UC Irvine
UC Irvine Previously Published Works

Title
Development and Assessment of a General Theory of Cervical Carcinogenesis Utilizing a Severe Combined Immunodeficiency Murine–Human Xenograft Model

Permalink
https://escholarship.org/uc/item/5101g19s

Journal
Gynecologic Oncology, 77(1)

ISSN
0090-8258

Authors
Tewari, Krishnansu Sujata
Taylor, Jason A
Liao, Shu Y
et al.

Publication Date
2000-04-01

DOI
10.1006/gyno.2000.5729

Copyright Information
This work is made available under the terms of a Creative Commons Attribution License, availalbe at https://creativecommons.org/licenses/by/4.0/

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Gynecologic Oncology **77,** 137–148 (2000)
doi:10.1006/gyno.2000.5729, available online at http://www.idealibrary.com on IDEAL®

Development and Assessment of a General Theory of Cervical Carcinogenesis Utilizing a Severe Combined Immunodeficiency Murine–Human Xenograft Model

Krishnansu Sujata Tewari, M.D.,*,† Jason A. Taylor, B.S.,* Shu Y. Liao, M.D.,‡ Philip J. DiSaia, M.D.,† Robert A. Burger, M.D.,† Bradley J. Monk, M.D.,† Christopher C. W. Hughes, Ph.D.,* and Luis P. Villarreal, Ph.D.*,[1]

**Department of Molecular Biology and Biochemistry, School of Biological Sciences, University of California, Irvine, Irvine, California 92697; †Division of Gynecologic Oncology, Department of Obstetrics & Gynecology, University of California, Irvine–Medical Center, Orange, California 92868; and ‡Department of Surgical Pathology, St. Joseph's Hospital, Orange, California 92868*

Received August 11, 1999

Objective. **Currently, we lack a theoretical explanation for why squamous cell cervical cancer develops predominantly in specific sites (i.e., along the squamocolumnar junction). We therefore implanted human cervical tissues containing the transformation zone in severe combined immunodeficiency (SCID) mice and studied morphology, steroid effects, gene expression, and human papillomavirus (HPV) factors.**

Methods. **Normal and dysplastic human cervical tissues (3 × 2 mm) were placed subcutaneously in SCID-*beige* mice and later assessed by *in situ* hybridization for HPV 16/18 DNA and by immunohistochemistry for expression of CD31, keratin, proliferating-cell nuclear antigen, HPV 16 E6, p53, and Notch-1 (a binary cell fate determination protein). Some normal tissues were implanted with either a 90-day release 1.7-mg 17β-estradiol pellet or a 5-mg tamoxifen pellet; others were infected prior to implantation with human recombinant adenovirus 5 vector containing a human cytomegalovirus promoter-driven β-galactosidase gene and later assessed by X-gal staining.**

Results. **Murine and human vessels formed anastomoses by 3 weeks. For at least 11 weeks, normal tissue retained the transformation zone and normal cell-type-specific keratin expression and exhibited normal proliferation; Notch-1 was present only in the basal cell layer. Dysplastic tissues exhibited koilocytosis, increased levels of cellular proliferation, and aberrant keratin, p53, and Notch-1 expression; HPV 16/18 DNA and HPV 16 E6 protein were detected for at least 6 weeks. Squamous metaplasia of normal cervical epithelium resulted from estrogen exposure, and a predominant columnar differentiation pattern was associated with tamoxifen administration. Through stable adenovirus infection, β-galactosidase was expressed for at least 6 weeks.**

Conclusions. **This small manipulatable xenograft model maintains normal and dysplastic human cervical epithelium through neovascularization. Neoplastic tissue retains HPV 16/18 DNA and a premalignant phenotype, including elevated levels of cellular proliferation and aberrant keratin, p53, and Notch-1 expression. These attributes constitute essential features of a biologic model through which one may study HPV-mediated human disease and may be superior to cell culture and transgenic murine systems. Furthermore, this may serve as a model for gene therapy. Finally, we suggest that the normal cervical epithelium is maintained through putative interactions between the Notch locus and cell cycle growth regulators such as p53 and pRb. Neoplastic cervical epithelium may arise through disruption of this pathway. This theory may be testable in our animal model.** © 2000 Academic Press

INTRODUCTION

The ability to study the molecular and physiologic mechanisms which underly the development of cervical neoplasia is frustrated by the lack of an intact and manipulatable biologic model. Such a model is required to develop and test the various hypotheses which have been advanced to explain cervical carcinogenesis.

Among the predominant theories is loss of cell cycle regulation through tumor suppressor gene mutation. Mutation of p53 has been associated with the disruption of apoptosis and stimulation of angiogenesis in a variety of human malignancies, including cervical carcinoma. However, because the vast majority of cervical cancers contain integrated human papillomavirus (HPV) 16 or 18 DNA, the ability to assess viral factors would be a prerequisite for any model [1–3]. Indeed, the protein products of the HPV 16/18 oncogenes E6 and E7 have been shown to cause inactivation and degradation of p53 and pRb, respectively, and may also be involved in reprogramming the host immune system so that HPV infection is unrecognized.

As the premalignant and malignant lesions of the uterine cervix arise from the transformation zone (an area of immature metaplasia between the mature stratified squamous epithelium

[1] To whom correspondence should be addressed at Department of Molecular Biology and Biochemistry, The Center for Viral Vector Design, University of California, Irvine, California 92697. Fax: 949-824-8551. E-mail: lpvillar@uci.edu.

0090-8258/00 $35.00
Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

of the exocervix and the columnar epithelium of the endocervix) an ideal cervical cancer tissue model should maintain this squamocolumnar junction. Although many anatomical sites may proliferate (e.g., the epidermis), the transformation zone is atypical relative to many tissues, in that it is still making an embryo-like binary cell fate decision. Thus this is distinct from the transition zones at the gastroesophageal junction or in the anal canal where active metaplasia may no longer occur.

The Notch gene family consists of four mammalian genes (Notch 1–4) which maintain an evolutionarily conserved mechanism that effects binary cell fate decision-making processes [4]. Specifically, the Notch proteins do not participate exclusively in cell division (i.e., proliferation), but are also involved in cell fate changes. In normal human cervical epithelium, the Notch-1 protein is found exclusively in the basal cell layer [5]. Molecular interactions at the Notch-1 locus on the long arm of chromosome 9 may be crucial to the biology of the transformation zone.

Many biological mechanisms may be evaluated in cell culture; however, such systems are usually unable to address important *in vivo* phenomena such as tissue interactions, cytokine signal transduction, angiogenesis, and host immune surveillance.

Murine Models

Several distinct animal models have been developed for studying cervical neoplasia. Because HPV does not infect mouse tissues, the study of the virus has required that specific murine strains be genetically engineered to carry viral sequences of interest. In this fashion, working with transgenic mice expressing the HPV 16 oncoproteins, E6 and/or E7, several investigators have elucidated molecular mechanisms associated with the induction of epidermal hyperplasia, angiogenesis, and DNA damage [6–9]. Recently, Arbeit and colleagues utilized transgenic mice expressing the HPV 16 oncoproteins, E6 and E7, from a keratin 14 promoter and demonstrated synergistic effects between the viral oncoproteins and chronic estrogen exposure in the development of squamous carcinogenesis along multiple sites (cervix and vagina) of the murine female reproductive tract [10]. Although this is clear evidence of the ability of these viral oncogenes to induce cancer *in vivo,* these results do not offer sufficient insight that can explain the underlying mechanism which restricts disease to the cervical transformation zone. HPV is known to infect all areas of the human female genital tract, yet only infection of the transformation zone can account for a cervical cancer problem of global epidemic proportions; malignant disease develops less frequently in other lower genital tract sites such as the vagina and vulva. Therefore, transgenic models, although important, may produce artificial experimental observations that do not sufficiently correlate with clinical experience. Furthermore, the lack of human tissue interactions would seem to limit these models.

Some investigators have attempted to examine *human* cervical tissues in murine systems. In 1985, Kreider and co-workers reported a xenograft model for the study of human papillomavirus infections [11]. Human uterine cervix fragments were infected with a lysate prepared from condylomata acuminata and placed under the renal capsule of nude (i.e., athymic) mice. Three months later, these implants demonstrated some histologic features of HPV infection and some even contained HPV 11 DNA. However, the absence of the human cervical transformation zone and failure to support HPV cervical-cancer-associated viral subtypes (e.g., HPV 16 or 18) represent a significant drawback of this model.

Discovered in 1980, severe combined immunodeficiency (SCID) mice lack B and T lymphocytes. The animals have limited adaptive immune responses which precludes the rejection of human xenogenic tissue grafts. Several centers have reported the isolation and/or propagation of high-risk HPV subtypes in xenografts derived from human foreskin and/or human condyloma [12–15]. For example, using ballistic particle bombardment, Brandsma and colleagues inoculated primary human foreskin that had been engrafted onto SCID mice with DNA from high oncogenic risk HPV [14]. Although the resulting xenograft gave rise to papillomatous changes including koilocytosis and capsid protein expression, the model did not incorporate viral infection and utilized nonjunctional keratinocytes. Using neonatal human foreskin implants to passage virus in SCID mice, Bonnez and colleagues propagated HPV 16, the major papillomavirus subtype associated with cervical neoplasia [15].

We have established a murine–human xenograft model which utilizes SCID-*beige* mice. In addition to lacking B and T lymphocytes, the strain is devoid of functional natural killer cells and harbors macrophage defects. Our preliminary results have recently been reported in abbreviated form [16]. We have expanded upon our original work and in this paper we review our methodology and some early results in greater depth as well as present our more recent observations through which we postulate a molecular theory for cervical carcinogenesis.

Primary Objectives

Experimental sequences I–VII. Our objectives were to establish a murine system which supports implanted human cervical tissues and maintains characteristic morphologic features; to determine whether human papillomavirus is retained; to effect change within the system.

Secondary Objectives

Experimental sequences VIII and IX. Our objectives were to evaluate molecular interactions at the level of the squamocolumnar junction through steroid hormone exposure and specific cell cycle growth regulators and signaling pathways.

MATERIALS AND METHODS

Animals

C.B.-17 ICR SCID-*Bg* mice (Harlan Sprague–Dawley, Indianapolis, IN) were used at ages 4–6 weeks. The animals were housed in microisolator cages and were fed sterilized water and mouse chow. All experimental protocols were approved by the University of California, Irvine Institutional Animal Care and Use Committee.

Human Tissue

Fresh human cervical tissue was obtained from patients treated by members of the Department of Obstetrics & Gynecology under protocols approved by the University of California, Irvine Institutional Review Board.

1. Normal cervical tissue. This tissue was retrieved from discarded premenopausal hysterectomy specimens.

2. Dysplastic cervical tissue. This tissue was obtained from diagnostic cervical cold-knife conization procedures. Tissue lying between two circumferential points with demonstrable severe dysplasia by frozen-section analysis was provided.

The tissues were transported in Hank's balanced salt solution supplemented with penicillin (500 units/mL), streptomycin (500 μg/mL), and nystatin (200 units/mL). Implants were prepared in $3 \times 2 \times 2$ mm sections containing the squamocolumnar junction and used within 4 h of harvest. One section from each specimen (labeled Day 0) was placed in 10% buffered formalin solution and embedded in paraffin.

Xenograft Creation

Implantation procedure. Mice were transferred to a laminar flow hood and anesthetized by inhalation of methoxyflurane. The abdominal surface was prepped with 95% ethanol. A 1-cm incision was made in the lateral wall of the abdomen above the peritoneum. The human cervical implant was inserted into a subcutaneous pocket created by blunt dissection in the adipose tissue. The skin was reapproximated using 4-O vicryl suture.

Tissue retrieval. At specific time points the mice were euthanized by carbon monoxide inhalation. The implants and adjacent mouse tissue were excised *en bloc* and either embedded in paraffin or sucrose saturated for frozen-section analysis.

Experimental Variations (Manipulating the System)

Steroid hormone administration. Initially, some mice (n = 5) underwent implantation with both normal human tissue and a contralateral, subcutaneous 90-day time-released 1.7-mg 17β-estradiol pellet (Innovative Research of America, Sarasota, FL). In later experiments, some mice (n = 5) also underwent implantation of normal tissue with a contralateral, subcutaneous 5-mg tamoxifen tablet (Innovative Research of America).

Recombinant adenoviral vector infection. Prior to implantation, some normal human cervical tissues were infected *ex vivo* for 1 h with 1×10^9 plaque-forming units/mL of a human adenovirus 5 recombinant containing the β-galactosidase gene driven by the human cytomegalovirus immediate early promoter. The incubation was performed in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY) containing 2% fetal bovine serum for 1 h prior to implantation.

Tissue Analysis

Tissue sections of 4–6 μm from each series of experiments were cut from the paraffin blocks and stained with hematoxylin–eosin using standard procedures. Unstained tissue samples were also provided from each experimental sequence for either immunohistochemical analyses or *in situ* hybridization studies. The X-gal stain was performed on tissues prepared by frozen section immediately following retrieval from mice harboring recombinant viral vector-infected normal tissue xenografts.

CD31. The excised normal and dysplastic tissue xenografts with surrounding tissue were double stained for the human (light blue stain) and mouse (brownish–red stain) endothelial cell marker, CD31, utilizing standard protocols for mouse anti-human CD31 (Becton–Dickinson Co., San Jose, CA) and rat anti-mouse CD31 (PharMingen, San Diego, CA), respectively.

Keratin. Monoclonal mouse anti-human keratin 5/6 antibody (Boehringer Manheim Corporation, Indianapolis, IN) and monoclonal mouse anti-human keratin 8 antibody (Novocastra Laboratories, Newcastle upon Tyne, UK) were used to examine differential keratin expression in normal and dysplastic tissues.

Proliferating cell nuclear antigen (PCNA). A monoclonal mouse anti-human PCNA antibody (Dako Corp., Carpinteria, CA) was used to assess cellular levels of proliferation in normal and dysplastic implants.

p53. Using an Immunocruz staining system (Santa Cruz Biotechnology, Santa Cruz, CA), normal and dysplastic implants (n = 3) were stained with a mouse anti-human p53 monoclonal antibody.

HPV 16 E6 and Notch-1. Using Immunocruz staining systems, normal and dysplastic implants (n = 3) were stained with goat anti-human HPV 16 E6 and goat anti-human Notch-1 polyclonal antibodies.

In situ hybridization. HPV DNA was detected using the Dako GenPoint catalyzed signal amplification system for *in situ* hybridization (Dako Corp.). Dysplastic implants were retrieved after 6 weeks and tested with biotinylated HPV wide-spectrum and subtype 16/18 specific DNA probes. Formalin-fixed cells from the cervical carcinoma cell line, carrying one

to two chromosomally integrated copies of the HPV type 16 genome per cell, served as positive controls and normal human cervical tissue implants which had been excised from SCID-*Bg* mice at 6 weeks were used as negative controls.

X-gal staining. For the β-galactosidase assay, 9-μm frozen tissue sections were fixed in a phosphate-buffered saline solution containing 0.2% gluteraldehyde and 2% formaldehyde for 90 s. The substrate was developed using 0.1% X-gal (Life Technologies, Inc.) overnight at room temperature.

RESULTS

Vascularization of Implanted Human Tissue

The xenografts and surrounding tissue were double stained for the human (light blue stain) and mouse (brownish–red stain) endothelial cell marker, CD31. Both human and mouse vasculature was present at the boundary of the xenograft where mouse vessels entered the implants, forming anastomotic links with the human vessels. Examples of chimeric vessels containing both human and mouse endothelial cells are present in normal tissues from Day 23 (Fig. 1A) and dysplastic xenografts from Day 77 (Fig. 1B).

Morphology of Normal Human Cervical Tissue

The squamocolumnar junction and stratified epithelium present at preimplantation Day 0 (Fig. 2A) were both present in normal human cervical tissues for up to 11 weeks (Fig. 2B).

Morphology of Dysplastic Human Cervical Tissue

For up to 28 weeks, dysplastic tissues maintained koilocytotic changes (Fig. 3A) and a premalignant phenotype (Fig. 3B).

Differential Keratin Expression of Normal and Dysplastic Tissues

Normal tissues exhibited the expected pattern of keratin expression at the squamocolumnar junction from K5 positive/K8 negative (squamous epithelium, Fig. 4A) to K8 positive/K5 negative (columnar epithelium, Fig. 4B). The keratin expression of dysplastic tissue (Figs. 4C and 4D), however, was more variable, with a section at Day 147 revealing a focus of K5-positive squamous metaplasia below K5-negative columnar cells (Fig. 4C).

Cellular Proliferation of Normal and Dysplastic Tissues

Normal tissues from preimplantation Day 0 (Fig. 5A) and Day 42 demonstrated immunoreactivity for PCNA only in the basal cell layers. In contrast, implanted dysplastic tissue stained strongly for PCNA in both the columnar cells and the suprabasalary cells of the stratified squamous epithelium for at least 191 days postimplantation (Fig. 5B).

The Maintenance of HPV in Dysplastic Tissue

Specific signals (brownish stain) were concentrated in the human epithelium, both in stratified squamous and in columnar cells (Figs. 6A and 6B). Signals were not detected in surrounding human stromal tissue or in adjacent murine tissue.

Estrogen Administration

At Day 24, all ($n = 3$) exposed implants demonstrated regions of squamous metaplasia and hyperplastic change (Fig. 7A), which were not present at Day 0. Implants which had been derived from the same surgical specimens ($n = 3$) but had not

FIG. 1. Neovascularization. Chimeric blood vessels containing both human (blue) and murine (brown–red) CD31 proteins. Both images taken at 40×. (A) Day 77, normal cervical tissue, with blood vessel in longitudinal section (arrow); (B) Day 23, dysplastic tissue, with blood vessel in cross-section (arrow).

FIG. 2. Preservation of the stratified epithelium and squamocolumnar junction of normal human cervical tissues. Hematoxylin and eosin. (A) Day 0, prior to implantation; the squamocolumnar junction is indicated by the arrow (20×); (B) Day 77, the squamocolumnar junction is indicated by the arrow (40×).

FIG. 3. Maintenance of dysplastic features. Hematoxylin and eosin; both images taken at 40×. (A) Day 79, koilocytotic changes consistent with HPV infection (arrow); (B) Day 191 with a focus of dysplastic cells (arrow).

FIG. 4. Differential kerratin expression of normal and dysplastic human cervical epithelium. Immunohistochemistry for cytokeratin proteins; all images taken at 40×. (A–B) Day 24, normal cervical tissue: K5–6 expression present in the stratified squamous epithelium and K8 expression present in the columnar cells; the squamocolumnar junction is indicated by the arrow. (C–D) Day 147, dysplastic cervical tissue, with K5–6 expression below the columnar layer at a site of squamous metaplasia and K8 expression appropriate for columnar cells.

FIG. 5. Cellular proliferation levels of normal and dysplastic human cervical epithelium. Immunohistochemistry for proliferating cell nuclear antigen; all images taken at 40×. (A) Day 0, normal stratified squamous epithelium with proliferating basal layer; (B) Day 191, dysplastic focus remains mitotically active throughout the stratified layers.

FIG. 6. Retention of HPV 16 and/or 18 DNA in dysplastic implants. *In situ* hybridization; both images taken at 40×. The signals are concentrated in both stratified squamous (A) and columnar (B) epithelium of the human tissue (Day 42). Signals were not detected in surrounding human stromal tissue or in murine tissue which had been excised en bloc.

FIG. 7. Biologic and pharmacologic manipulation of normal tissues. Both images taken at 40×. At Day 24, following implantation with a 90-day release 1.7-mg 17β-estradiol pellet normal human cervical tissue exhibited a disorganized structure and an increase in stratified nuclear epithelium, consistent with hyperplasia and a squamous metaplasia and hyperplastic changes (A). X-gal substrate turns blue at Day 46 in the presence of β-galactosidase expressed from normal human cervical tissue infected *ex vivo* with a recombinant adenoviral vector prior to implantation in the mouse; the diffuse staining pattern is evidence of widespread recombinant viral expression with the implant (B).

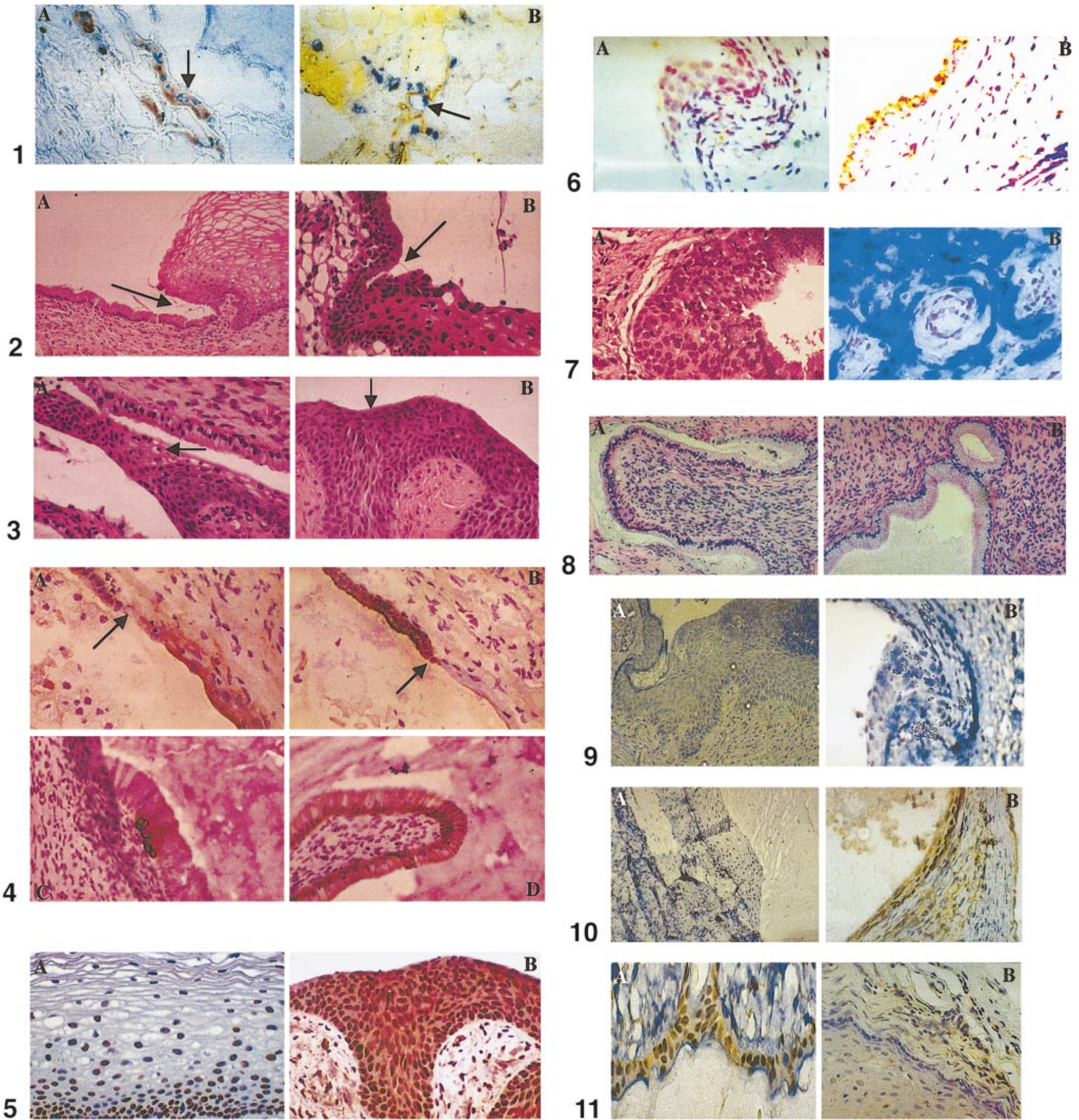

FIG. 8. Estrogen and tamoxifen in parallel. Both images taken at 40×. At Day 49, squamous metaplastic changes and hyperplasia were also observed in normal tissues which had been implanted with a 90-day release 1.7-mg 17β-estradiol pellet (A). By contrast, normal cervical implants which had been implanted with a 5-mg tamoxifen pellet revealed exclusively columnar differentiation at Day 49 (B).

FIG. 9. Immunostaining for the HPV 16 E6 oncoprotein. HPV 16 E6 expression was not detected in normal tissues at Day 50 (A, 20×); dysplastic tissues at Day 42 contained HPV 16 E6 in the stratified epithelium (B, 40×).

FIG. 10. Immunostaining for the p53 tumor suppressor gene product. Wild-type p53 expression was not detected in the normal cervical epithelium (A, 20×); up to 50% of the cells in dysplastic tissue expressed p53 (B, 20×).

FIG. 11. Immunostaining for the Notch-1 protein. In normal cervical tissues, Notch-1 expression was restricted to the basal cell layer of the squamocolumnar junction (A, 40×); Notch-1 expression was observed throughout the stratified epithelial layers of dysplastic implants (B, 20×).

undergone implantation with an estrogen pellet did not exhibit these changes.

Recombinant Adenoviral Vector Gene Transfer

At Day 46, using 0.1% X-gal blue stain, the excised implant demonstrated expression of the β-galactosidase gene product (Fig. 7B). No evidence of expression was exhibited by neighboring murine tissue or contralateral implants.

Parallel Estrogen and Tamoxifen Administration

Once again, squamous metaplasia and hyperplastic change resulted from estrogen exposure (Day 49, Fig. 8A). Interestingly, implants which had been exposed to tamoxifen revealed columnar differentiation exclusively (Day 49, Fig. 8B). This columnar epithelia exhibited "closed loop patterns" which were distributed throughout three retrievable implants implanted with tamoxifen (two other implants which had undergone implantation with tamoxifen were not found when the mice were euthanized and dissected).

HPV 16 E6 Expression in Normal and Dysplastic Tissues

The viral oncoprotein HPV 16 E6 was not detected in normal cervical implants at Day 50 (Fig. 9A). HPV 16 E6, however, was detected in the stratified squamous epithelium of dysplastic tissue at Day 42 (Fig. 9B).

p53 Expression in Normal and Dysplastic Tissues

As expected, the relatively short half-life of p53 did not permit its detection in normal tissues by immunohistochemistry at Day 50 (Fig. 10A). On the other hand, up to 50% of the cells in dysplastic implants at Day 42 ($n = 3$) had measurable levels of p53 by immunostaining (Fig. 10B).

Notch-1 Expression in Normal and Dysplastic Tissues

Consistent with the observations of others [5], Notch-1 expression was relegated only to the basal cell layer of the transformation zone in normal tissue at Day 50 (Fig. 11A). In the dysplastic tissue from Day 42, Notch-1 expression was detected throughout all layers of the stratified epithelium (Fig. 11B). These findings are also in agreement with previous published investigations [5, 9].

The dysplastic tissues utilized for the *HPV 16 E6–p53–Notch-1 experimental sequence* were taken from the Day-42 dysplastic implants in which HPV 16/18 DNA had been detected.

DISCUSSION

I. Human Papillomavirus and Cervical Cancer

The presence of HPV infection in the majority of cervical neoplasms has been considered evidence of an etiologic role for HPV in the development of cervical dysplasia and carcinoma [1–3]. Because the effect of HPV has generally been limited to immortalization, the precise role of the virus in the multistage process of cervical carcinogenesis has been difficult to study. Despite data from transgenic murine systems suggesting that the viral genes, E6 and E7, possess essential activity to effect a fully transformed phenotype [6–10], some have argued that HPV infection is not causative, but either represents an essential cofactor or even an opportunistic pathogen in a host whose immune system is compromised by disease. This area of controversy has led to a considerable effort invested into creating a mouse model with implanted human tissue that could propagate anogenital HPV. Indeed, several SCID mouse systems have been developed to permit isolation and propagation of both low and high oncogenic risk HPV subtypes [12–15]. However, these systems have yet to address the causative role of HPV infection.

Although the site of HPV integration appears to be random with respect to the host genome, there exists a consistent pattern with respect to the site of disruption of the circular viral genome during the process of integration; specifically, the E6 and E7 open reading frames are consistently retained. It has been postulated that the inactivation of p53 and pRb may disrupt control of cellular proliferation and apoptosis, but why this might especially apply to the transformation zone is unknown. Perhaps the recently identified p53 polymorphisms which render the molecule exquisitely susceptible to degradation by HPV 16/18 E6 represent a conditional molecular phenotype that is more prevalent/active at the transformation zone [17]. Alternatively, cellular differentiation along the basal cell layer may provide a window vulnerable to viral manipulation.

II. Establishment of This SCID Mouse Model

While some animal models have involved the engraftment of human tissues into immunocompromised mice, most have used primary human foreskin cultured keratinocytes which may not be susceptible to the specific oncogenic stimuli which act at the cervical squamocolumnar junction [13–15]. As it is at the transformation zone where neoplasia develops, its absence from the keratinocyte models is problematic. In reports where cervical tissue had been successfully implanted below the renal capsule of mice, the implants were devoid of columnar epithelium and not well differentiated [11]. Because *proliferation* is not synonymous with *differentiation,* we proposed a small animal model which contained the human cervical transformation zone and ascertained the viability and morphologies of the implants at several points in time. To be valid, such a model must preserve very specific cellular interactions and molecular events that are particular to this site in human tissue.

Summary of observations: Experiments I–VII. Before neovascularization ensued, the viability of the implants was pred-

icated on diffusion of nutrients. Thus, the implants were prepared in sections ranging from 1 to 3 mm^3 and placed subcutaneously, in proximity to rich capillary beds lying beneath the skin.

Microvessel density based on quantification of CD31 immunostaining has been described for both precancerous and invasive carcinomas of the cervix. Dellas and co-workers observed a significant increase in microvessel counts in dysplastic and invasive lesions compared to normal cervical epithelium [18]. In our model, dual species-specific CD31 immunostaining revealed that the establishment of a blood supply to the implant occurred via anastomotic vascular channels. The origin of the stimulus which initiates angiogenesis, whether it be human or murine, remains unknown.

In addition to supporting a stratified epithelium and maintaining the squamocolumnar junction of normal cervical tissues over time, the model is also one in which dysplastic tissues retain their premalignant phenotype and HPV 16 or 18 DNA. Moll and colleagues described the differential expression of the cytokeratins across the transformation zone of both normal and neoplastic cervical epithelia [19]. Normal endocervical cells (along with ovarian mesothelium, oviduct, and endometrium) expressed the cytokeratin polypeptides 7, 8, 18, and 19. In contrast, tonofilaments of the normal stratified squamous epithelia of the exocervix (and vagina) were observed to contain cytokeratins 4–6, 13–16, and 19. Nonkeratinizing and keratinizing squamous cell carcinomas of the cervix displayed more complex patterns of keratin expression, similar to what we observed in dysplastic implants in which both squamous and columnar epithelia were found to express cytokeratin 8.

Proliferating cell nuclear antigen is a cofactor for DNA polymerase δ and is present in varying amounts throughout the cell cycle of proliferating cells, especially during the late G_1 and S phases. With findings that were consistent with our own results, Mittal and co-workers evaluated normal and abnormal cervical squamous epithelia and observed that PCNA was exclusively expressed in the parabasal and basal layers of the normal ectocervix; in contrast, PCNA expression occurred in higher layers of the epithelium of dysplastic tissues [20]. Additionally, Raju observed that the percentage of PCNA-positive cells was significantly higher in premalignant and malignant lesions of the uterine cervix than in nonneoplastic lesions [21].

Thus, the importance of our model lies in the retention by normal tissues of a stratified epithelium and the squamocolumnar junction, as well as the maintenance by dysplastic implants of premalignant features (including viral DNA and expression of important markers) over time. The opportunity to effect change within the system also exists as is evidenced by both hormonal manipulation and the introduction and expression of an exogenous gene. These attributes constitute many of the essential features of a biologic model through which one may study HPV-mediated human disease.

III. Steroid Hormone Studies

Once our primary objectives had been met and the model well established, we pursued additional lines of investigation as they related to the squamocolumnar junction.

The role of estrogen in the development of cervical dysplasia has been debated [22]. The emergence of widespread oral contraceptive use by women in the 1960s and 1970s was associated with a large increase in the incidence of cervical dysplasia. Early birth control pills contained high-dose estrogens and a possible link between oral contraceptives and the development of cervical dysplasia was initially entertained but soon abandoned when it was observed that oral contraceptive users were at increased risk of HPV exposure due to a lack of concommittent use of barrier methods. More contemporary reports, however, have documented that synergy may exist between steroid hormones and HPV in the pathogenesis of cervical disease; specifically, *in vitro* studies have demonstrated that estrogen can enhance transcription of HPV 16 E6 and E7 oncogenes [23].

In our animal model, the induction of squamous metaplasia and hyperplastic change in normal tissue xenografts via concommittent high-dose estrogen exposure is noteworthy. Interestingly, the observed metaplastic changes of the cervical transformation zone that occur at puberty have been attributed to the effects of high circulating levels of estrogen present during that period.

It is known from past published experiences that estrogen induces proliferation and squamous differentiation of the cervical and vaginal epithelium during the mouse estrous cycles, while progesterone and retinoids maintain the simple columnar epithelium of the endocervix and uterine horns. Celli and co-workers observed induction of retinoid receptors when ovariectomized adult mice were exposed to a single dose of estrogen [24]. The observed estrogen-associated receptor expression pattern suggests that the retinoid receptors are required to coordinate specific genetic programs that result in cervical epithelial growth and differentiation.

Of additional interest is the observation that, despite the presence of estrogen and progesterone receptors, cervical cancer is not considered a hormone-responsive tumor. Nevertheless, the anti-estrogen, tamoxifen, has been reported to induce progesterone receptors and inhibit cell growth in many cervical carcinoma cell lines. Vargas and colleagues evaluated 19 untreated women with invasive cervical cancer for histopathologic changes induced by orally administered tamoxifen [25]. Pre- and posttamoxifen biopsies were retrieved and the investigators observed a statistically significant decrease in the number of mitotic figures in the latter specimens. Their data demonstrate that certain cervical carcinomas may experience a change in proliferation and differentiation levels following tamoxifen administration.

In our model, the predominance of columnar epithelium

among tamoxifen-exposed implants is consistent with the above-cited observations and decidedly confirmatory. In point of fact, via upregulation of progesterone receptors, tamoxifen may indirectly maintain the columnar epithelium of the human reproductive tract. Whether this manifests through properties inherent in the anti-estrogen itself or occurs secondary to the absence of estrogen is unknown. Nevertheless, the therapeutic implications should prompt further study with tamoxifen.

IV. Notch-1 Signaling and Proposal of a Unified Theory

The induction of opposing cellular changes across the cervical epithelium by estrogen and the anti-estrogen, tamoxifen, lends support to the existence of a differential "switch" or putative signaling pathway along the basal layers of the squamocolumnar junction. The presence and description of such a pathway may provide sufficient insight to account for the near-exclusive development of cervical neoplasia at the transformation zone.

The Notch-1 locus encodes a large transmembrane protein and represents an evolutionary conserved cell interaction mechanism that is involved in controlling the progression of immature cells to a more differentiated state. The extracellular domain contains 36 epidermal growth-factor-like repeats [4].

The Notch pathway plays a fundamental role in controlling the fates of undifferentiated, proliferative cell populations and is subjected to various developmental control mechanisms. For example, activation of the Notch-1 receptor via one of its ligands, *Jagged,* may block or delay the progression of immature cells toward a more committed state. Furthermore, inactivating Notch-1 via the inhibiting protein *Numb* leads to the acquisition of an incorrect fate [4]. In *Drosophila,* Notch proteins are highly expressed in the proliferative epithelia of imaginal discs [4]. In contrast, most adult tissues are devoid of Notch expression, with the exception of the ovary and testis, the only sites in adult flies where immature cells may be found. In addition, studies involving embryonic muscle patterning in *Drosophila* have revealed that the asymmetric segregation of *Numb* during division of muscle progenitor cells results in two sister cells with different identities [26]. The daughter cell which inherits *Numb* is committed to becoming a founder embryonic muscle cell by virtue of having a nonfunctioning Notch pathway; the sister in which Notch signaling is active postpones terminal differentiation as an adult muscle precursor. Thus, Notch represents a marker for development, not just proliferation.

Cervical tissues contain squamous and columnar epithelia which are constantly renewed at the level of the transformation zone from precursor cells. These reserve cells often replace the overlying columnar cells with squamous epithelium via squamous metaplasia. Notch-1 signaling in this location represents a binary cell fate decision.

Zagouras and colleagues considered a putative role for Notch at the cervix and observed that Notch-1 expression in normal cervical tissue was restricted to the reserve cells lying fully beneath the differentiated columnar epithelium and to metaplastic areas [5]. *In situ* and invasive squamous cell carcinoma tissues were also found to express high levels of Notch-1. The investigators concluded that Notch-1 activity in the human uterine cervix is consistent with the notion that the pathway is not needed in terminally differentiated tissues, but is associated with proliferative cell populations that are thought to be responsive to developmental signals. Modulation of the Notch pathway may provide a general way to influence the fate of developmentally immature cells [4, 5, 27].

If the Notch locus controls squamous and columnar differentiation at the level of the transformation zone, what controls Notch, and how might HPV oncoproteins affect this process? In an effort to identify changes in proliferative cells as they progress from cervical intraepithelial neoplasia to invasive cervical carcinoma, Daniel and co-workers studied the Notch pathway and the levels of HPV 16 E6–E7 transcripts [27]. Notch-1 proteins were not detected in 7 of 9 mildly dysplastic lesions, but membrane- and cytosolic-localized Notch-1 proteins were discovered in 9 of 12 specimens with severe dysplasia. Furthermore, in all invasive carcinomas evaluated (n = 15), a clear, widespread strong *nuclear* staining of Notch-1 was evident. Finally, the progression from mildly dysplastic lesions to invasive cancer was associated with increases in the levels of HPV 16 E6–E7 transcripts. Syal and colleagues investigated the functional significance of the consistent presence of nuclear forms of Notch-1 in invasive cervical tumors and observed in transformation assays that Notch-1 synergizes with the HPV 16 oncogenes E6 and E7; in cell culture, nuclear Notch-1 and the Notch-1 ligand, Jagged-1, generated resistance to apoptosis on matrix withdrawal [28].

The above results suggest that HPV has evolved to somehow interact with the Notch pathway at a time when cell commitment decisions are being made and that the virus is able to override the normal control over the Notch-1 locus and enforce a persistent pattern of asymmetric signaling that is in direct contradistinction to the wild-type binary pattern. Viral mechanisms of adaptation are very precise and it seems unlikely that the HPV 16/18 E7 oncoprotein's ability to both inactivate pRb and indirectly interact with the Notch-1 locus constitutes *coincidental* viral properties, especially in the setting of cervical cancer where pRb mutation and aberrant Notch-1 expression are demonstrable. Thus it is plausible that the binding of HPV 16 E7 to pRb is critical in the virus' attempt to overcome normal control of the Notch-1 locus. Indeed, inactivation/mutation of pRb appears to be a rite of passage for the vast majority of cancer cells, including those of cervical carcinomas.

How could pRb be involved in regulation of the Notch-1 locus? While a molecular link between pRb and Notch-1 is purely speculative, the results of Daniel *et al.* and Syal *et al.*

[27, 28], would suggest that a significant event occurs in the nucleus in invasive tumors where Notch-1 is present at relatively high levels. Histone deacetylase-1 modulates chromatin architecture through the removal of highly charged acetyl groups from core histones, causing a tighter association between DNA and nucleosomes and thereby impairing the access of transcription factors to DNA-recognition elements. Studies by Brehm *et al.* and Magnaghi-Jaulin *et al.* have established both a physical and a functional link between pRb and HDAC1 [29, 30], the disruption of which is believed to contribute to the road to carcinogenesis.

Transcriptional repression of genes that regulate a cell's irreversible commitment to enter the S phase requires the formation of the pRb–HDAC1 complex. If pRb is mutated/inactivated so that it cannot bind HDAC1, then cellular proliferation genes and possibly even those that determine cell fate choices (i.e., Notch) may be aberrantly expressed. Such speculation is supported by the observed predominance of nuclear Notch-1 in cervical carcinomas. We may invoke p53 involvement by recalling that pRb is a downstream target of p53 through phosphorylation by p21.

Because chromatin regulation is implicated in cell fate control, the pRb/HDAC1 complex would represent a potential target of transforming viruses that alter cell fate. Therefore, if a molecular signaling cascade exists between pRb and the Notch-1 locus through HDAC1, then one may construct a general unified theory or *molecular algorithm* in which elevated HPV 16/18 E6 and E7 expression manifest their oncogenic potential through inactivation and/or degradation of p53 and pRb, respectively, resulting in a stable alteration of cell commitment. One likely consequence would be that the viral genes can act to prevent the apoptosis that normally follows terminal differentiation, thus leading to hyperplasia. The ability of E7 to inhibit terminal commitment is supported by transgenic murine studies in which the expression of HPV 16 E7 in lens epithelial cells at the time of their commitment leads to the failure of the cells to withdraw from the cell cycle and undergo morphological terminal differentiation; this activity of E7 is dependent on its ability to bind pRb [31].

The modulation of p53 and pRb is predicated on the ability of HPV 16/18 to produce adequate levels of E6 and E7 during the period when binary cell fate changes are being made. Under normal viral conditions, the expression of E6 and E7 is inhibited by the early viral gene, E2. However, if the E2 sequences are disrupted by a random genetic event (e.g., viral integration into the host genome), then transcriptional repression of E6 and E7 (which are consistently preserved upon integration) would be ameliorated. A hypothetical algorithm depicting these proposed molecular events culminating in aberrant Notch-1 expression appears in Fig. 12.

Loss of Notch-1 regulation is not likely to be sufficient for the development of dysplastic lesions and/or carcinoma of the cervix. Abolition of apoptosis and initiation of angiogenesis through p53-dependent or p53-independent pathways as well as exposure to estrogen and tobacco and even immune modulation may represent cofactors which are essential to neoplastic transformation.

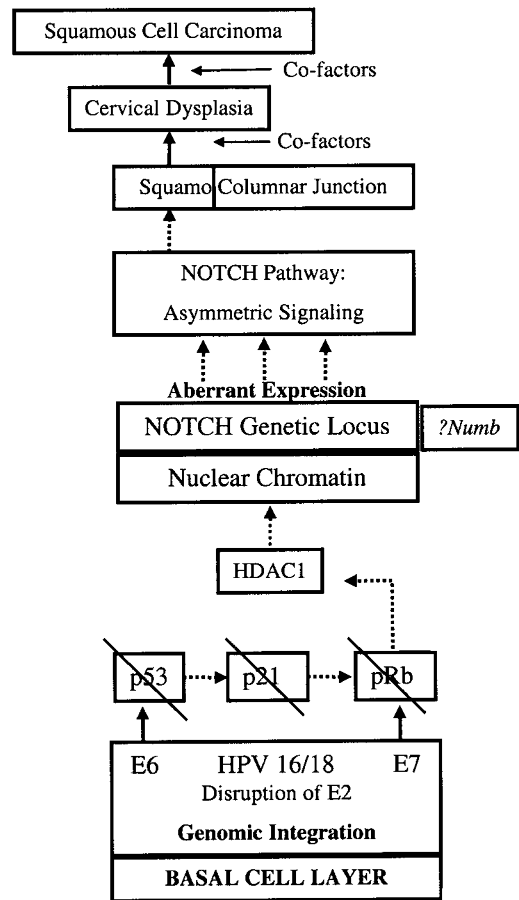

FIG. 12. Aberrant Notch expression (i.e., unregulated transcription) and cervical neoplasia in the presence of integrated oncogenic HPV and additional cofactors.

HPV 16 E2 protein may be essential to maintaining episomal infection in the basal cell layer of the cervix. Daniel *et al.* believe that the presence of detectable episomes in benign cervical lesions implies that integration of the virus correlates with, or is the cause of, progression to invasiveness [27]. In some cases of HPV infection, if the virus were to remain episomal or did not experience disruption of the E2 reading frame upon integration, then women harboring even oncogenic strains would not be destined to develop cervical cancer. This concept through which a nonmalignant cervical epithelium is maintained despite the presence of high-risk HPV infection requires both the appropriate regulation of the Notch-1 pathway and the adequate expression of E2 and low levels of E6 and E7 (Fig. 13).

Because of the limited period of Notch expression and relatively short half-life of wild-type p53 and resultant molec-

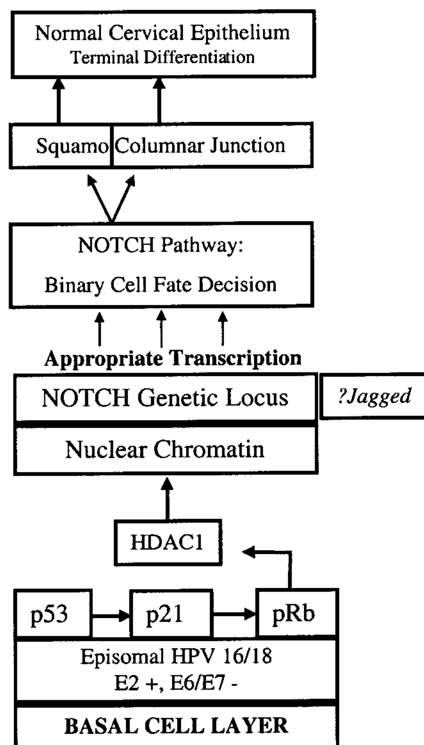

FIG. 13. Appropriate Notch expression (i.e., regulated transcription) and normal cervical epithelium in the presence of episomal oncogenic HPV.

ular instability, a *window of susceptibility* should exist which would limit the attempts to delineate the proposed interactions at the Notch locus. Nevertheless, if this hypothesis is viable, an intact animal model such as ours allows experimental assessment.

V. Further Applications

Of course, the ability to implant invasive cervical cancer tissues into SCID-*beige* mice will permit other important oncogenic phenomena such as tumor angiogenesis, local invasion, and possibly metastasis to be studied *in vivo*. Chemoprevention and pharmacotherapeutics may prove feasible by subjecting mice harboring dysplastic and invasive implants to investigational systemic therapy.

Angiogenesis. It is generally believed that solid tumor growth is angiogenesis dependent [32, 33]. Bremer and coworkers retrospectively evaluated 114 women with Stage IB to IIA cervical cancer and documented a statistically significant correlation between microvessel density and both lymph node metastases and overall survival [33]. The hypothesis that angiogenesis represents an early molecular event may be tested in our model through immunostaining at successive time points for a variety of cellular markers, including vascular endothelial cell growth factor (VEGF), the VEGF receptors, and the angioproteins and their receptors.

Apoptosis. First described in 1972, apoptosis is now known to play a major role in embryogenesis, tissue homeostasis, and neoplasia [34]. The process is one of single cell deletion requiring the active participation of the cell in its own demise (i.e., cellular suicide) [35, 36]. The cytotoxic signal is transmitted by the intracellular death domain of molecules of the TNF-receptor superfamily, the "death receptors" [36]. The apoptosis stimulator, Bax, and the apoptosis inhibitor, Bcl-2, also participate in cellular transformation and cancer growth [34–36]. Death receptors, Bcl-2, and Bax expression represent additional lines of investigation which we are intent on pursuing in this xenograft model. It would be interesting to determine whether we can reestablish terminal differentiation in this system.

Gene therapy. Hamada and co-workers have explored the potential for gene therapy using recombinant adenoviral vectors. These investigators have documented in cell culture the growth inhibition of human cervical cancer cells by recombinant infection of the HPV 16 genome in antisense orientation [37]. They have also observed growth inhibition of human cervical cancer cells as well as induction of apoptosis following infection in cell culture of a recombinant adenovirus carrying wild-type p53 [38, 39]. It should be possible to evaluate these novel recombinant adenoviral vectors using human cervical tissue.

Adoptive immunotherapy. Finally, the existence of a xenograft containing dysplasia or even invasive carcinoma represents only one aspect of a solid tumor system. SCID mice have the unique property whereby they may undergo reconstitution with a human immune system [40, 41]. Thus, we may test the specificity of various HPV 16/18-epitope-directed vaccines. Through intraperitoneal injection of prevaccination and primed human peripheral blood mononuclear cells harvested from women with demonstrable dysplasia, the ability to achieve immunologic rejection of the xenograft may be assessed. "Adoptive immunity" allows for the study of immune surveillance in HPV infection and cancer progression.

CONCLUSION

It is anticipated that further work using this SCID mouse xenograft model will provide additional insight into the molecular mechanisms which underly the foundations of HPV-associated cervical neoplasia. Through the *unification of physics,* theoretical physicists seek to explain the physical universe through one grand unified theory. We also would like to construct a unified carcinogenesis theory, albeit on a much smaller (i.e., molecular) level. Using the cervix as a model for a general theory of carcinogenesis is attractive as cervical cancer represents a global epidemic with established risk factors based on sound epidemiologic data. Furthermore, the cervix is anatomically accessable for study and has a lengthy premalignant disease state that is detectable.

Although an evolutionary argument has not yet been developed, the observed conservation of the Notch family of genes in differentiation strategies among multiple species (e.g., *Drosophila,* mouse, man) should prompt further study of conserved phylogenetic systems involving normal development and neoplasia, with the expectation that the *cen*ancestor, or common ancestor, of primitive and advanced organisms will emerge.

ACKNOWLEDGMENTS

C.C.W.H. and L.P.V. contributed equally to this work. This work was supported by a grant awarded to C.C.W.H. from The Chao Family Comprehensive Cancer Center of the University of California, Irvine–Medical Center, a grant awarded to L.P.V. from the Cancer Research Coordinating Committee of the University of California, and The Center for Viral Vector Design of the University of California, Irvine. The authors express their gratitude to Tony Velazquez, B.S., for technical and computer-assisted support.

REFERENCES

1. Burger RA, Monk BJ, Kurosaki T, Anton-Culver H, Vasilev SA, Berman ML, Wilczynski SP: Human papillomavirus type 18: association with poor prognosis in early stage cervical cancer. J Natl Cancer Inst 88:1361–1368, 1996
2. Lombard I, Vincent-Salomon A, Validire P, Zafrani B, De la Rochefordiere A, Clough K, Favre M, Pouillart P, Sastre-Garau X: Human papillomavirus genotype as a major determinant of the course of cervical cancer. J Clin Oncol 16:2613–2619, 1998
3. Bosch FX, Manos MM, Munos N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, Shah KV: Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. J Natl Cancer Inst 87:796–802, 1995
4. Weinmaster G: The ins and outs of Notch signaling. Mol Cell Neurosci 9:91–102, 1997
5. Zagouras P, Stifani S, Blaumueller CM, Carcangiu ML, Artavanis-Tsakonas S: Alterations in Notch signaling in neoplastic lesions of the human cervix. Proc Natl Acad Sci USA 92:6414–6418, 1995
6. Griep AE, Lambert PF: Role of papillomavirus oncogenes in human cervical cancer: transgenic animal studies. Proc Soc Exp Biol Med 206: 24–34, 1994
7. Gulliver GA, Herber RL, Liem A, Lambert PF: Both conserved region 1 (CR1) and CR2 of the human papillomavirus type 16 E7 oncogene are required for induction of epidermal hyperplasia and tumor formation in transgenic mice. J Virol 71:5905–5914, 1997
8. Smith-McCune K, Zhu YH, Hanahan D, Arbeit J: Cross-species comparison of angiogenesis during the premalignant stages of squamous carcinogenesis in the human cervix and K14-HPV 16 transgenic mice. Cancer Res 57:1294–1300, 1997
9. Song S, Gulliver GA, Lambert PF: Human papillomavirus type 16 E6 and E7 oncogenes abrogate radiation-induced DNA damage responses in vivo through p53-dependent and p53-independent pathways. Proc Natl Acad Sci USA 95:2290–2295, 1998
10. Arbeit JM, Howley PM, Hanahan D: Chronic estrogen-induced cervical and vaginal squamous carcinogenesis in human papillomavirus type 16 transgenic mice. Proc Natl Acad Sci USA 93:2930–2935, 1996
11. Kreider JW, Howett MK, Wolfe SA, Bartlett GL, Zaino RJ, Sedlacek T, Mortel R: Morphological transformation in vivo of human uterine cervix with papillomavirus from condylomata acuminata. Nature 317:639–641, 1985
12. Renz JF, Lin Z, de Roos M, Dalal AA, Ascher NL: SCID mouse as a model for transplantation studies. J Surg Res 65:34–41, 1996
13. Bonnez W: Murine models of human papillomavirus-infected human xenografts. Papillomavirus Rep 9:27–38, 1998
14. Brandsma JL, Brownstein DG, Xiao W, Longley BJ: Papilloma formation in human foreskin xenografts after inoculation of human papillomavirus type 16 DNA. J Virol 69:2716–2721, 1995
15. Bonnez W, Darin C, Borkhuis C, De Mesy Jensen K, Reichman RC, Rose RC: Isolation and propagation of human papillomavirus type 16 in human xenografts implanted in the severe combined immunodeficiency mouse. J Virol 72:5256–5261, 1998
16. Taylor JA, Tewari K, Liao SY, Hughes CCW, Villarreal LP: Immunohistochemical analysis, human papillomavirus DNA detection, hormonal manipulation, and exogenous gene expression of normal and dysplastic human cervical epithelium in severe combined immunodeficiency mice. J Virol 73:5144–5148, 1999
17. Storey A, Thomas M, Kalita A, Harwood C, Gardiol D, Mantovani F, Breuer J, Leigh IM, Matlashewski G, Banks L: Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. Nature 393:229–234, 1998
18. Dellas A, Moch H, Schultheiss E, Feichter G, Almendral AC, Gudat F, Torhorst J: Angiogenesis in cervical neoplasia: microvessel quantitation in precancerous lesions and invasive carcinomas with clinicopathological correlations. Gynecol Oncol 67:27–33, 1997
19. Moll R, Levy R, Czernobilsky B, Hohlweg-Majert P, Dallenbach-Hellweg G, Franke WW: Cytokeratins of normal epithelia and some neoplasms of the female genital tract. Lab Invest 49:599–610, 1983
20. Mittal KR, Demopoulos RI, Goswami S: Proliferating cell nuclear antigen (cyclin) expression in normal and abnormal cervical squamous epithelia. Am J Surg Pathol 17:117–122, 1993
21. Raju GC: Expression of proliferating cell nuclear antigen in cervical neoplasia. Int J Gynecol Pathol 13:337–341, 1994
22. Piper JM: Oral contraceptives and cervical cancer. Gynecol Oncol 22:1–14, 1985
23. Khare S, Pater MM, Tang SC, Pater A: Effect of glucocorticoid hormones on viral gene expression, growth, and dysplastic differentiation in HPV 16-immortalized ectocervical cells. Exp Cell Res 232:353–360, 1997
24. Celli G, Darwiche N, De Luca LM: Estrogen induces retinoid receptor expression in mouse cervical epithelia. Exp Cell Res 226:273–282, 1996
25. Vargas Roig LM, Lotfi H, Olcese JE, Lo Castro G, Ciocca DR: Effects of short-term tamoxifen administration in patients with invasive cervical carcinoma. Anticancer Res 13:2457–2463, 1993
26. Ruiz-Gomez M, Bate M: Segregation of myogenic lineages in Drosophila requires Numb. Development 124:4857–4866, 1997
27. Daniel B, Rangarajan A, Mukherjee G, Vallikad E, Krishna S: The link between integration and expression of human papillomavirus type 16 genomes and cellular changes in the evolution of cervical intraepithelial neoplastic lesions. J Gen Virol 78:1095–1101, 1997
28. Syal R, Sarin A, Krishna S, Rangarajan A: Notch-1 synergises with HPV oncogenes and intersects the ras pathway in epithelial tumorigenesis. Abstract presentation, The International Centre for Genetic Engineering and Biotechnology and Program in Animal Virology, 1998 Virology Symposium, New Delphi, India
29. Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T: Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature 391:597–601, 1998
30. Magnaghi-Jaulin L, Groisman R, Naguibneva I, Robin P, Lorain S, Le Villain JP, Troalen F, Trouche D, Harel-Bellan A: Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature 391: 601–605, 1998

31. Griep AE, Foley JD, McCaffrey J, Nguyen M, Stolen C: Modulation of apoptosis in the mouse lens by HPV 16 E6 and E7. Abstract presentation, The Imperial Cancer Research Fund, 1999 Tumor Virus Meeting on Papoviruses, Papillomaviruses and Adenoviruses, Cambridge, England

32. Rawazoula P, Zolota V, Hatjicondi O, Sakellaropoulos G, Kourounis G, Maragoudakis ME: Assessment of angiogenesis in human cervical lesions. Anticancer Res 16:3861–3864, 1996

33. Bremer GL, Tiebosch AT, Van Der Putten HW, Schouten HJ, De Haan J, Arends JW: Tumor angiogenesis: an independent prognostic parameter in cervical cancer. Am J Obstet Gynecol 174:126–131, 1996

34. Sheets EE, Yeh J: The role of apoptosis in gynaecological malignancies. Ann Med 29:121–126, 1997

35. Kokawa K, Shikone T, Otani T, Nakano R: Apoptosis and the expression of Bax and Bcl-2 in squamous cell carcinoma and adenocarcinoma of the uterine cervix. Cancer 85:1799–1809, 1999

36. Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME: Apoptosis signaling by death receptors. Eur J Biochem 254:439–459, 1998

37. Hamada K, Sakaue M, Alemany R, Zhang WW, Horio Y, Roth JA, Mitchell MF: Adenovirus-mediated transfer of HPV 16 E6/E7 antisense RNA to human cervical cancer cells. Gynecol Oncol 63:219–227, 1996

38. Hamada K, Zhang WW, Alemany R, Wolf J, Roth JA, Mitchell MF: Growth inhibition of human cervical cancer cells with the recombinant adenovirus p53 *in vitro.* Gynecol Oncol 60:373–379, 1996

39. Hamada K, Alemany R, Zhang WW, Hittelman WN, Lotan R, Roth JA, Mitchell MF: Adenovirus-mediated transfer of a wild-type p53 gene and induction of apoptosis in cervical cancer. Cancer Res 56:3047–3054, 1996

40. Mosier DE, Gulizia RJ, Baird SM, Wilson DB: Transfer of a functional human immune system to mice with severe combined immunodeficiency. Nature 335:256–259, 1988

41. Murray AG, Petzelbauer P, Hughes CCW, Costa J, Askenase P, Pober JS: Human T-cell-mediated destruction of allogeneic dermal microvessels in a severe combined immunodeficient mouse. Proc Natl Acad Sci USA 91:9146–9150, 1994