypes or between changes in serum progesterone levels and the proportion of CD8+ Foxp3+ Tregs in peripheral blood samples.[31] The effect of smoking on the generation of tolerogenic T cells is also controversial.[32–34] Note that all of the above studies assess peripheral circulating rather than local cervical Tregs. Our data on the latter cells revealed no correlations between cervical Treg prevalence and either menstrual phase or smoking.

In this study, we focused on PD-1+ CD4+ T cells as well as Foxp3+ Tregs as engagement of PD-1 by its ligands on T cells is critical to the differentiation of naïve T cell into Foxp3+ iTregs. Furthermore, Tregs and the PD-1/PD-L pathway are integral in terminating immune responses and augmenting the suppression of anti-tumor T-cell responses. In short, the PD-1 pathway controls the development, maintenance, and function of iTregs at mucosal sites. Here, we show that PD-1+ T cells are more frequently found among cervical T cells than among PBMCs and that the prevalence of PD1+ T cells in CIN lesions (likely reflecting cervical iTregs) correlates inversely with spontaneous regression of CIN. Assessment for other tolerogenic T-cell subsets (e.g., Foxp3-IL10+ Tr1, Foxp3-TGF-β+ Th3) in this study, while potentially informative, was limited by the number of cervical lymphocytes that could be isolated from a single cytobrush sample.

In summary, even the study population is small and the results are limited, our flow cytometric analyses demonstrate for the first time that a prevalence of CD4+ CD25+ Foxp3+ Tregs infiltrating into CIN lesions significantly correlates with regression of CIN regardless of HPV subtype. Conversely, a high prevalence of lesional cervical Tregs may be responsible for CIN persistence as well as HPV infections and might function as a useful predictive biomarker for progression of CIN.

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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

ADLDKLNIDS IIQR [15]llev
LFEYGGFPPE SNYLFLGDYV DR [96]gkqs
IKYPENFFLL R [122]gnhe
YPENFFLLR [122]gnhe
AHQVVEDGYE FFAK [260]rqlv
KKPNATRPVT PPR [314]gmit

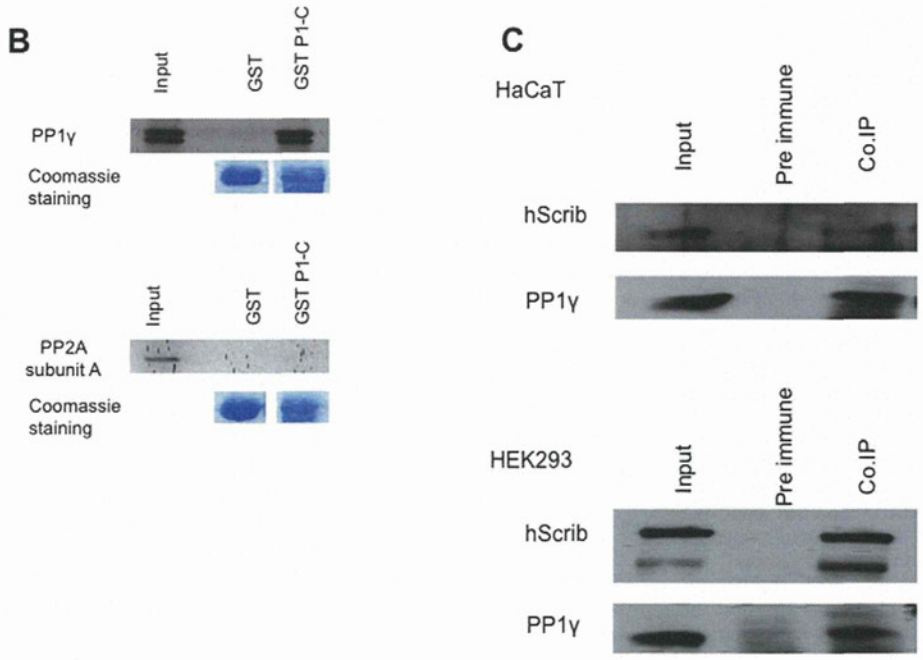

Figure 1. Interaction between hScrib and PP1γ in vivo. A) Results from the mass spectroscopy analysis of hScrib containing immunoprecipitates identified 6 peptides (indicated) corresponding to PP1γ. B) In vitro translated PP1γ (upper panels) and PP2A subunit A (lower panels) were incubated for 1 hour 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

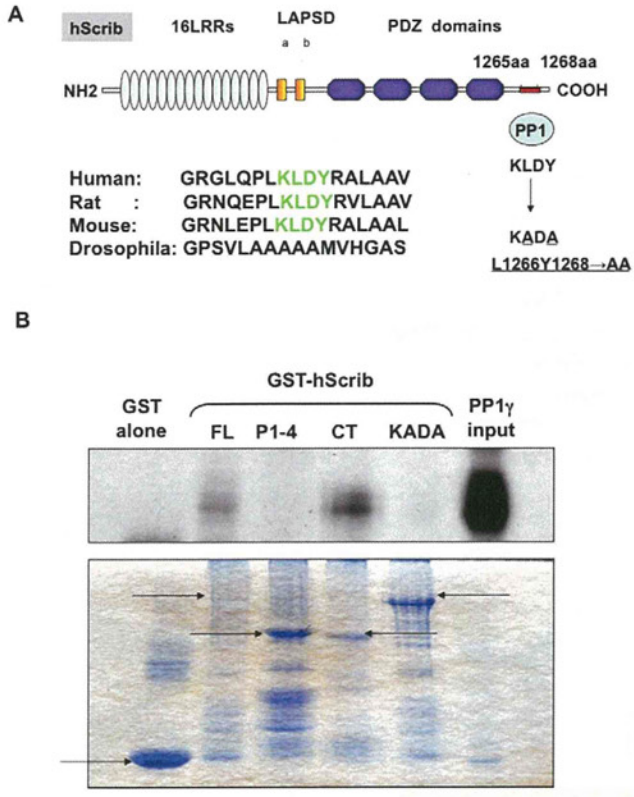

Figure 2. hScrib contains a consensus PP1-binding motif. A) The schematic shows the arrangement of the functional domains on the hScrib protein, highlighting the LRR, LAPSD and PDZ domains. The putative PP1-binding site, the RVXF (the consensus sequence is K/R/H/N/S V/I/L X F/W/Y) motif is also shown, 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

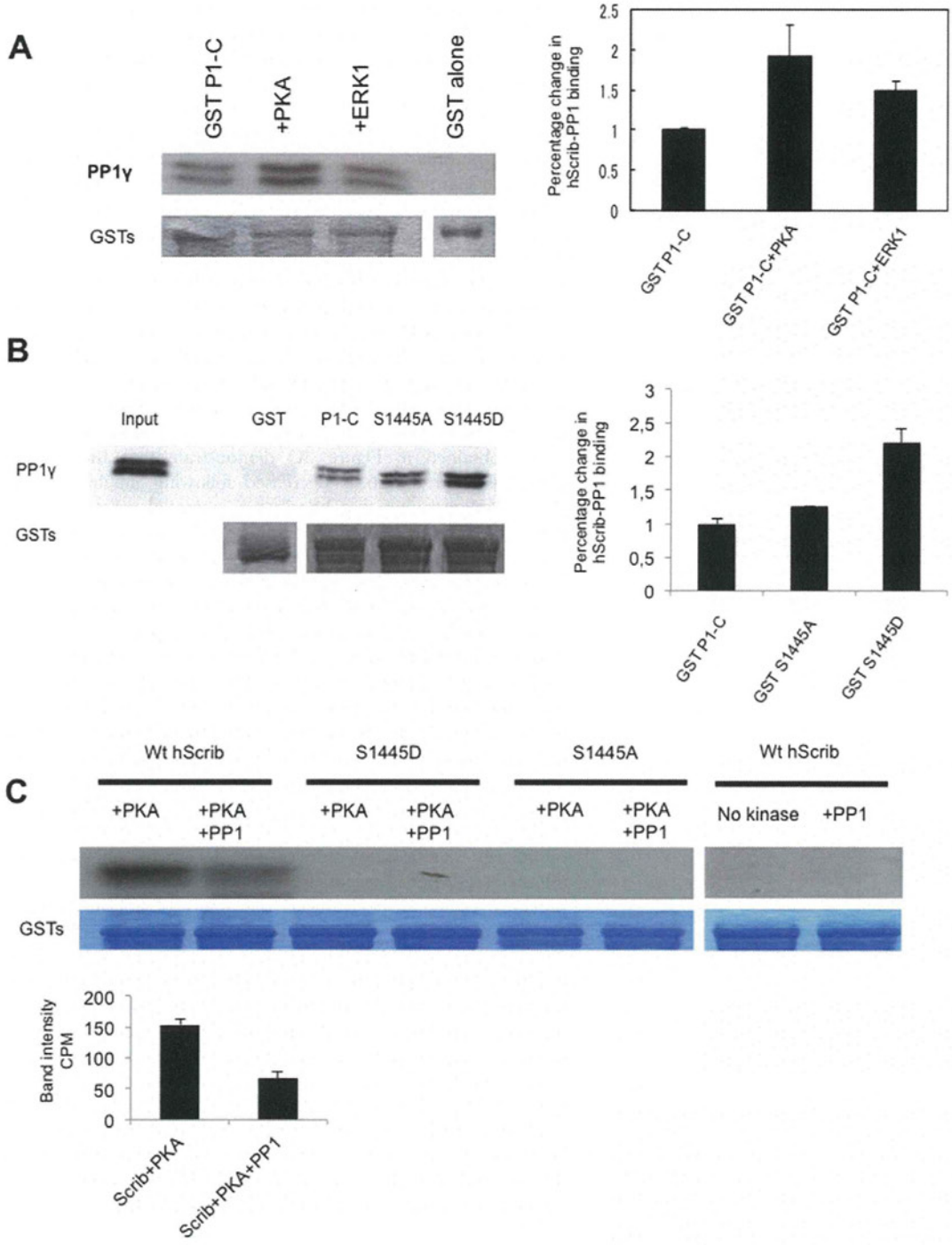

Figure 3. hScrib is a substrate of PP1γ. A) Purified GST-hScrib fusion protein was in vitro phosphorylated with purified PKA or ERK1 as described previously (19) and then incubated with PP1γ for 20 mins at 30°C. Bound PP1γwas detected by western blotting with anti PP1γ antibody. The lower panel shows 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.
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