AN IN VITRO STUDY OF ONCOGENESIS OF PRIMARY CERVICAL CELLS BY HUMAN PAPILLOMAVIRUS 16 AND CIGARETTE SMOKE CONDENSATE

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BY

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ABSTRACT

Previous epidemiological studies indicated that cigarette smoking females are at increased risk of developing cervical cancer. However, because of the confounding variables of these studies, and since other studies have given conflicting results, convincing evidence is lacking. Furthermore, the molecular role of cofactors, such as hormones, in the multistage carcinogenic process is still unclear.

To examine the role of human papillomavirus (HPV) and cigarette smoking in the carcinogenesis of cervical cancer, I studied two HPV16 immortalized endocervical cell lines previously established in this lab, HEN-16 and HEN-16-2. Both lines were treated with cigarette smoke condensate (CSC) at 75, 100, 125 μg/ml. After 6-12 months' treatment with CSC, 10^7 cells of treated and untreated immortalized cells with equal passages were injected into female nude mice. Four to six weeks later, only CSC-treated cells but not CSC-untreated immortalized cells formed palpable tumors, which were subsequently used to derive tumor cell lines, HEN-16T and HEN-16-2T.

Further characterization of the immortalized cell lines and CSC-transformed cell lines indicated that the CSC-treated

tumorigenic cells a) displayed distinct morphologies in monolayer and organotypic (raft) cultures; b) proliferated faster in DMEM, a medium containing physiological calcium levels; c) showed *in vitro* anchorage-independency; d) contained and expressed similar levels of HPV16; e) expressed similar levels of some cellular genes associated with enhanced malignancy, c-*myc*, H-*ras*, p53, WAF1, GADD45, GADD153, and fibronectin, compared with immortalized cells. However, f) HEN-16T and HEN-16-2T expressed higher levels of B-*myb* and PCNA; and g) obtained enhanced resistance to growth inhibition by tansforming growth factor-β1 (TGF-β1) and retinoic acid (RA). On the other hand, the two immortalized cell lines displayed higher levels of p53, B-*myb*, PCNA and lower levels of Waf1 and fibronectin, compared with primary endocervical cells.

In addition, two novel cDNAs, designated PA4 and PA9, which were specifically expressed at higher levels in immortalized cells and primary endocervical cells, respectively, were identified and cloned using differential display assays. Further characterization of these two novel cDNAs will be very helpful for us to fully understand the molecular pathogenesis of cervical cancer.

In summary, this study provided the first *in vitro*

evidence that cigarette smoke can enhance the risk of developing cervical cancer in HPV-infected patients. Furthermore, the *in vitro* system will be very useful to investigate the mechanism of multistep carcinogenesis of cervical cancer.

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I would also like to thank my other committee members, Dr. Laura Gillespie and Dr. Martin Mulligan, for their expertise, invaluable comments and enthusiastic support throughout this program.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
AP1	Activator protein 1
ATL	Adult T cell leukemia
BCIP	4-bromo-5-chloro-3-indolylphosphate
BL	Burkitt's lymphoma

bp	base pairs
BRL	Bethesda Research Laboratories
BSA	Bovine serum albumin
^{0}C	degree Celsius
CAT	Chloramphenicol acetyl transferase
cdk	cyclin dependent kinase
cDNA	Complementary DNA
CIN	Cervical intraepithelial neoplasia
Cip1	Cdk-interacting protein 1
CMV	Cytomegalovirus
CPM	Counts per minute
CSC	Cigarette smoke condensate
DCC	Deletion in colon cancer
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonulease I
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EBV	Epstein-Barr virus
FCS	Fetal calf serum
FITC	Fluorescein isothiocynate
FN	Fibronectin
EGF	Epidermal growth factor

GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
KBM	Keratinocyte basic medium
KGM	Keratinocyte growth medium
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HEN	Human endocervical cells
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSC	Herpes simplex virus
HTLV	Human T-cell leukemia virus
INF	Interferon
IL	Interleukin
Kb	Kilobase
kDa	Kilodalton
LCR	Long control region
mRNA	Messenger RNA
MSEE	Methanesulfonic acid ethyl ester
NCR	Noncoding region
NBT	Nitroblue tetrazolium
NF-IL6	Nuclear factor for interleukin 6
NMU	Nitrosomethylurea
NPC	Nasopharyngeal carcinoma
ORF	Open reading frame

PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
RA	Retinoic acid
RACE	Rapid amplification of cDNA ends
Rb	Retinoblastoma gene
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Revolutions per minutes
RT	Reverse transcriptase
Sid1	Senescent cell-derived inhibitor 1
SCC	Squamous cell carcinoma
SV40	Simian virus 40
TEMED	Tetra-methylethylenediamine
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TPA	12-o-tetradecanoylphorbol-13-acetate
URR	Upstream regulator region
UV	Ultraviolet
Waf1	Wild-type p53 activated-fragment 1

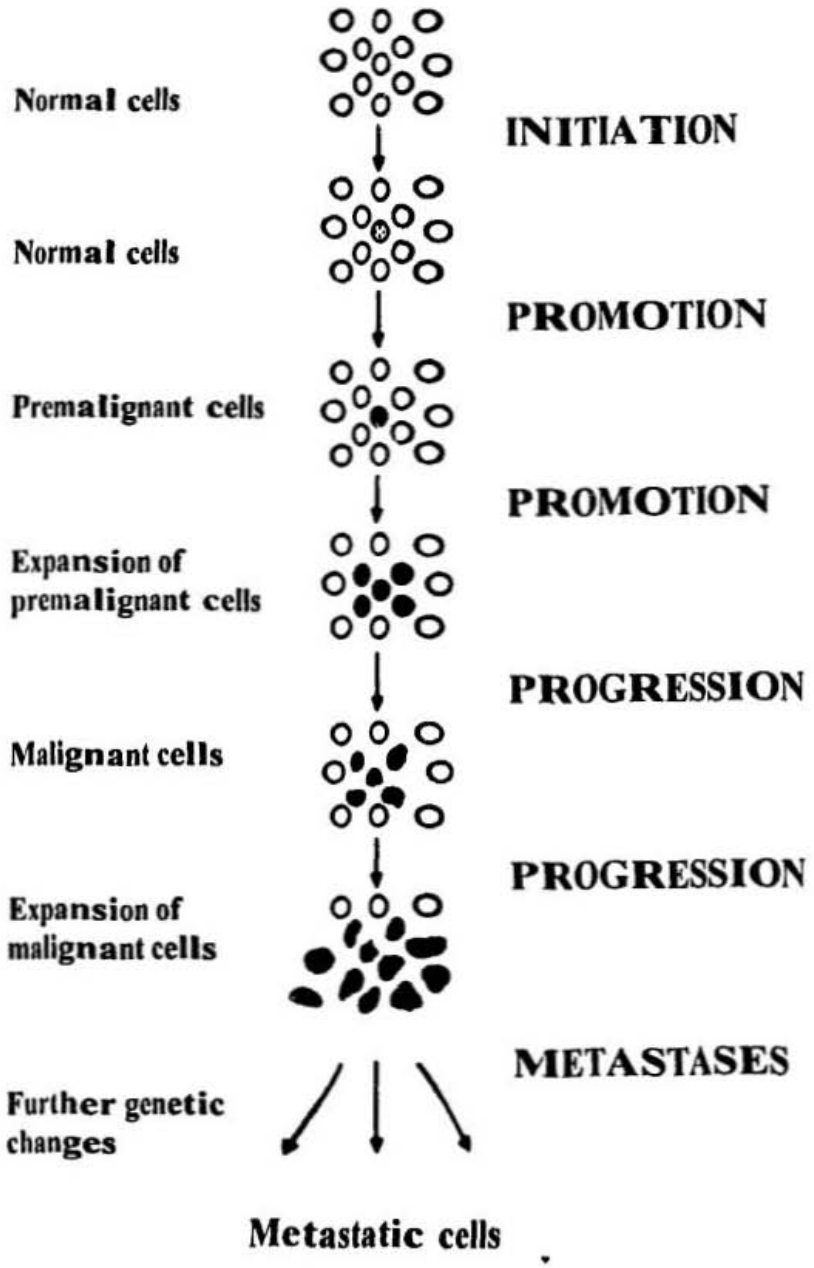
CHAPTER 1

INTRODUCTION

1.1 Multistage carcinogenesis

In 1954, Armitage and Doll studied the age and incidence of 17 common types of cancers and concluded that carcinogenesis was at least a six or seven stage process (Armitage and Doll, 1954). This lead to the concept of multistage carcinogenesis of cancers. Since then, this concept has been proven clearly and improved significantly by epidemiological and molecular genetic studies. As shown in Figure 1, multistage carcinogenesis can be divided into four steps: initiation, promotion, malignant progression and metastasis. However, in some cases, not all of these steps can be identified clearly. The initiation step is believed to be caused usually by genotoxic agents, especially physical factors, such as ultraviolet (UV) light and irradiation, chemical carcinogens and viruses, which cause genetic changes in a single cell. After initiation, the cell still may have the normal phenotype. However, when the cell becomes exposed to some tumor promoting reagents and experiences further genetic alterations, such as mutation, activation or inactivation of oncogenes and tumor suppressor genes, a single cell acquires some heritable form of growth advantage and

Figure 1 Multistage carcinogenesis of cancer. The symbols represent: ○, normal cells; ⊗, normal cells with genetic changes; ●, premalignant cells; ◕, malignant cells. See the text for details.


Normal cells
INITIATION
Normal cells
PROMOTION
Premalignant cells
PROMOTION
Expansion of premalignant cells
PROGRESSION
Malignant cells
PROGRESSION
Expansion of malignant cells
METASTASES
Further genetic changes
Metastatic cells

expands to form a clonal malignant tumor. Finally, additional changes allow the outgrowth of clones with metastatic potential to other tissues. Here, I will discuss how individual factors function in the multistage carcinogenesis of human cancer.

1.1.1 Physical factors

UV light and ionizing radiation are recognized as the two most important physical causative factors in the development of cancer (Adam and Cox, 1991).

UV light has wavelengths between 200 nm and 400 nm and is composed of UV-A (200-280 nm), UV-B (280-320 nm) and UV-C (320-400 nm), each having different effects on the skin and eye. Generally, the shorter the wavelength, the more destructive the radiation. Thus, UV-A has the most damaging effects on human cells. Since almost all the UV-A is absorbed by the oxygen and ozone of the atmosphere, it is a minor factor. However, a considerable amount of UV-B penetrates the atmosphere, especially in the Arctic and Antarctic regions where a severe seasonal depletion of ozone layer is observed. Since the spectrum of UV-B is very close to the absorption spectrum of DNA, it can be easily absorbed by DNA molecules and cause damage to DNA. The damaged DNA is normally repaired by the DNA repair system in the cells. However, when

the repair system is also impaired, UV-B may cause further genetic changes and, finally, induce cancer. UV light is believed to be one of the major causes of malignant melanoma and other forms of skin cancers (Stern and Lang, 1988).

Ionizing radiation consists of electromagnetic radiation, such as X- and γ-rays, and subatomic particles, such as alpha particles, electrons, neutrons, and protons. Epidemiological studies indicate that ionizing radiation is believed to be one of the causative factors for the development of leukemia and skin, bone, breast, lung, thyroid, stomach, colon, bladder, esophagus, and liver cancer (BEIR IV, 1988). Furthermore, cellular and molecular biological studies have confirmed that ionizing radiation can be involved in the initiation and promotion process of multistage carcinogenesis of cancer by causing cell inactivation, chromosome damage and mutation of genes, such as *ras*, directly or by interacting with water to form hydroxyl radicals that can damage DNA (Adam and Cox, 1991).

1.1.2 Chemical factors

Epidemiological and animal toxicological studies have led to the general acceptance that chemical carcinogens are one of

Table 1. Chemical carcinogens associated with human cancers*

Chemicals	Cancers/sites
Alcohol	Oral cavity, pharynx, larynx, esophagus, liver
Aflatoxin	Liver
Alkylating agents	Leukemia
Arsenic & its compounds	Skin, lung, liver angiosarcoma periungular
Betal quid	Oral cavity
Chlorambucil	Leukemia
Cyclophosphamide	Bladder, leukemia
Diethystilboestrol	Cervix/vagina, breast, testis
Estrogens, steroidal	Endometrium, breast
Melphalan	Leukemia
Nickel & its compounds	Nasal sinus, lung
Nitrites	Colon
Progestin	Cervix
Tobacco smoke	Lung, bladder, oral cavity, larynx, pharynx, esophagus, pancreas, renal pelvis
Vinyl chloride	Angiosarcoma, lung

*Adapted partially from IARC, 1987.

the most important causative factors for the development of human cancer. Table 1 lists some of the chemical carcinogens that can cause human cancers.

From the Table 1 we notice that a specific chemical causes only certain types of tumors. For example, arsenic and its compounds can induce skin, lung and liver cancer; however, chlorambucil only causes leukemia. The mechanism for this carcinogen-specific is unknown.

One of the most convincing pieces of evidence that implicate chemical carcinogens in multistage carcinogenesis came from studies on the formation of mouse skin tumors (Berenblum and Shubik, 1949; Balmain and Brown, 1988). In these experiments, a single chemical carcinogen, such as MNNG or NMU, was first applied to the skin of mouse. This resulted in the initiation of an unknown number of cells which would persist for a very long time without showing any apparent changes if they were left without further treatment. However, if a second class of chemical agent, a tumor promoter, such as tetradecanoyl phorbol 13-acetate (TPA), was applied at the same time or even a year later, benign tumors called papillomas appeared. A small portion of these papillomas developed into fully malignant tumors with or without further applications of tumor promoters. Further molecular biological studies indicated that the tumors were derived from the clonal

expansion of cells bearing the *ras* proto-oncogene which had been activated by mutation after treatment with the first chemical carcinogen (Balmin and Brown, 1988). Therefore, chemical carcinogens are believed to be involved in the initiation and promotion of cancers by activating proto-oncogenes or inactivating tumor suppressor genes.

1.1.3 Viral factors

Viruses have been implicated in the etiology of up to 20% of all cancers in the world (Vousden and Farrel, 1994). Thus, studying the relationship between viruses and human cancers has become one of the most extensively studied areas in cancer research.

Until now, only five viruses, including three DNA viruses, Epstein-Barr virus (EBV), hepatitis B virus (HBV), human papillomavirus (HPV) and two RNA viruses, human T-cell leukemia virus I (HTLV I) and human immunodeficiency virus (HIV) are well known to be the factors associated with several human cancers.

EBV is a member of human herpesvirus family, which infects about 90% of the world population (Keiff and Liebowiz, 1990). Previous studies indicate that EBV may be the causative agent of two human neoplasms, Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) (Keiff and Liebowiz, 1990). The

epidemiological association of EBV with BL was further supported by the ability of EBV to immortalize human lymphocytes in culture (Hammerschmidt and Sugden, 1989). However, until now, the molecular mechanism for EBV in the etiology of BL and NPC is still not clearly defined due to the large 180 kbp size of the EBV genome and the lack of cell culture systems for virus propagation (Vousden and Farrel, 1994).

HPVs are a large group of epitheliotropic papovaviruses which infect a wide variety of cutaneous and mucosal epithelia including those in the skin, mouth, cervix, vulva, larynx and respiratory tract (Howley, 1991). Of the 70 types of HPVs that have been identified so far, most are related to benign genital or skin warts. Only a few of them, especially HPV16 and 18, are involved in the development of human cancers, such as cervical, anal and laryngeal carcinomas (de Villiers,1994). The association of HPVs with cervical cancer, the second most common female malignancy in the world, has been extensively studied (for recent reviews see zur Hausen, 1994; zur Hausen and de Villiers, 1994a,b). This will be discussed in more detail in section 1.2 and 1.3.

Hepatitis B virus (HBV) is a 3.2 kb partially double stranded and partially single stranded circular DNA virus. It

is a member of the hepatitis viruses and infects at least 300 million people in the world (Beasley and Hwang, 1991). Epidemiological studies indicate that HBV is a major risk factor for the development of hepatocellular carcinoma (HCC) (Beasley et al., 1981). Despite the close association between HBV incidence and hepatocellular carcinoma, the exact mechanisms by which HBV contributes to malignant progression remain to be elucidated. It has been reported that HBV can integrate into the human DNA genome and persist for life. This integration is thought to allow HBV to escape the immune response and initiate the subsequent development of HCC (Beasley et al.,1981). In addition, integration of HBV near some genes, such as *c-myc*, cyclin A or retinoic acid receptor β may cause the deregulation of the genes, which is related to the multistage carcinogenesis of HCC (Nagaya et al., 1987). Furthermore, HBV X protein can interact with the p53 tumor suppressor gene and inactivate the transactivating function of the gene (Feitelson et al., 1993). All of the above observations indicate that HBV may initiate the carcinogenesis of hepatocellular carcinoma by interacting with some cellular gene products.

HTLV-1 is a retrovirus which infects from 0.025% (in the U.S.A.) to 30% (in Japan), of the human population of

different countries during their life time (Cann and Chen, 1990). Epidemiological studies link this retrovirus to a type of cancer called adult T cell leukemia (ATL). Since only 2% of the people who are infected by HTLV-I develop ATL and HTLV-I can only immortalize peripheral blood lymphocytes, HTLV-I has been only regarded as the initiating agent in the carcinogenesis of ATL (Hollsberg and Hafler, 1993). Other changes such as chromosomal rearrangements or somatic mutations are necessary for the full transformation of primary lymphocytes (Hollsber and Hafler, 1993).

HIV is another retrovirus and is linked to acquired immunodeficiency syndrome (AIDS) (Gallo and Montagnier, 1988). The hallmark of AIDS is the gradual loss of T4 cell lymphocytes and the subsequent development of an immunodeficient state. The absence of T cells leads to the expansion of polyclonal B cell populations and, finally, the formation of lymphomas. Molecular genetics studies have shown that rearrangement and activation of the c-*myc* locus is one of the major pathomechanisms in the development of lymphomas (Knowles et al., 1988). Benign hyperplastic lymph nodes obtained from patients infected by AIDS contained an intact c-*myc* gene locus and oligoclonal IgH, whereas frequent monoclonal c-*myc* gene rearrangement and oligoclonal IgH gene rearrangement have been observed in lymphoid neoplasms

(Knowles et al., 1988), suggesting that infection of HIV is only an initiating agent in the multistep process in AIDS patients and that the alteration of other cellular genes such as c-*myc* may also contribute to this process. In addition, a higher incidence of several types of tumors, such as rectal, cervical, oral, testicular cancers, has also been described in AIDS patients, suggesting the possible role of this virus in the oncogenesis of these cancers due to immunosuppression (Brau, 1994).

In summary, all these DNA viruses and RNA viruses can be regarded to be necessary but not sufficient agents in the multistep carcinogenesis of cancers. Other changes are critical in tumor progression and metastasis.

1.1.4 Cellular factors

Molecular genetic studies indicate that development of cancer is largely a consequence of abnormal expression or function of specific cellular genes (Solomon et al., 1991). Specific cellular genes are either activated or inactivated in the process of multistage carcinogenesis. Genes whose activation or abnormal function of the gene product can induce neoplastic transformation of nonmalignant cells are called oncogenes. On the contrary, genes for which inactivation of the gene product can induce neoplastic transformation of

benign cells are called tumor suppressor genes.

Until now, at least 50 different oncogenes have been identified that induce changes necessary for malignant cell growth. Table 2 lists oncogenes amplified in some of the most common tumors.

A certain kind of cancer is usually caused by deregulation of several of the oncogenes in this table, suggesting that multiple genetic alterations are required for or involved in the multistage carcinogenesis of the cancer.

While oncogenes previously were regarded as the major cellular factor in human cancers, with the development of molecular genetics within the past 10 years, an increasing number of reports have shown alterations in tumor suppressor genes (Solomon et al., 1991). Thus, it is now believed that mutation and deletion of chromosomes for tumor suppressor genes plays a central role in the genesis and progression of human cancers (Weinberg, 1989; Levin, 1993). Table 3 shows the correlation between human cancers and deletion of chromosomes or tumor suppressor genes.

Comparing Table 2 with Table 3, one basically can perceive that both oncogenes and tumor suppressor genes may be involved in a specific type of cancer. For example, in colon cancer, both the activation of *erb*B1, L-*myc*, N-*myc*, H-*ras* and

Table 2: Oncogenes amplified in human cancers*

Cancer	Oncogenes
Bladder	H-*ras*, K-*ras*, *int*-2
Brain	*erbB*1, *sis*
Breast	*erbB*2, H-*ras*, c-*myc*, *int*-2
Cervical	c-*myc*, H-*ras*, *erbB*2
Colorectal	H-*ras*, K-*ras*, c-*myb*, c-*myc*
Gastric	*erbB*1, *hst*, c-*myb*, c-*myc*, N-*ras*, *yes*
Lung	*erbB*1, c-*myc*, L-*myc*, N-*myc*,H-*ras*, K-*ras*
Melanoma	H-*ras*
Neuroblastoma	c-*myc*, N-*myc*
Ovarian	*erbB*2, K-*ras*
Pancreas	K-*ras*, c-*myc*
Prostate	c-*myc*
Stomach	c-*myc*, *int*-2, *hst*
Testicular	c-*myc*

* Cole and Kelekar, 1987; Cooper, 1990; Davies ant Vousden, 1993; Mitra et al., 1994.

Table 3. Deletion of chromosomes and loss of human tumor suppressor genes in human cancers*

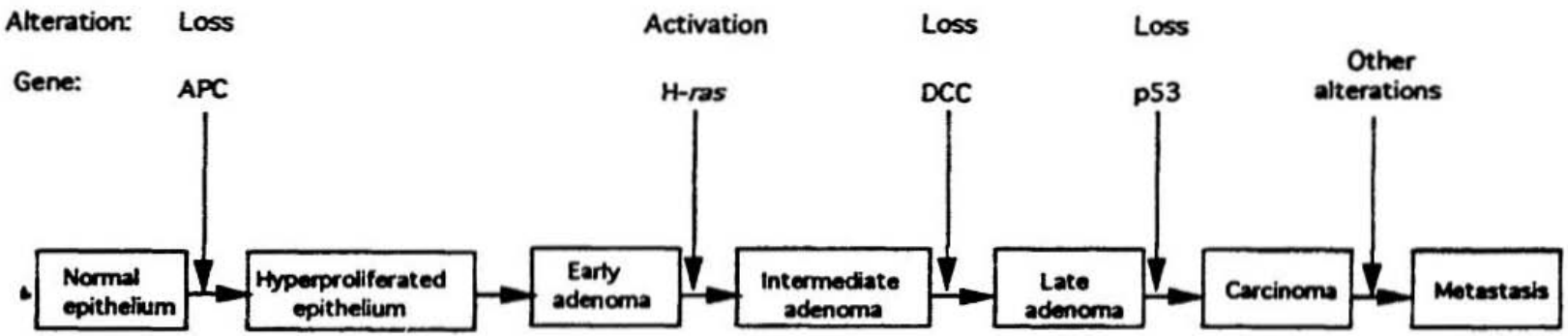
Tumor/site	Chromosome deletion	Loss of tumor suppressor gene
Bladder	13q14	Rb
	17p13	p53
Breast	5q21	APC
	13q14	Rb
	17p13	p53
	17	BRCA1
	18q11	DCC
Cervix	3p,11q,17q	?
Colon	5q21	APC
	17p13	p53
	18q11	DCC
Liver	4,16q	?
Lung	3p25	VHL
	5q21	APC
	13q14	Rb
	17p13	p53
Melanoma	9p21	MTS1
	1p,6q, 9q	?
Neuroblastoma	1p, 14q	?
Neurofibromatosis	17q11	NF1
	22q12	NF2
Osteosarcoma	13q14	Rb
	17p13	p53
Prostate	11p, 16q	?
Stomach	1q, 12q	?
Wilms	11p13	WT1
	11p15	H19

*Weinberg, 1989; Solomon et al., 1991; Levin, 1993; Yokoya et al., 1993.

K-*ras* oncogenes and the inactivation of APC, Rb, p53 and VHL tumor suppressor genes were found. However, how are these oncogenes and tumor suppressor genes involved in the oncogenesis of a certain cancer? Is their function altered simultaneously or subsequently during the process of carcinogenesis? Studies on the multistep carcinogenesis of colon cancer have shed some light on this questions.

Figure 2 shows the genetic changes occurring during evolution of a typical colon carcinoma (Fearon and Volgelstein, 1990). While the tumor suppressor gene, APC, was inactivated by somatic mutations at the earliest stages of colon carcinogenesis, H-*ras* activation and DCC loss were only found in most of the late adenomas. Inactivation of p53 is detected only in the full carcinoma (Figure 2). This model clearly illustrates that alterations of oncogenes and tumor suppressor genes may occur at different stages of carcinogenesis. However, there are also some examples of activation of certain oncogenes and inactivation of certain tumor suppressor genes occurring at the same stage of carcinogenesis (McDonald and Ford, 1991). Thus, cooperation between oncogenes, such as H-*ras* and c-*myc*, or between oncogenes and tumor suppressor genes, such as inactivation of Rb and activation of c-*myc*, may also be necessary for the multistep carcinogenesis of cancer.

Figure 2 Alterations associated with multistage carcinogenesis of colon cancer. Activation of specific oncogenes and inactivation of specific tumor suppressor genes are statistically associated with 4 of the 6 steps in tumor progression (Fearons and Volgelstein, 1990).


Alteration:
Loss
Activation
Loss
Loss
Other alterations
Gene:
APC
H-ras
DCC
p53
Normal epithelium
Hyperproliferated epithelium
Early adenoma
Intermediate adenoma
Late adenoma
Carcinoma
Metastasis

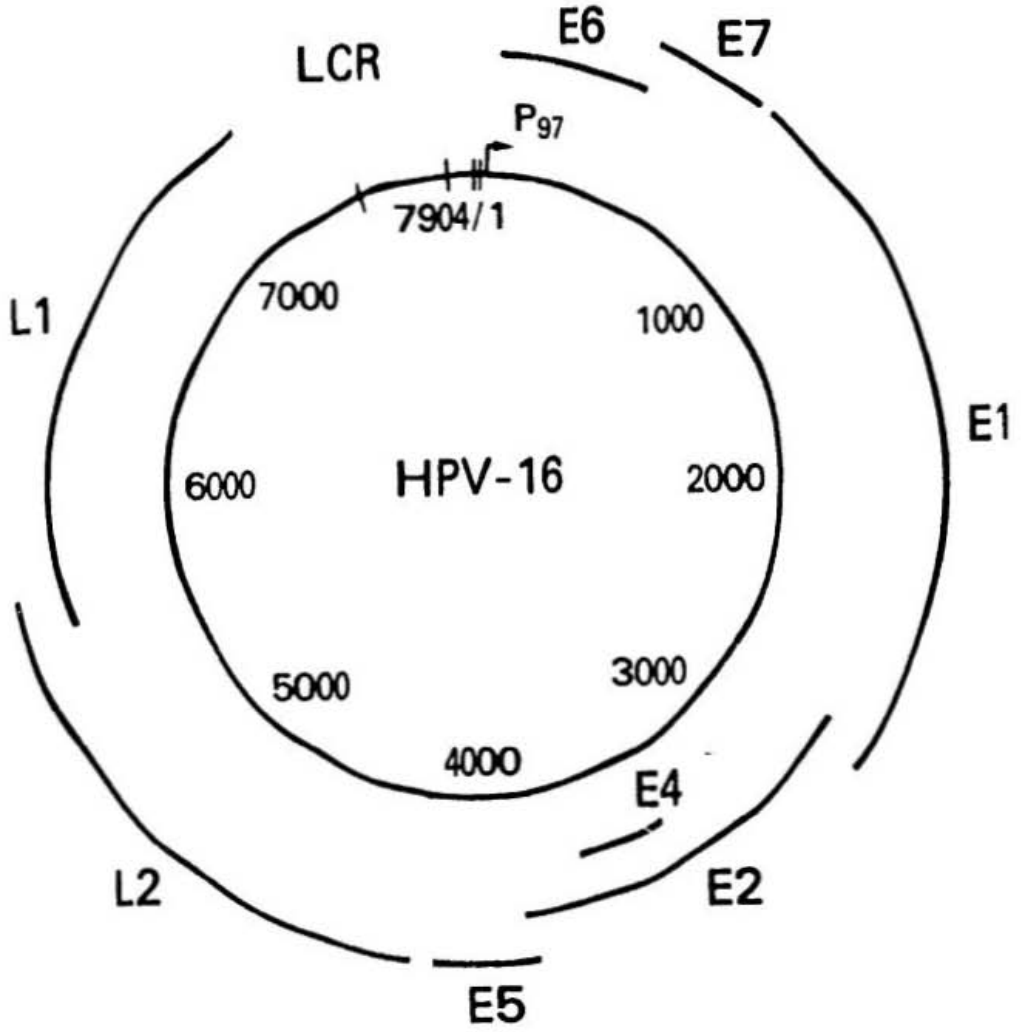
1.2. General features of HPVs

Human papillomaviruses are small, naked, icosahedral double-stranded circular DNA of about 8,000 bps. They belong to the sub-family papillovirinae (Howley, 1991).

Until now, approximately 70 types of HPVs have been identified based on a comparison of the DNA sequences of E6, E7, and L1 open reading frames (ORFs). Over 30 types are associated with anogenital lesions (de Villiers, 1994). These HPVs can be further classified as either high risk, such as HPV 16, 18, 31, 33, 39 and 52, or low risk, such as HPV 6 and 11, based on the relative tendency of the lesions to progress into genital carcinomas (de Villiers, 1989). Generally, high risk HPVs are frequently found in high grade cervical intraepithelial neoplasia (CIN), which has a higher tendency for malignant conversion. In contrast, low risk HPVs are commonly found in benign genital condylomas and nasal and laryngeal papillomas, which have a lower tendency for malignant conversion (Shah and Howley, 1990).

The 8,000 bp genome of HPVs is generally organized into three distinct regions: early region (E), late region (L), and long control region (LCR) as shown in Figure 3. The early region is composed of 6 ORFs, designated E1, E2, E4, E5, E6, E7, which are involved in viral DNA replication and transcription and cellular transformation and maturation. The

Figure 3. Genomic organization of HPV16 (Adapted from Munger et al., 1992). All papillomaviruses contain a double-stranded circular DNA genome of approximately 8 kb. The major transcriptional promoter for HPV16 is designated P97. Transcription occurs only in a clockwise manner. Nucleotide positions are indicated in the inner circle. The early ORFs deduced from the DNA sequence are designated E1 to E7, and the late ORFs are designated L1 and L2. The viral long control region (LCR) contains DNA elements that regulate HPV transcription and replication by interacting with viral and cellular transcription factors and replication factors. Short vertical lines in the LCR represent the locations of the E2 binding sites, $ACCN_6GGT$. Note that overlap between some of the early ORFs and late ORFs is observed in HPV16 and other HPVs.


LCR
E6
E7
P97
7904/1
7000
1000
L1
E1
6000
HPV-16
2000
5000
3000
4000
E4
L2
E2
E5

late region is composed of two ORFs, designated L1 and L2, which encode the viral capsid proteins. The LCR, also called the noncoding region (NCR) or upstream regulatory region (URR), is found between the 3' end of the late region and 5' end of the early region and provides binding sites for several cellular and viral transcription and replication factors (Howley, 1991; Hoppe-Seyler and Butz, 1994).

The basic features and functions of each ORF and their proteins are listed in Table 4, and they will be further discussed in succeeding sections.

1.3. Molecular oncogenesis of primary cervical cells

1.3.1 An *in vivo* and *in vitro* model of multistage oncogenesis of cervical cells

As stated above, the development of cancer is a multistage process, in which multiple genetic changes are required for the full transformation of normal cells. This multistage nature of cancer also clearly can be observed in the oncogenesis of human cervical cells both *in vivo* and *in vitro*. Figure 4 illustrates a model of the *in vivo* and *in vitro* oncogenesis of cervical cancer. In this model, high risk

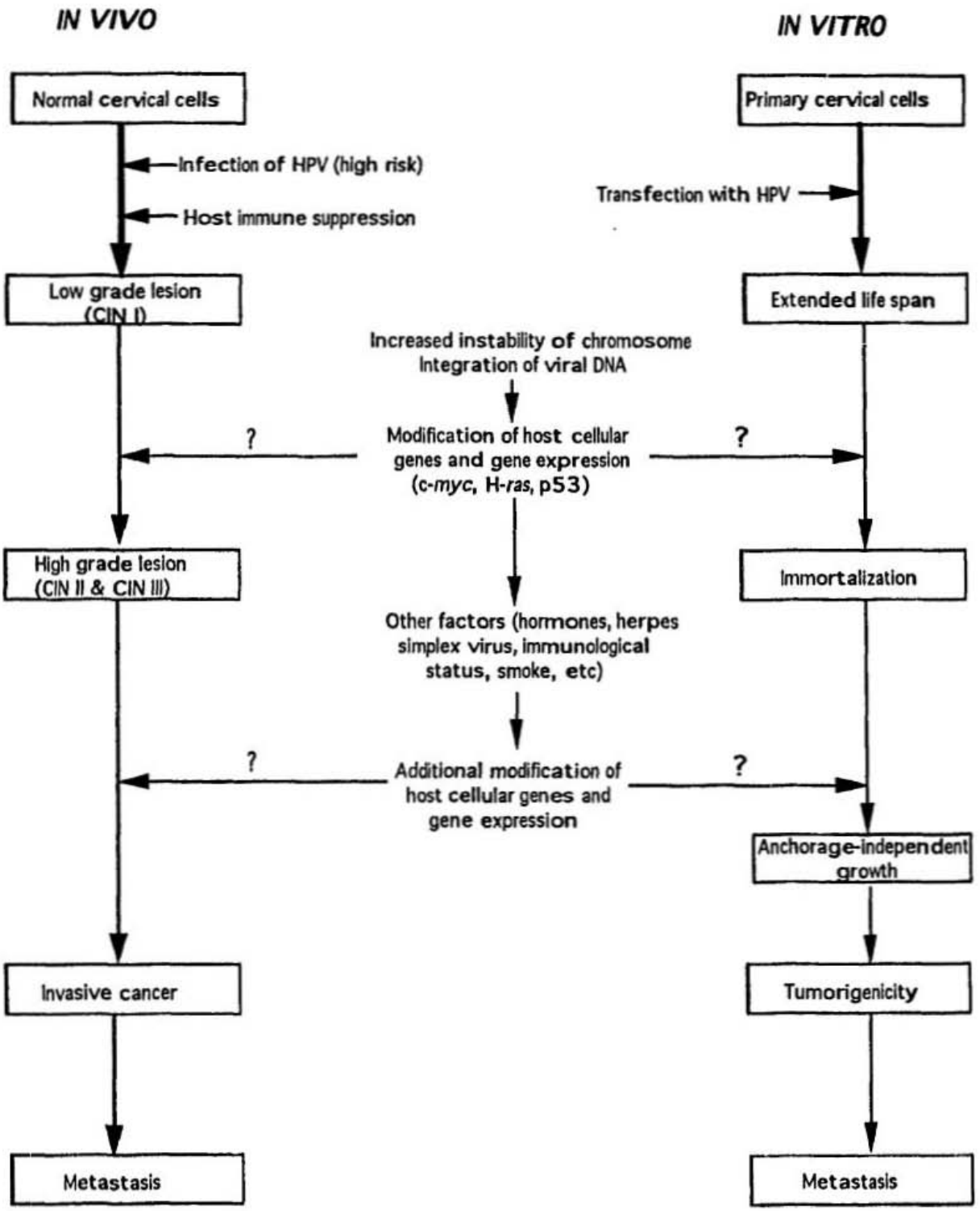
Table 4 Basic features and functions of HPV ORFs and proteins *

ORF	Protein size (KDa)	Features	Functions
E1	68-72	1. Conserved fairly well among HPVs	1. Increase immortalization capacity of HPV genome
		2. Binds to minimal replication origin	2. Required together with E2 for viral replication
		3. Interacts with E2 protein	
		4. Usually can be deleted in HPV-containing carcinomas	
E2	48	1. Contains DNA binding and transactivating domain	1. Transactivate or repress HPV expression
		2. Forms dimers	2. Required together with E1 for viral replication
		3. Interacts with E1 protein	
		4. Usually deleted in HPV-containing carcinomas	
E4	16-20	1. Significant divergence among HPVs	1. Viral maturation
		2. Most abundant protein in HPV-infected cells	2. Collapse of keratin cytoskeleton
		3. Interacts with cytokeratins	
		4. Expressed in differentiating keratinocytes	
E5	10	1. Usually deleted or disrupted in cervical cancers	1. Weak oncogenesis
			2. Enhanced EGF-mediated mitogenic signal transduction
E6	18-19	1. Produces unspliced E6 and E6* and E6**, internally spliced mRNA	1. Required together with E7 for immortalization and transformation
		2. Zn++-binding protein	2. Trans-activation
		3. Interacts with cellular protein such as p53	

ORF	Protein size (KDa)	Features	Functions
E7	21	1. Zn++-binding phosphoprotein	1. Required together with E6 or alone for immortalization and transformation
		2. Interacts with cellular proteins such as pRb	2. Stimulation of DNA synthesis
			3. Induction of chromosome abnormalties
			4. Transactivation of adenovirus E2 promoter
L1	55	1. Most highly conserved ORF among HPVs	1. Major virion capsid protein
		2. Highly glycosylated	
		3.Transcribed and translated only in terminally differentiated keratinocytes	
L2	70	1.Rather poorly conserved among HPVs	1. Minor virion capsid protein
		2.Transcribed and translated only in terminally differentiated keratinocytes	
LCR	-	1. Contains sequences for HPV promoter, enhancer and origin of DNA replication	1. Regulation of HPV mRNA expression and processing
		2. Contains late polyadenylation site	2. Regulation of HPV replication
			3. Dtermination of immortalization/transformation

* Howley, 1988, 1991; zur Hausen, 1991; Munger et al., 1992; Mansur and Androphy, 1993; Scheffner et al., 1994.

Figure 4. ***In vivo*** **and** ***in vitro*** **models of the multistage oncogenesis of cervical cancer.** Each step will be described in detail in the text.


IN VIVO
IN VITRO
Normal cervical cells
Primary cervical cells
Infection of HPV (high risk)
Host immune suppression
Transfection with HPV
Low grade lesion (CIN I)
Extended life span
Increased instability of chromosome
Integration of viral DNA
?
Modification of host cellular genes and gene expression (c-myc, H-ras, p53)
?
High grade lesion (CIN II & CIN III)
Immortalization
Other factors (hormones, herpes simplex virus, immunological status, smoke, etc)
?
Additional modification of host cellular genes and gene expression
?
Anchorage-independent growth
Invasive cancer
Tumorigenicity
Metastasis
Metastasis

HPVs are regarded as the initiating agent for the development of cervical cancer. The presence of HPV is responsible for CIN I low grade lesions *in vivo* and an extended life span in cell culture (*in vitro*). Further modifications of cellular genes result in CIN II and III high grade lesions *in vivo* or immortalization *in vitro*. However, these events are not enough to transform the normal cervical cells into invasive tumors. Other factors, such as hormones and smoking are required to make further changes in other cellular genes to transform some of the high grade neoplasias or immortalized cells to form invasive tumors. I will discuss this process in detail in the next section.

1.3.2 Role of HPV in oncogenesis of cervical cells

Cervical cancer is the second most common cancer among women in the world (Howley, 1988). It was recognized as a sexually transmitted disease more than a century ago and since then numerous infectious agents have been suggested to play a causative role in cervical cancer (for review see zur Hausen and de Villiers, 1994). In 1976, zur Hausen first proposed a hypothesis that links human papillomaviruses to cervical carcinoma. Over the subsequent 20 years, a large body of epidemiological and molecular biological evidence has accumulated to firmly support a role for HPVs in the

development of cervical cancer (for reviews see Howley, 1988; zur Hausen, 1991,1994; zur Hausen and de Villiers, 1994a,b; Garland et al., 1992; Davies and Vousden, 1993; Lowy et al., 1994; Ponten et al., 1995). Here, I briefly discuss the *in vivo* and *in vitro* evidence that support the role of HPV in the etiology of cervical cancer.

1.3.2.1 *In vivo* evidence

The *in vivo* data linking HPV with carcinoma of the cervix are as follows:

1) HPV DNA, especially high risk HPVs such as HPV16 and HPV18, can be detected in over 90% of cervical carcinoma biopsies (zur Hausen, 1991).

2) HPV DNA of the same types can be detected both in primary cervical cancer and metastatic lesions (Lowy et al., 1994).

3) In morphologically benign lesions, the viral genome of HPV is usually maintained in an episomal state while in malignant lesions and high grade intraepithelial neoplasia it is often integrated into the host cell chromosomal DNA. Integration generally occurs within the E1 or E2 ORFs, leaving the ORFs of the transforming oncogenes, E6 and E7, intact and actively transcribed in HPV-positive cancer cells (Cullen, 1991). Only a small percentage of malignant tumors harbor

episomal DNA (Cullen, 1991).

4) The interval between primary infection of HPV and the appearance of cervical cancer is usually several decades. In addition, only a small percentage of women with clinically apparent HPV infection will eventually develop cervical carcinoma, suggesting that HPV is necessary but not sufficient for malignant progression (zur Hausen, 1994; Ponten et al., 1995).

1.3.2.2 *In vitro* evidence

Most of the evidence for the role of HPV in the oncogenesis of cervical cancer comes from the *in vitro* studies, which are summarized in the following:

1) Transfection with the high risk HPVs, such as HPV16 and HPV18, or their transforming genes, E6 and E7, can initiate immortalization of ectocervical and endocervical cells (Pirisi et al., 1987; Woodworth et al., 1988; Tsutsumi et al., 1992). The E6 and E7 ORFs of HPV16 and HPV18 are sufficient to immortalize cervical cells *in vitro* (Hawley-Nelson et al., 1989; Kaur et al., 1989, Tsutsumi et al., 1992). The E7 gene of HPV16 and HPV18, when placed under the control of a strong promoter, can immortalize human cervical epithelial cells with low efficiency (Halbert et al., 1991); the E6 gene of HPV alone can not immortalize human cervical

cells, but can enhance the efficiency of immortalization by the E7 gene (Halbert et al., 1991) or immortalize human mammary epithelial cells (Band et al., 1990). Other genes of HPV, such as E1, E2, E4, E5, can not immortalize cervical cells (Pirisi et al., 1987). However, disruption of E1 or E2 can enhance the immortalization capacity of the HPV genome (Durst et al., 1987; Munger et al., 1989; Sang et al., 1992).

2) The E6 and E7 oncoproteins of HPVs are responsible for the immortalization of genital cells by HPVs, since continued expression of the E6 and E7 is necessary for the infinite proliferation of cervical carcinoma cells (Munger et al., 1989a; Mansur and Androply, 1993).

3) The immortalized cells usually contain integrated HPV DNA, express viral transcripts, are defective in their ability to terminally differentiate and resemble moderate or high grade CIN (Woodworth et al., 1990; Sun et al., 1992).

4) The E7 protein from high risk HPVs can bind to the tumor suppressor gene, Rb (Dyson et al., 1989), and the E6 protein can bind to p53 and accelerate its degradation (Werness et al., 1989; Munger et al., 1989b; Scheffner et al., 1990). Inactivation of the tumor suppressor genes, Rb and p53, by HPV is necessary but not sufficient for the immortalization and tumorigenicity of primary cervical cells *in vitro* (Chen et al., 1993; Jewers et al., 1992). P53 is usually wild-type in

HPV-positive cervical carcinoma cell lines and mutated in HPV-negative cervical carcinoma cell lines (Park et al., 1994).

5) Although *in vitro* E6-E7-immortalized cervical cells are nonmalignant in nude mice (Hawley-Nelson et al., 1989, Watanable et al., 1989), additional transfection with the v-*ras* oncogene or with Herpes Simplex virus can convert them to tumorigenic cells (Durst et al., 1990; DiPaolo et al., 1987, 1990, 1994). Furthermore, co-transfection of HPV 16 and 18 with an oncogene, such as c-*myc* or H-*ras*, can transform rodent cells and primary human fibroblasts and keratinocytes *in vitro* (DiPaolo et al., 1989; Durst et al., 1990; Pei, 1993), suggesting HPVs may cooperate with oncogenes in the oncogenesis of cancer.

1.3.3 Chromosome abnormalities as cause of cervical cancers

Cytogenetic analysis of cervical tumors has shown that chromosomes 1, 3, 11, and 17 are often abnormal (Teyssier, 1989), and that transformation is associated with non-random abnormalities of chromosomes 1, 11, 19, and 20 (DiPaolo et al., 1993). In addition, aneuploidy is often observed in HPV-immortalized cells (Smith and Perira-Smith, 1990). Recent studies indicate that E7, but not E6, is responsible for the chromosomal instabilities in HPV-positive cells (Hashida and

Yasumoto, 1991). Thus, chromosomal loss, duplication, or rearrangement might lead to inactivation of some tumor suppressor genes such as p53, DCC or activation of cellular oncogenes such as c-*myc*, which suggests a role of abnormalities in the mechanism of oncogenesis of cervical cancers (referred to Section 1.1.4).

1.3.4 Cellular genes involved in multistage oncogenesis of cervical cells

1.3.4.1 Cellular genes regulated by HPVs

As we know from Table 4, the HPV E6 and E7 products are zinc binding proteins, implying that they may modulate cellular gene expression by binding to their promoter regions. In addition, the E6 and E7 proteins also contain protein-protein binding and transactivating domains, which may be also very important for the transforming activity of HPVs.

The amino terminal half of the E7 protein contains regions of similarity with the transforming proteins of the DNA tumor viruses, adenovirus E1a and SV40 large T antigen, implying that these proteins may act by a common mechanism (Phelps et al., 1986). As expected, previous *in vitro* studies demonstrated that all the three proteins can form a complex with a tumor suppressor gene product, Rb, which is a negative

regulator of cell growth (Munger et al., 1992). Since pRb induces G1 growth inhibition by binding and inactivating the transcription factor, E2F, binding of E7 to the hypophosphorylated form of Rb results in the release of E2F from pRb. This permits the activation by E2F of oncogenes such as *c-myc*, *c-fos*, or B-*myb*, and consequently the progression of the cell into S phase of the cell cycle (Scheffner et al., 1994). In addition, E7 has been shown to interact with other cellular proteins, such as p107, p130, histone H1 kinase, $p33^{cdk2}$, cyclin A and B-*myb* (Tommasino et al., 1993; Arroyo et al., 1993; Davies et al., 1993; Lam et al., 1994). The activation, such as for B-*myb*, or inactivation, such as for Rb, of these proteins by the E7 protein must play an important role in the oncogenesis of cervical cells.

Unlike E7, E6 does not display sequence similarity with the transforming proteins of other DNA tumor viruses. However, a clear functional similarity is found in that HPV E6, adenovirus E1b and SV40 large T antigen all target another tumor suppressor gene protein, p53 (Munger et al., 1992). Interaction of the HPV E6 protein with p53 through a cellular protein, E6-AP, results in the rapid degradation of the p53 protein (Scheffner et al., 1990; HuiBregtse et al., 1991), abrogation of the transactivating activity of p53 (Lechner et al., 1992) and loss of the p53-mediated cellular response to

DNA damage (Kessis et al., 1993; Hickman et al., 1994), which are responsible for the subsequent accumulation of genetic changes associated with cervical tumorigenesis. Furthermore, E6 has recently been shown to interact with at least seven other cellular proteins, designated pp212, pp182, p100, p81, p75, p53 and p33 (Keen et al., 1994). The role of the interaction of E6 with these proteins needs to be further characterized.

In addition, cellular genes that are up- or down-regulated by p53, such as Rb and p107, may be also important in the etiology of cervical cancer. However, the roles of these gene products in the oncogenesis of cervical cancer have not been well characterized.

1.3.4.2 Cellular genes regulating HPV expression

From the above discussion, it can be concluded that the expression of HPV plays a central role in the oncogenesis of HPV-associated cervical carcinoma. Therefore, it is of particular interest to understand the regulatory mechanism resulting in the activation of HPV oncogene expression in this carcinogenesis process.

The 500-1000 bp LCR of HPVs is known to function as a transcriptional regulatory element in almost all the HPV-containing cells. It is the major determinant of the

differential immortalization activities of some HPVs such as HPV16 and HPV18 (Romanczuk et al., 1991). Previous studies indicated that, in addition to the binding of E1 and E2 to the LCR, cellular proteins are the main factors that participate in the transcriptional control of HPV oncogene expression (zur Hausen, 1994). A number of cellular transcription factors have been identified that bind to the LCR regions of HPV16 (AP1, NF1, Oct-1, TEF-1, Sp1, and GR) and HPV18 (AP1, NF1, Oct-1, KRF-1, Sp1, GR, YY1) (For review see Hoppe-Seyler and Butz, 1994). The binding of these transcription factors can either activate (e.g. AP1, NF1, and GR) or repress (e.g. Oct-1 and YY1), the transcription of HPVs. In addition, other proteins, such as nuclear factor for interleukin 6 expression (NF-IL6) and TGF-β1, have also been shown to suppress HPV expression (Bran et al., 1990, 1992; Kyo et al., 1993). The regulation of HPV by these factors will be discussed further in section 1.3.7.

Several observations have indicated the possible role of deregulation of the HPV LCR in the carcinogenesis of primary cervical cells. For example, May et al. (1994) found that deletions or mutations in the HPV16 promoter region resulted in the loss of a YY1 binding site in some cervical tumors containing episomal HPV DNA. Chloramphenicol acetyl transferase (CAT) assays on the promoter activity of HPV16 in

cervical cancer-derived HT3, SiHa and CaSki cells showed that the CAT activity increased 5- to 6-fold under the control of this partially mutated or deleted LCR. Since YY1 was shown to serve as a negative regulator of HPV expression, deletions or mutations within HPV promoter may alter the regulation of E6 and E7 expression, allowing escape from the repression by YY1.

Loss of some parts of chromosome 11 have been reported in several cervical carcinomas (Teyssier, 1989). In addition, it has been shown by several researchers that transfer of chromosome 11 into cervical carcinoma cell lines can suppress their tumorigenicity (Sexon et al., 1986), indicating that a suppressor gene may exist on chromosome 11. The inactivation of this gene might cause cervical cancer. The tumor suppressor gene was proposed to act through the inhibition of HPV expression by binding the LCR (Schegget and van der Noordaa, 1994).

1.3.4.3 Cellular genes not interacting with HPV

From the above discussion, it seems that HPV has become the most important factor in the etiology of cervical cancer. However, the long latency between HPV infection and tumor emergence (zur Hausen, 1991), non-malignant characteristics of HPV-immortalized cells (Hawley-Nelson et al., 1989), and the existence of cell lines derived from cervical carcinomas

(C33A, H3T) or cervical carcinoma specimens devoid of HPV (Park et al., 1994), all suggest that HPV is not sufficient for the full transformation of primary cervical cells and that deregulation of cellular genes other than HPV may be also involved in the multistage carcinogenesis of cervical cells.

One cellular gene that is not related to HPV is a tumor suppressor gene DCC. The DCC gene product is a cell surface protein which may be responsible for cell-cell communication (Gao et al., 1993). The inaction of DCC by mutations or deltions has been reported to be responsible for several human cancers including colon, breast and lung cancer (Gao et al., 1993; Thompson et al., 1993). Cytogenetic analysis of the HPV-18-immortalized keratinocyte cells exposed to nitrosomethlurea (NMU) detected a deletion of part of the long arm of chromosome 18, which harbors DCC. Further analysis indicated that the expression of DCC in NMU-transformed cells was greatly decreased (Klingelhuz et al., 1993), indicating that inactivation of DCC expression may be important in the NMU-induced carcinogenesis. However, the role of DCC in the oncogenesis of primary cervical cell has not been clearly identified.

By checking 22 protooncogenes for amplification in 50 primary, untreated squamous cell carcinomas of uterine cervix, Mitra et al. (1994) observed that amplification of erbB2

(HER2/neu) was found in 14% of the cases. In addition, 2 tumors with erbB2 amplification showed additional restriction fragments, suggesting that the amplification of erbB2 may play an important role in tumorigenesis.

In addition, deregulation of several other gene products, such as the proliferation cell nuclear antigen (PCNA), fibronectin, integrin and the TGF-α receptor, in immortalized cells has also been reported (Li et al., 1992; Hodivala et al., 1994; Karakitsos et al., 1994; Noda et al., 1994; Shin et al., 1994). Studying the regulation of these cellular genes, especially in HPV-negative cells, will be very helpful for us to fully understand the multistage oncogenesis of cervical cancer.

1.3.5 Role of co-factors in multistage carcinogenesis of cervical cells *in vivo* and *in vitro*

Several co-factors have been identified by epidemiological and molecular biological approaches.

1.3.5.1. Hormones

Previous epidemiological studies indicated that chronic use of oral contraceptive pills which contain steroid hormones is an important risk factor for the development of cervical cancers (Hildesheim et al., 1990). In experimental

studies, the steroid hormones, dexamethasone and progesterone, have been shown to be essential for the expression of HPV16 in primary cervical cells (Mittal et al.,1993). Most importantly, these hormones have also been shown to enhance the expression of HPV16 from three GREs of the enhancer and to enhance the immortalization and transformation efficiencies of high risk HPVs (Pater et al., 1988; Crook et al., 1988; Pater et al., 1990; Pater et al., 1994; Mittal et al., 1993). Thus, cooperation of HPV and hormones may plays an important role in the oncogenesis of primary cervical cells.

1.3.5.2 Other infectious agents

Various sexually transmitted agents, including herpes simplex virus (HSV), cytomegalovirus (CMV) and HIV have been regarded as the co-factors in cervical cancer (Herrington, 1995). Recent experimental studies indicate that HSV 6 not only infected genital epithelial cells, but also upregulated the expression of HPV (DiPaolo et al, 1994), and transformed HPV-immortalized human genital cells into tumors (DiPaolo et al, 1990), suggesting that HSV has a potential role in the tumorigenesis of cervical cells. Several reports also show that squamous intraepithelial lesions of the cervix progressed more rapidly into cervical cancers in HIV-infected women (Maiman et al., 1990), suggesting that HIV may act as a

co-factor and promote the malignant progression of cervical lesions. Further *in vitro* studies also indicate that the HIV-1 regulatory protein, *tat*, in combination with the E2 protein of HPV16, can transactivate the HPV 16 P97 promoter in cervical carcinoma cells. *tat* can also reverse E2-mediated repression of the HPV16 promoter (Vernon et al., 1993).

1.3.5.3 Immune response

In immunocompetent individuals the cell-mediated immune response influences the persistence or regression of HPV infections. When HPVs infect the cells, the host usually infiltrates the lesions with inflammatory cells, including macrophages, cytotoxic T-lymphocytes, and natural killer cells. These cells can secrete a variety of regulators known as cytokines and lymphokines, including TNF α and β, TGF-β, interleukin (IL) 1, 2, 3, 4, 5, 6, 7 and 8 and interferon (INF) α, β and γ, which can repress the growth of HPV-infected cells and HPV expression. Therefore, the infiltration of macrophages, T-lymphocytes, and natural killer cells is often observed to be followed by regression of HPV infection (Fierlbeck et al., 1989). However, a disorder of the immune response should lead to persistence or progression of viral infection. Therefore, depression of immune response has been

regarded as a co-factor for the oncogenesis of cervical cells.

Several *in vivo* and *in vitro* experiments have been carried out to understand the role of the immune response in the carcinogenesis of cervical cancer.

Several *in vivo* studies demonstrated an assciation between impaired immune function and the development of cervical cancers. Firstly, patients with histologically proven invasive squamous cell carcinoma of the cervix have been observed to have immune defects characterized by a decrease in T cell numbers and CD4+ helper cells, and impaired function of Langerhans's cells, compared with healthy controls (Castello et al., 1986). Secondly, women who have been immunosuppressed and have HPV infection have been observed to develop invasive cervical cancer rapidly (Sillman et al., 1984). Thirdly, the incidence of invasive cervical cancer increases with advancing age, indicating that immune senescence, which increases with age may contribute to this cancer (Mandelblatt, 1993). Finally, women who are infected by both HIV and HPV have a markedly increased incidence of cervical cancer. Women with concurrent HPV and HIV infection are 42 times more likely to have CIN than women without either virus (Feingold et al., 1990).

The following *in vitro* experiments studied mainly the basic mechanism underlying the immune response to viral

infection using molecular biological approaches. Firstly, it has been shown that TNF-α, TGF-β, IL-1 and IFN-γ can repress HPV expression at the transcriptional level in HPV-immortalized cells (Woodworth et al., 1992; Braun et al., 1990; Kyo et al., 1994). However, in some tumor cell lines, insensitivity to TNF-α and TGF-β1 has also been observed (Braun et al., 1990, 1992; Rosl et al., 1994), indicating that escape from the immune response may be a mechanism for the tumorigenesis of cervical cells. Secondly, cocultivation experiments with activated macrophages revealed that nonmalignant fibroblast-Hela hybrid cells respond to macrophages by suppressing HPV gene expression. However, this response was not detectable in malignant fibroblast-Hela cells, indicating that the crucial step in malignant conversion involved a disruption of an immune response pathway that normally suppresses HPV transcription in immortalized cells(Rosl et al., 1994). Thirdly, reduced expression of class MHC class I genes in HPV16-positive cervical carcinoma cell lines was observed (Cromme et al, 1993), indicating that insufficient presentation of antigenic domains of viral oncoproteins due to diminished expression of MHC class I molecules may be a cause for malignant progression *in vivo*.

1.3.6 Tobacco smoke and cervical cancer

Epidemiological investigations and animal studies over the past several decades have clearly delineated that tobacco, particularly cigarette smoking, is one of the major cause of human cancers including lung, oral cavity, pharynx, larynx, esophagus, urinary bladder, renal pelvis and pancreas (IARC, 1986). The most important of these is lung cancer. Experimental studies have shown that tobacco smoke contains more than 6000 chemicals which include toxic agents, such as nicotine, ammonia, formic acid and carbon monoxide; carcinogens, such as benzene, benzo[a]pyrene, N-nitroso compounds, formaldehyde, 2-nitropropane and vinyl chloride; and tumor promoter agents, such as phenol. The role of some of these carcinogens in the carcinogenesis have been clearly demonstrated (for review see Hoffmann and Wynder, 1986).

An association between cigarette smoking and cervical cancer was first noted in 1966 (Naguib et al., 1966). In 1977, a specific hypothesis regarding the causitive role of cigarette smoking in cervical cancer was proposed (Winkelstein, 1977). Since then, increasing epidemiological investigations have been carried out. The evidence that supports the hypothesis is as follows: 1) The relative risk for the development of invasive cervical cancer is higher in cigarette-smokers than in non-smokers. With an increase in the

number of cigarettes smoked per day by smokers, the relative risk factor also increased (Brinton, 1990). 2) Accumulation of tobacco metabolites in cervical fluids has been observed in cigarette smokers (Sasson et al., 1985; McCann et al., 1992). 3) Some chemicals contained in cigarette smoke, such as benzo[a]pyrene and NMU, have been shown to cause malignant transformation of human HPV-immortalized keratinocytes cells from the foreskin and mouth (Li et al., 1992; Garrett et al., 1993; Klingelhutz et al., 1993). However, there are some results that argue against the role of cigarette smoking in cervical cancer: 1) No association between smoking and cervical cancer was found by Layde(1989). 2) Some other factors, such as the number of sexual partners, which may also contribute to cervical cancer, can confound the statistics of the association between cigarette smoking and cervical cancer (Eluf-Neto, 1994; Philips and Smith, 1994). 3) There is no direct evidence which proves that cigarette smoke causes cervical cancer as demonstrated for other cancers (Masui et al., 1986). Therefore, the role of cigarette smoking in the carcinogenesis of cervical cancer is undetermined. Further *in vitro* experiments are required before making conclusions.

1.4 Objective of this study

From the above discussion, it can be concluded that HPV

and some co-factors are very important in the multistep carcinogenesis of cervical cells. However, there are few *in vitro* systems that demonstrate the multistage nature of the development of cancer. In addition, the mechanism underlying the cooperative and sequential role of HPV and co-factors in the oncogenesis of cervical is still poorly understood. Understanding the complex interaction between host cells and HPV is critically essential in cervical cancer research.

The objectives of my study were to further the understanding of the molecular mechanism of the cooperation of HPV and cigarette smoking in the oncogenesis of cervical cells. For this purpose, I first established an *in vitro* system, in which primary human endocervical cells (HEN) were immortalized by HPV and subsequently transformed into tumors by treating the immortalized cells with CSC, to mimic the multistage oncogenesis of cervical cells *in vivo*. Then, the morphology, growth characteristics, viral and cellular genes expression, and response to cytokines or retinoic acid (RA) were studied in primary cells and immortalized and CSC-transformed tumor cell lines. In addition, a new method called mRNA differential display was employed to identify novel genes involved in this oncogenesis process.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

KGM and DMEM were purchased from GIBCO-BRL and ICN respectively. GIBCO-BRL was the supplier for fetal calf serum (FCS), goat serum and trypsin-ethylenediamine tetraacetic acid (trypsin-EDTA). Penicillin-streptomycin and Collagen type I were obtained from ICN and Collaborative Biomedical Products, respectively. Primary endocervical tissues for preparation of endocervical cells were kindly provided by Dr. M. Parai of the Grace Hospital, St. John's, Newfoundland. HEN-16 and HEN-16-2 were established by Dr. K. Tsusumi. CaSki and SiHa cell lines have been cultured in this lab.

Restriction endonuclease were ordered from GIBCO-BRL with their respective 10X reaction buffers. Superscript reverse transcriptase with 10X buffer and 0.1 M DTT was also purchased from GIBCO-BRL. Promega was the supplier of T4 kinase, Taq polymerase and rRNasin ribonuclease inhibitor. DNase I (RNase free) was obtained from Boehringer Mannheim.

$\alpha[^{32}P]$dCTP, $\gamma[^{32}P]$ATP, and $\alpha[^{35}S]$dATP were purchased from Amersham. Nick columns were obtained from Pharmacia Biotechnology. GIBCO-BRL was the supplier for Random Primer DNA Labelling System and BioNick Labelling System.

GIBCO-BRL supplied TGF-β1 and all the markers including the 1 kb and 100 bp DNA ladders and the 0.24-9.5 kb RNA ladder, while Sigma Chemical Co. supplied TNF-α and RA. Low melting point agarose, agarose, acrylamide, N,N'-methylenebisacrylamid, urea, 10 mM dNTP (dGTP, dATP, dTTP and dCTP) and dextran were all purchased from GIBCO-BRL. Baker Inc, Bio-Rad, Carnation, and Pharmacia were the suppliers of 2-mercaptoethanol,N,N,N',N'-tetra-methylethylenediamin e (TEMED), skim milk powder, and dextran sulphate, respectively.

Wizard PCR Preps DNA Purification System and the f-mol DNA Sequencing System were purchased from Promega. GIBCO-BRL, Invitrogen, Pharmacia Biotechnology, and United States Biochemical Co. supplied the *In Situ* Hybridization and Detection System, TA Cloning Kit version 2.0, QuickPrep mRNA Purification Kit, and Sequenase Version 2.0 Sequencing Kit, respectively.

HPV16 DNA was kind gift from Dr. H. zur Hausen. JE/MCP-1 plasmid was purchased from American Type Culture Collection (ATCC). Plasmids for B-*myb*, fibronectin, GADD45 and GADD153, p53, and Waf1/Cip1 were kindly provided by Drs. A. Sala (Thomas Jefferson University, USA), J.R. Smith (National Cancer Institute, USA), J. Fornace, Jr. (National Cancer Institute, USA), S. Benchimol (Ontario Cancer Institute,

Canada), and W. Harper (Baylor College of Medicine, USA), respectively. Proliferating cell nuclear antigen (PCNA) 40-mer probe was purchased from Oncogene Sciences. All other synthetic oligonucleotides used for primers and probes were ordered from General Synthesis and Diagnostic, Toronto. DAKO Co., Gibco BRL, and Jackson Immunoresearch Lab, Inc. were the suppliers of PCNA monoclonal mouse antibody (PC10), anti-human fibronectin, mAb (Clone I), and goat anti-mouse IgG, fluorescein isothiocynate (FITC)-conjugate, respectively. CSC was a kind gift of Dr. M.H. Billimoria of Imperial Tobacco Limited, Canada.

BioTrace HP membrane for Northern and Southern blots and NA45 DEAE membranes were obtained from Gelman Sciences and Schleicher & Schuell, respectively. Kodak was the supplier of X-ray film. Eight-well tissue culture chamber slides, 35 mm, 60 mm and 100 mm tissue culture plates and 0.5 ml eppendorf micro test tube for PCR were obtained from NUNC and Fisher, respectively.

The Isotemp Vacuum Oven Model 281 and Incubator Model 60 were purchased from Fisher and Precision Scientific, respectively. Pharmacia LKB Biotechnology was the supplier of Ultraspectrophotometer II and Ultroscan XL Laser Densitometer. The Laborlux fluorescence microscope was purchased from Leitz Inc, Germany. Bio/CAN scientific was the supplier of the

Hybraid Thermal Reactor for PCR.

2.2 Cell culture

Primary and HPV16-immortalized endocervical cells were maintained in serum-free KGM medium containing 1% penicillin/streptomycin. However, CSC-treated tumor cells and established cervical carcinoma cell lines (CaSki and SiHa) were routinely cultured in DME medium containing 10% FCS, 1% penicillin/streptomycin, and 0.4 μg/ml hydrocortisone. Fibroblast cells used for raft formation were maintained in E medium containing 10% FCS and 1% penicillin/streptomycin.

2.2.1 Monolayer culture

All the cells were maintained at 37 °C in an incubator containing 5% CO_2. For DNA and RNA extraction purposes, the cells were usually cultured in 100 mm tissue culture plates. For the purpose of growth measurement, the cells were cultured in 60 mm plates. When the cells reached confluency, the medium was aspirated from the plates and the cells were washed with PBS. Then 2 ml of trypsin-EDTA was added into each plate, which was left in the 37 °C incubator for 15 minutes. For the cells cultured in KGM, 8 ml of PBS containing 10% FCS was added to the trypsinized cells to quench the activity of

trypsin. The cells were then suspended and centrifuged at 1,000 rpm for 10 minutes. The pellet of cells was then resuspended with KGM, dispensed into new plates at the ratios of 1:10, 1:4 and 1:2. For the cells cultured in DMEM containing 10% FCS, 8 ml of DMEM was added directly into the trypsinized cells, which was further suspended in medium and dispensed into new plates. The cells were usually maintained at 37 ^{0}C by passage every three days or when the plates were confluent.

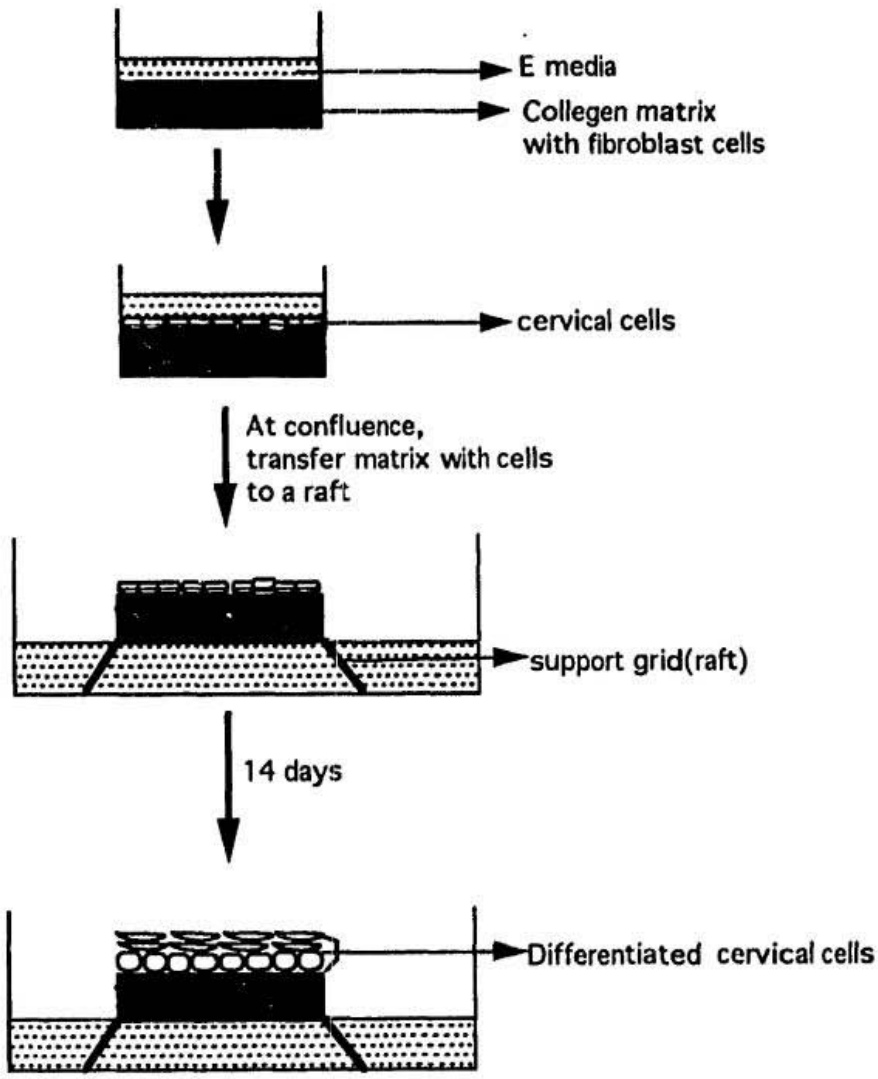
For *in situ* hybridization and indirect immunofluorescence experiments, the cells were usually cultured in 8 well tissue culture chamber slides. When they reached 70% confluency, the chamber frame was release from the slides, the cells were briefly washed with PBS and the slides were processed differently depending on the purpose of experiments.

2.2.2 Organotypic (raft) culture

The procedure of organotypic raft culture was basically according to Meyers et al. (1992) and is clearly illustrated in Figure 5.

The cervical cells and 3T3 J2 fibroblast cells were cultured before starting the raft culture. Usually, for each raft, 3 X 10^5 fibroblast cells and 3 X 10^5 cervical cells were required. When the fibroblast cells were ready, the raft gel

Figure 5. Organotypic (raft) culture system. See description in the text.


E media
Collegen matrix
with fibroblast cells
cervical cells
At confluence,
transfer matrix with cells
to a raft
support grid(raft)
14 days
Differentiated cervical cells

was made by mixing the fibroblast cells with collagen mix containing type I collagen, reconstruction buffer (268 mM $NaHCO_3$, 200 mM HEPES and 50 mM NaOH), and 10X E medium (8:1:1; volume:volume:volume), dispensing 3 ml of collagen/fibroblast mix into each 35 mm plate, and leaving the plates at 37 °C to solidfy for 30-60 minutes. Subsequently, 2 ml of E media was added into each plates. The plates were kept in 37 °C incubator for over 2 days to regain the morphology of the fibroblast cells. When the cervical cells were ready, 3 X 10^5 trypsinized cells were seeded into each plate containing collagen/fibroblast gel raft. After the cells attached to the gel in 4 hrs to overnight, the media was replaced with fresh E media. The rafts were raised onto stainless steel grids and allowed to grow at the air-liquid interface for 12-14 days, during which the medium was changed every two days. The culture was then fixed in 4% paraformaldehyde and embedded in paraffin for further analysis.

2.3 Tumorigenesis of HPV16-immortalized cells by CSC

2.3.1 Treatment of HPV16-immortalized cells with CSC

HEN-16 and HEN-16-2 maintained in KGM medium were first adapted to grow in DMEM. Then, at each passage, they were cultured either in normal DME medium or DME medium containing 75, 100 or 125 µg/ml CSC, respectively. One day later, the

CSC-containing medium was replaced by fresh normal DME medium. The cells were not treated with CSC again until the next passage. The tumorigenicity was checked in nude mice every two months.

2.3.2 *In vivo* tumorigenicity assay

After every two months of CSC treatment, the cells were trypsinized and resuspended in 1X PBS. For each cell lines, 1 X 10^7 CSC-untreated, CSC-treated, and SiHa cells in 0.1 ml were injected into each of 2 2-3-month-old female immunocomprimised nude mice, and the tumor incidence was monitored weekly for 4-8 weeks. Upon tumor formation, the size of the tumors was measured, the mice were sacrificed, and the tumors were excised. The tumors were placed in KBM containing 25 mg/ml fungizone and 50 I.U. penicillin and streptomycin for 20 minutes with one change in 10 minutes. The tumors were subsequently cut into two half: one half was fixed in 4% paraformaldehyde and used for pathological analysis; another half was dissected into pieces, trypsinized at 37 ^{0}C for 20 min, centrifuged, and then cultured in DMEM medium at 37 ^{0}C.

2.3.3 Pathological analysis

The fixed tumors were embedded in paraffin, sectioned in a microtome, and stained with hematoxylin and eosin (Sun et

al., 1992). The stained sections were examined by light microscopy under 100X and 400X magnification for tumor morphology and invasion. The selected sections showing tumor morphology were photographed with Kodak Tmax 400 film for documentation.

2.4 Measurement of growth rate and saturation density of cervical cells

The exponentially growing primary, immortalized and CSC-transformed cells were trypsinized, centrifuged, and resuspended in 2 ml of medium. The cell numbers were counted with a hemocytometer, and about 2 X 10^4 cells were dispensed into each of three 60 mm plates for each cell line. The cell numbers were counted every two days for 8 days.

The saturation density of each cell lines was measured by counting the cell number 5 days after the cells reached confluence.

2.5 Soft agar or anchorage independent growth assays

The 0.7% agarose underlying gel was prepared by adding an equal volume of 2X DMEM containing twice the amount of DEM components and 20% FCS into low melting point agarose dissolved in sterilized distilled water, dispensing 2.5 ml into 60 mm plates, and leaving the plates at room temperature

until the gel solidified. Subsequently, the immortalized (HEN-16 and HEN-16-2), CSC-transformed (HEN-16T and HEN-16-2T), and positive control SiHa cells were trypsinized, resuspended in 2X DMEM, and counted. Then, the 0.35% agarose overlaying gel was prepared by mixing about 1 X 10^5 cervical cells in 1.25 ml of 2X DMEM with 1.25 ml of agarose in sterilized distilled water, pouring the mixture into 60 mm plates containing the 0.7% gel, tempering the plates at 4 ^{0}C for 5 minutes, and incubating at 37 ^{0}C. One week later, DMEM was carefully added onto the surface of the soft agar to provide nutrients. The colony formation was monitored every two days for 2-4 weeks. Generally, triplicate assays were carried out for each cell line. Representative photographs were taken for documentation.

2.6 Labelling of probes

2.6.1 Radioactive probes for Northern and Southern blot hybridization assays

Radioactive cDNA probes were generally prepared using the Random Primer DNA Labelling System (GIBCO-BRL). Briefly, about 50 ng of cDNA were incubated in random primer buffer [0.67 M HEPES, 0.17 M Tris-HCl, 17 mM $MgCl_2$, 33 mM 2-mercaptoethanol, 1.33 mg/ml BSA, 18 OD_{260} units/ml oligodeoxyribonucleotide primers (hexamers), pH6.8] with 0.5 mM each of dATP, dGTP,

dTTP, 50 μCi of α[^{32}P]dCTP, and 3 units of Klenow fragment at 25 °C for 2 hrs. The mixture was passed through a NICK column (Pharmacia Biotechnology) to remove the unincorporated α[^{32}P]dCTP. The labelled probe was denatured at 100 °C for 5 minutes and immediately used for hybridization.

The oligonucleotides were usually labelled by the 5'-end labelling method as described by Sambrook et al.(1989). In brief, about 10 pmol of oligonucleotide probe was incubated in kinase buffer (0.1 M Tris-HCl, pH9.5, 5 mM $MgCl_2$) with 10 mM DTT, 50 μCi of γ[^{32}P]ATP, and 1 unit of T4 polynucleotide kinase at 37 °C. Thirty minutes later, 4 volumes of 0.1 M EDTA was added to stop the reaction. The unincorporated γ[^{32}P]ATP was removed by ethanol precipitation. The labelled oligonucleotide probes were used directly for hybridization.

Generally, the specific activity for both cDNA and oligonucleotide probes was usually more than 8 X 10^8 cpm/μg DNA. The probe activity used for hybridization was usually 1-4 X 10^6 cpm/ml hybridization buffer.

2.6.2 Non-radioactive probes for *in situ* hybridization assays

The BioNick Labelling System (GIBCO-BRL) was used for labelling non-radioactive probes for *in situ* hybridization.

Generally, 1 μg of cDNA was incubated in 1X dNTP mix (Biotin-7-dATP, dCTP, dGTP, dTTP) and 1X enzyme mix at 16 °C for 1 hr. One tenth volume of stop solution was then added to stop the reaction. Unincorporated nucleotides were removed by ethanol precipitation twice. The biotinylated probes were usually less than 500 bp in size and could be stored at -20 °C in Tris-EDTA (TE) buffer for at least one year. Generally, 2.5 ng of labelled probe was used for *in situ* hybridization.

2.7 Protocol for mRNA expression assays

2.7.1 Preparation of total RNA and mRNA

All the materials and reagents used in RNA work were first treated with 1% diethyl pyrocarbonate (DEPC). Total RNA was prepared basically according to the CsCl gradient centrifugation method described by Sambrook et al.(1989) with minor modifications. Briefly, the cells cultured in monolayer were first washed once with ice-cold 1X PBS, lysed with lysis solution, scraped from the plates with rubber policemen, and then removed from the plates with a syringe. The DNA in the lysed cells was subsequently sheared by passing quickly through a 18 G needle 10 times. The total RNA was purified from the lysed cells by centrifuging with a SW41 rotor at 30,000 rpm, 20 °C for 20 hrs. The supernatant was decanted and

the RNA pellet was dissolved in DEPC-treated water, ethanol precipitated, centrifuged, and then dissolved in DEPC-treated water. The concentration and quality of RNA was checked by measuring the absorbance of the samples at 260 nm and 280 nm. The concentration of the RNA was calculated according to the following formula: $\mu g/\mu l$=dilution factor X A_{260} X 40/1000. Generally, the A_{260}/A_{280} ratio should be between 1.4 to 2.0. The integrity of the RNA was checked by electrophoresis in a 1% neutral agarose gel. RNA can be stored at -70 °C for less than 1 year.

Purification of mRNA was performed using the QuickPrep mRNA Purification Kit (Pharmacia Biotechnology) according to the manufacturer's instructions with minor modification. In brief, about 500 µg of total RNA were first dissolved in 1.0 ml of extraction buffer (a buffered aqueous solution containing guanidinium thiocyanate and N-lauroyl sarcosine), mixed with 3 ml of dilution buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), and then centrifuged at 12,000g and 4 °C for 5-10 minutes. The supernatant was then loaded onto the surface of the resin of an oligo(dT) cellulose spun column and mixed gently with a rocking device for 30 minutes at room temperature. Subsequently, the column was centrifuged in a table top centrifuge at 350g for 2 min. The supernatant was

discarded, and the column was washed three times with high salt buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 M NaCl) and three times with low salt buffer (10 mM Tris-HCl, pH7.4, 1 mM EDTA, 0.1 M NaCl). The mRNA was eluted with 0.75 ml elution buffer at 65 °C. After ethanol precipitation, the mRNA was dissolved in 20 µl of DEPC-treated water, and the concentration was determined as described above. Usually, mRNA was 1-8% of the total RNA.

2.7.2 Northern blot assays

The method used for Northern blotting was similar to that described by Sambrook et al. (1989) with some changes. Briefly, 20 µg of total RNA or 3 µg of mRNA together with 4.5 µg of 0.2-9.5 kb RNA ladder were first mixed with sample buffer (20 mM MOPS, 0.5 mM EDTA, 1.5 mM formaldehyde, 50% formamide) and then separated on a 1% formaldehyde gel [1% agarose in 20 mM 3-(N-morpholino)propane-sulfonic acid (MOPS), 8 mM sodium acetate, and 0.66 mM formaldehyde] by electrophoresis at 40 volts overnight or when the bromophenol blue migrated approximately 8-10 cm. The RNA ladder lane was then cut from the gel, stained with ethidium bromide for 30-45 minutes, destained for 30 minutes, and photographed under UV light together with a fluorescent ruler. The gel was then

washed twice in DEPC-treated water to remove the formaldehyde. The RNA was then transferred by capillary blotting onto BioTrace HP membrane (Gelman Sciences) following the manufacturer's instruction. The membrane with transferred RNA was subsequently baked at 80 °C in vacuum oven for 1.5-2 hrs to immobilize the RNA on the membrane. The membrane-RNA was prehyridized in hybridization buffer (1% non-fat dry milk, 0.5 M NaH_2PO_4, 7% SDS, pH 7.2) for 2-3 hrs, and then hybridized with a ^{32}P-labeled probe in hybridization buffer overnight at 65 °C. The hybridized membrane was washed twice with 2X SSC, 0.1% SDS solution at room temperature for 15 minutes, twice with 0.1X SSC, 0.1% SDS at 42-65 °C for 10 minutes, depending on the probe used. After washing, the membrane was exposed to film for 5 hrs to 10 days. The probe was removed by boiling in 0.1X SSC, 0.1% SDS solution, and the membrane was reused for the next probe. γ-actin was usually used as an internal control.

2.7.3 RT-PCR

The method used for RT-PCR was basically similar to that described by Innis et al. (1990), with modifications.

The total RNA used for RT-PCR was first treated with RNase free DNase I. Then, 1 μg of DNA-free RNA was reverse

transcribed to cDNA in 1X RT buffer, 0.5 mM dNTP, 10 mM DTT, 40 units of rRNase ribonuclease inhibitor (Promega), and 1 unit of Superscript reverse transcriptase at 37 ^{0}C for 1 hr. One tenth of the RT product was further used for the PCR amplification using Hyraid thermal reactor (Bio/CAN Scientific). The conditions for PCR were as follow: 1X PCR buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.01% (w/v) gelatin], 0.5 mM dNTP, 0.2 mM of sense and antisense primers, and 2 unit of Taq polymerase (Promega) in 50 µl. The PCR program for the DCC gene was: 94 ^{0}C for 3 min for the first cycle, then 94 ^{0}C for 1 min, 56 ^{0}C for 2 min and 72 ^{0}C for 2 min for a total of 35 cycles, then 72 ^{0}C for 7 min for the last cycle and, finally, 25 ^{0}C on hold.

2.7.4 *In situ* hybridization assays

The *In situ* Hybridization and Detection System (GIBCO-BRL) was used for *in situ* hybridization.

The slides, coverslips, staining jars, and forceps were baked in 180 ^{0}C for 4 hr before use. All the solutions used before hybridization were treated with DEPC.

Cervical cells grown on slides were first fixed in fresh 4% paraformaldehyde at 22 ^{0}C for 30 min, and then transferred to 70% ethanol at 4 ^{0}C until use. When using, the slides were

immersed in 50% ethanol for 2 minutes, rinsed with 1X PBS, and then incubated in prewarmed 40 μg/ml proteinase K in 1X PBS at 37 ^{0}C for deproteinization. Five to 20 min later, the slides were rinsed with PBS, dehydrated through a graded ethanol series (3 min each in 50%, 70%, 90% and 100% ethanol). After being air dried at room temperature for 5-10 min, the slides were hybridized with denatured biotin-labelled probe in 1X hybridization buffer (2X SSC, 0.1 M sodium phosphate, pH 6.5, 1X Denhart's solution), 10% dextran sulphate at 42 ^{0}C overnight.

After hybridization, the slides were washed twice with 0.2X SSC, 0.1% SDS solution for 15 min, once with buffer 1 (0.1 M Tris-HCl, pH7.5, 0.1 M NaCl, 2 mM $MgCl_2$, and 0.05% Triton X 100), and blocked in blocking buffer [3% (w/v) bovine serum albumin in buffer 1] for 1 hr. The signals were detected by incubating the slides sequentially with streptavidin, biotin (AP), nitroblue tetrazolium (NBT) and 4-bromo-5-chloro-3-indolylphosphate (BCIP). A purple color should be visualized under the microscope for positive samples. The results shown are typical for cells observed in at least three independent experiments.

2.8 Protocols for DNA detection

2.8.1 Preparation of high molecular weight DNA

Protocols used for DNA extraction were as described by Sambrook et al. (1989). Briefly, cells grown in 100 mm plates were scraped from the plates in cold 1X PBS, centrifuged, and then incubated in extraction buffer (10 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 20 µg/ml pancreatic RNase, 0.5% SDS) at 37 ^{0}C for 1 hr. Proteinase K at 20 mg/ml was then added into each sample to a final concentration of 100 µg/ml. After 3 hrs incubation at 50 ^{0}C, the DNA was purified by three phenol extractions, ethanol precipitated, and dissolved in TE. The concentration and quality of DNA were determined as described for RNA. DNA concentration was calculated according to the following formula: µg/µl=dilution factor X A_{260} X 50/1000. The A_{260}/A_{280} ratio should be between 1.7 and 2.0. The integrity of DNA was checked on a 1% agarose gel.

2.8.2 Southern blot assays

Southern blot assays were performed according to the protocol provided by Gelman Sciences. Equal amounts of DNA, usually 10 µg, were digested with specific restriction enzymes, run by electrophoresis in 1% agarose gels, transferred to Biotrace HP membranes, and baked at 80 ^{0}C for 1.5-2.0 hrs. The conditions for prehybridization,

hybridization, and washing were the same as described for the Northern blot assays.

2.9 Indirect immunofluorescence assays

Indirect immunofluorescence assays were performed according to Bartek et al. (1990), with modifications.

The epithelia grown in raft culture were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned, and kept at room temperature until use. However, the cells grown on slides in monolayer culture were fixed in ice-cold 50% methanol : 50% acetone for 30 min, air dried, and left at room temperature until use.

The fixed epithelia grown on rafts were first incubated in xylene to remove the paraffin. Then, the monolayer and raft culture were immersed 3 times in 100% ethanol, 3 times in 70% ethano, once in dH_2O, and 3 times in 1X PBS. The raft epithelia were subsequently trypsinized in digestion solution (0.2% trypsin, 18 mM $CaCl_2$, 50 mM Tris-HCl, pH7.5). Then, all the cells grown in monolayer and raft culture were incubated in 20% goat serum for 1 hr, with the respective antibody in PBS at 1:20-1:100 dilution for 1 hr, and with FITC diluted in 20% goat serum at 1:40 for 1 hr. The staining was visualized by mounting in 3% glycerol, covering with coverslips, and examining on a Leitz Laborlux S Fluorescence Microscope

(Germany) in the dark. A green color should be visualized for positive samples. Representative photographs were taken with Kodak Tmax films for documentation. The results shown were typical for cells observed in at least three independent experiments.

2.10 Treatment of cervical cells with TNF-α, TGF-β1, and RA

The concentrations of TNF-α, TGF-β1 and RA for the treatment of cervical cells were 250 U/ml, 5 ng/ml, and 3 μM, respectively. For mRNA expression experiments, the cells were untreated or treated 1 day for TNF-α, 2 days for TGF-β1, and 3 days for RA. The medium without or with TGF-β1 or RA was changed daily. After treatment, RNA was extracted for Northern blot analysis. For growth inhibition experiments, 1 X 10^4 cells were seeded in 60 mm plates and were untreated or treated for 5 days with a daily change of medium. Five days later, the cells in each plate were counted with a haemocytometer. The percentage of growth inhibition by TNF-α, TGF-β1 and RA was then calculated as follow: % growth inhibition=100-[(cell numbers in treated dishes/cell numbers in untreated dishes) X 100)]. The results presented the mean of three experiments.

2.11 Differential display assays

Differential display assays were performed according to Liang et al. (1992a, b). Figure 6 shows schematically the techniques for identifying and cloning differentially expressed genes by the mRNA differential display method.

2.11.1 RT-PCR

The RT-PCR method used in differential display was basically similar to that described in Section 2.7.3., with several differences. First, $\alpha[^{35}S]$dATP was used in the PCR reaction to label the PCR products. Second, the dNTP concentration was reduced from 500 μM to 25 μM. Third, two specific groups of primers were used. One group of primers, designated $T_{12}MN$, were 3'-end primers composed of an oligo-dT 12-mer and 2 additional nucleotides. In my study, $T_{12}CA$, $T_{12}AC$, $T_{12}CG$, and $T_{12}AT$ were used. The second group of primers, the arbitrary primers (AP), was 5'-end primers composed of nine or ten oligonucleotides (9-mer or 10-mer). In my study, 14 primers designated AP1 to AP14 were used. The sequence for each arbitrary primer is listed in Table 5. Fourth, a low annealing temperature (40 °C) and short extension time (30 sec) were used in PCR. The PCR program was as follow: first, 94 °C

Figure 6. Schematic representation of mRNA differential display method. Dashed lines represent RNA, solid lines represent DNA and arrows represent the direction of RT and PCR. Boxed sequences are the primers. AP1 represents arbitrary primers containing 9 or 10 nucleotides. Only one set of primers, $T_{12}CA$ and AP1, is shown here. See text for details.

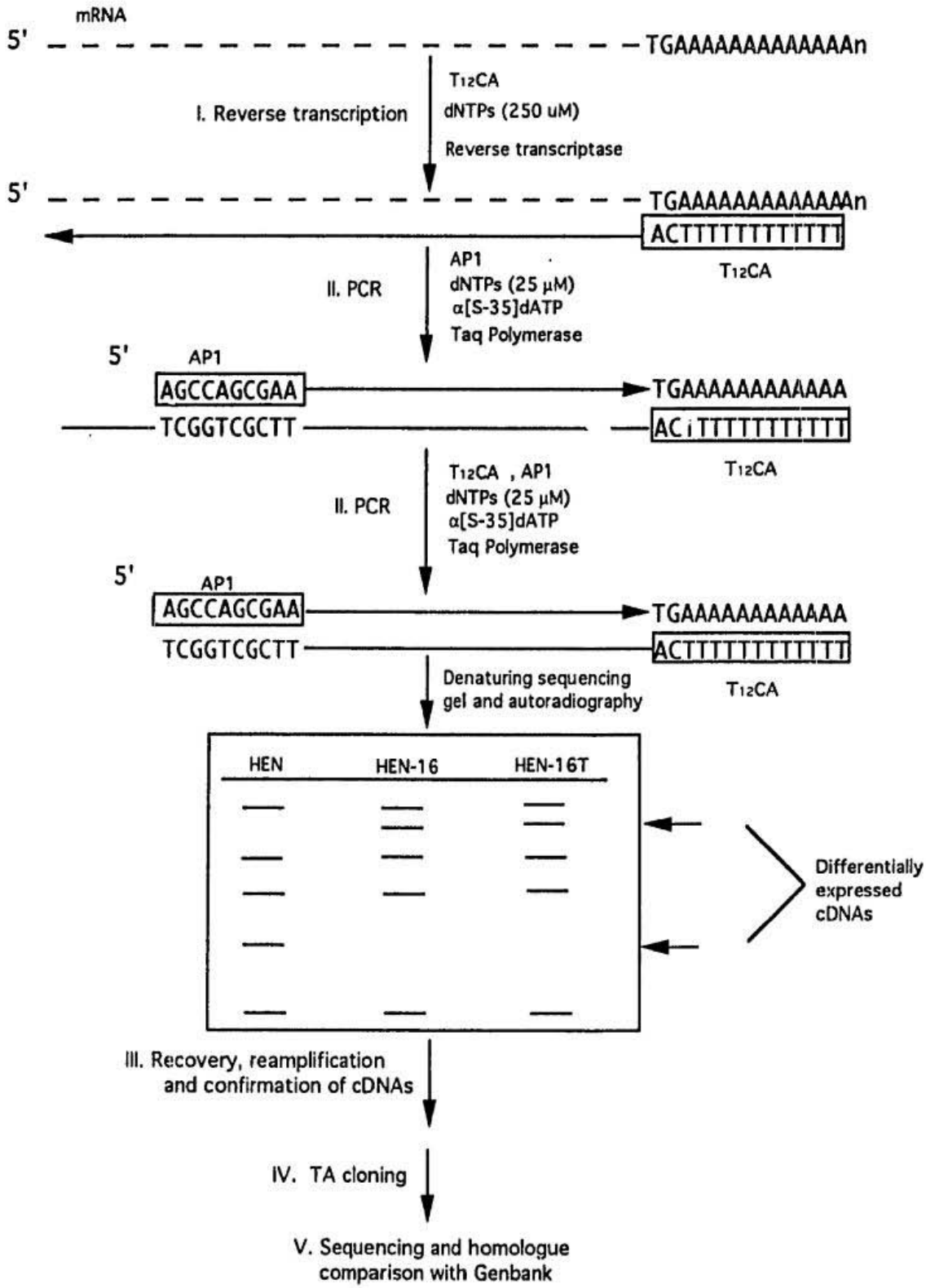

mRNA
5'
TGAAAAAAAAAAAAn
I. Reverse transcription
T12CA
dNTPs (250 uM)
Reverse transcriptase
5'
TGAAAAAAAAAAAAn
ACTTTTTTTTTTT
T12CA
II. PCR
AP1
dNTPs (25 µM)
α[S-35]dATP
Taq Polymerase
5'
AP1
AGCCAGCGAA
TGAAAAAAAAAAAA
TCGGTCGCTT
AC i TTTTTTTTTTT
T12CA
II. PCR
T12CA , AP1
dNTPs (25 µM)
α[S-35]dATP
Taq Polymerase
5'
AP1
AGCCAGCGAA
TGAAAAAAAAAAAA
TCGGTCGCTT
ACTTTTTTTTTTT
T12CA
Denaturing sequencing
gel and autoradiography
HEN
HEN-16
HEN-16T
Differentially
expressed
cDNAs
III. Recovery, reamplification
and confirmation of cDNAs
IV. TA cloning
V. Sequencing and homologue
comparison with Genbank

Table 5 List of 5' primers used in differential display assays

Primers	Sequences (5' to 3')
AP1	A G C C A G C G A A
AP2	G A C C G C T T G T
AP3	A G G T G A C C G T
AP4	G G T A C T C C A C
AP5	G G T C C C T G A C
AP6	G G T C C C T G A C
AP7	G A A A C G G G T G
AP8	G T G A C G T A G G
AP9	G G G T A A C G C C
AP10	G T G A T C G C A G
AP11	C T G A T C C A T G
AP12	C T T G A T T G C C
AP13	C T G C T C T C A
AP14	C T C T A G C A T G

for 30 sec, 40 °C for 2 min and 72 °C for 30 sec for a total of 40 cycles; then, 72 °C for 5 min for 1 cycle; and, finally, 25 °C on hold. Fifth, the PCR products were separated on 6 % sequencing gels at 100 watts for 4 hr, dried without fixation to allow recovery of PCR products, and exposed to X-ray film 0.5 to 7 days.

2.11.2 Recovery, reamplification and confirmation of differentially expressed cDNA

The autoradiogram was oriented with the gel after developing the film. The differentially expressed cDNAs were identified by comparing duplicate of HEN, HEN-16, HEN-16T cDNA bands on the film (Figure 6). The bands were located and cut from the 3MM paper. The gel slice along with the 3 M paper was soaked in 100 ul H_2O for 10 min, boiled for 15 min, and centrifuged in the eppendorf tube to pellet the gel and paper debris. The supernatant was transferred to another tube, precipitated with sodium acetate, glycogen (10 mg/ml), and ethanol. The pellet was dissolved in 10 µl H_2O and 4 µl was used for reamplification. The reamplification was done using the same primer set and PCR conditions except the dNTP concentration was 25 µM.

To determine if the size of reamplified PCR products were

consistent with their size on the DNA sequencing gel, the cDNAs were subsequently extracted from the 1.5% agarose gel using Schleicher & Schuell NA45 DEAE membranes and protocols. The isolated cDNA was used as probe to verify the differential expression of mRNA on Northern blot assays as described in Section 2.7.2.

2.11.3 Cloning

The cDNAs that were differentially expressed on Northern blot assays were cloned into the PCR II vector using the TA Cloning System (Invitrogen) following the manufacturer's instructions. In brief, a single deoxyadenosine was added to the 3'-end of PCR cDNA molecules because of the non-template-dependent acivity of thermostable Taq polymerase during PCR. These cDNAs with 3' A-overhangs were then ligated into PCR II vectors which contained single 3' T-overhangs. The ligated plasmids with cDNA inserts were then transformed into TA Cloning OneShot competent cells and spread on LB plates containing 50 µg/ml ampicillin and covered by 40 mg/ml X-Gal for blue/white colony selection. The white colonies were subsequently selected and grown in 2 ml LB medium at 37 ^{0}C overnight. The plasmids were purified by the small-scale plasmid purification method described by Sambrook et al. (1989). The plasmids were digested with EcoRI to check the

size of cDNA inserts.

2.11.4 Sequencing and Genbank data bank search for homology

The positive clones that had inserts with the same size as the original cDNA were sequenced using the Sequenase Version 2.0 DNA Sequencing System (United States Biochemical Co.) or the f-mol DNA Sequencing System (Promega), following the manufacturers' instructions.

The sequences of the reproducibly differentially expressed cDNAs were compared with those in the DNA sequence databases, including GenBank Release 86.0; GenBank cumulative daily updates to the major release (February 1, 1995); EMBL DATA Library, Release 41.0; and EMBL Data Library cumulative daily updates to the major release (February 1, 1995), through the NIH BLAST e-mail Server.

CHAPTER 3

RESULTS

3.1 Tumorigenicity of CSC-treated cell lines

Two HPV16-immortalized endocervical cell lines, HEN-16 and HEN-16-2, were either untreated or treated with 75, 100 and 125 μg/ml of CSC and then injected into nude mice. SiHa cervical carcinoma cells served as a positive control. Table 6 summarizes the results for each cell line.

All the treatments except treatment of HEN-16 by 100 μg/ml of CSC led to the formation of tumors in nude mice. The tumors were generally apparent after 3 weeks and continued to grow until 2-3 months. Seven of the 10 nude mice injected with CSC-treated HEN-16 and 4 of the 6 nude mice injected with CSC-treated HEN-16-2 cells produced tumors. SiHa cells gave rise to tumors for all injected nude mice. None of the untreated immortalized cells induced tumors in nude mice after 2 months, indicating that the induction of tumors in nude mice by CSC-treated immortalized cells was due to CSC treatment. In addition, the occurrence and size of the tumors were not correlated with the concentration of CSC, suggesting that immortalized cells would have been transformed at lower levels

Table 6 Tumorigenicity of HPV16-immortalized and CSC-treated immortalized cells in nude mice

Cell lines	Passages	CSC treatment	Tumor incidence (No. tumor/no. injected)	Tumor size (cm)
HEN-16	75-81	-	0/10	-
	75-81	+	7/10	0.8-1.2
HEN-16-2	85-113	-	0/6	-
	85-113	+	4/6	0.6-2.0
SiHa	-	-	6/6	2.0

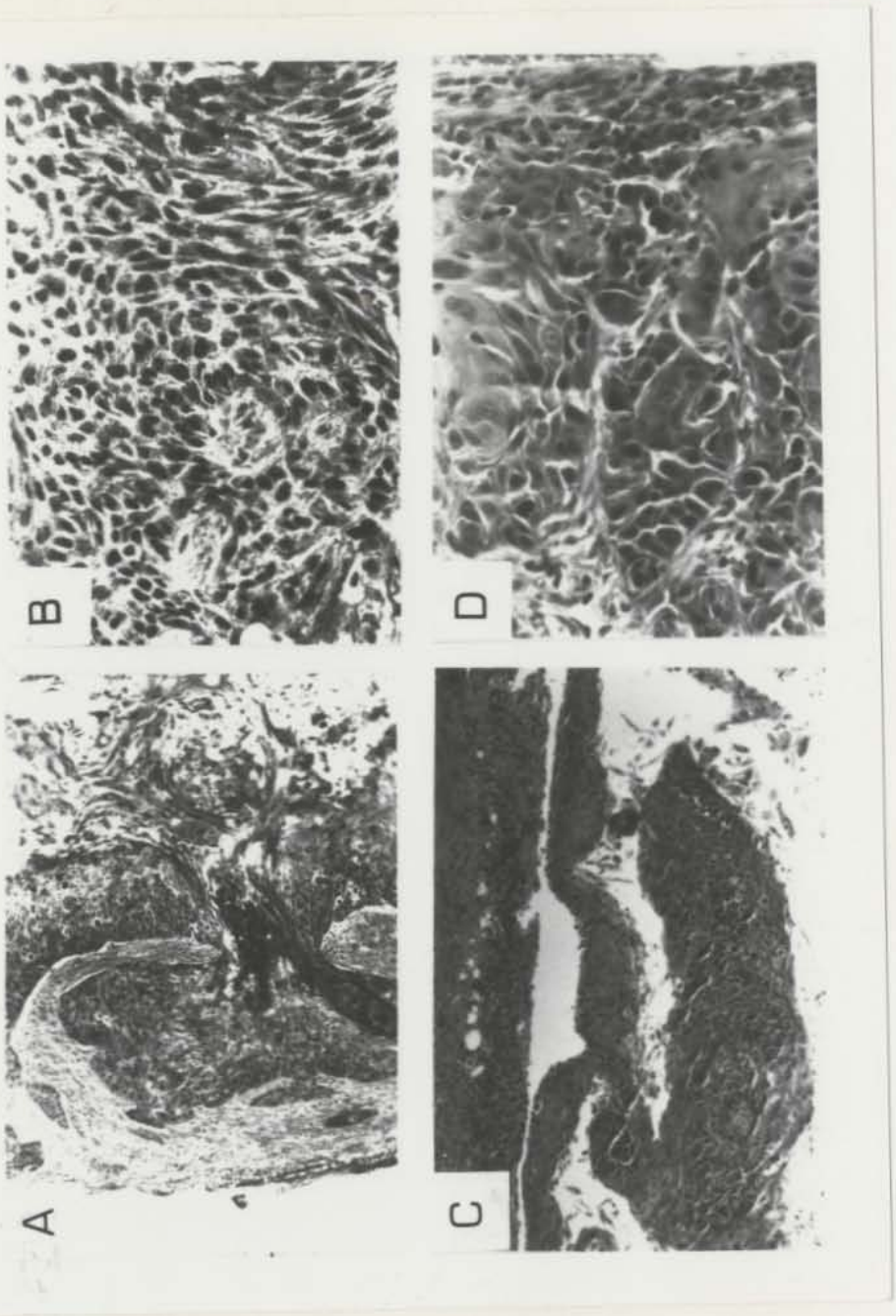
of CSC.

The tumors formed in nude mice were excised. Half of these were used to establish CSC-transformed cell lines, designated HEN-16T and HEN-16-2T, respectively, and used for other analyses. The other half of the tumors was used for pathological analysis. Figure 7 is an example of histology of tumors transformed after CSC-treatment of immortalized cells. All the tumors displayed the squamous cell carcinoma (SCC) phenotype, which was characterized by a local increase in cell number, loss of normal arrangement of cells, increase in nucleus/cytoplasm ratio and density of staining, and infiltration of tumor cells into or around the normal mouse tissues (Figure 7).

3.2 Morphology of primary cells and HPV16-immortalized and CSC-transformed cell lines

Compared with normal cells, tumor cells have obvious morphological abnormalities *in vitro*. Therefore, the morphology of primary, HPV16-immortalized untransformed, and

Figure 7. Histology of tumors formed by CSC-treated immortalized cell lines. Panels A and B, and C and D represent tumors used to produce HEN-16T and HEN-16-2T, respectively. The tumors formed in nude mice were excised, fixed in paraformaldehyde, paraffin-embedded, sectioned with a microtome, stained with hematoxylin and eosin, and observed with light microscopy (Original magnification: A,C, X100; B,D, X400).

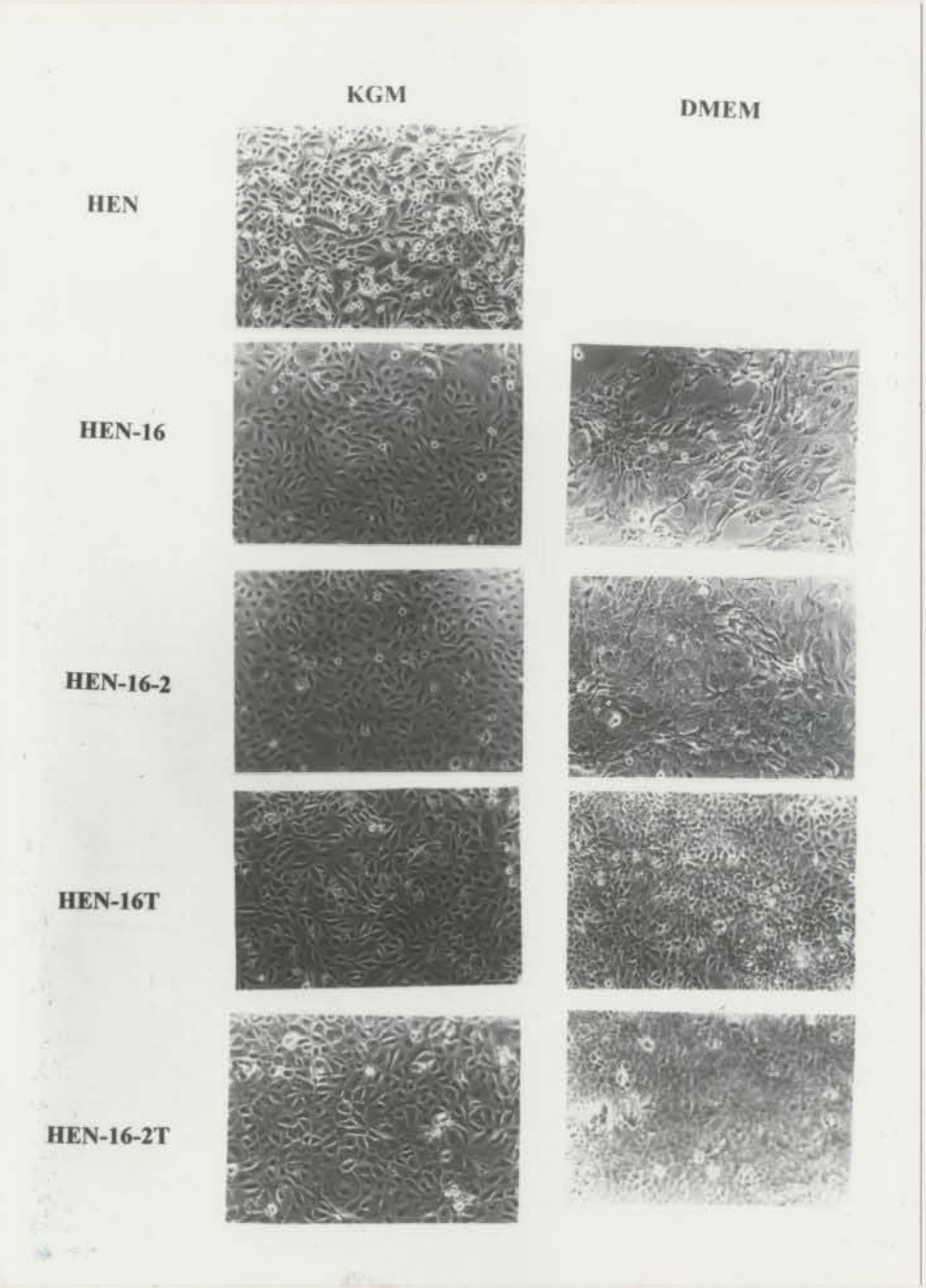

B
D
A
C

CSC-transformed cells was compared.

3.2.1 Monolayer culture

In monolayer cultures, cells were grown in either KGM or DMEM. In KGM, a serum-free medium that contains 0.15 mM calcium (see Appendix I), all the cells grew actively and formed monolayers of keratinocyte-like cells (Figure 8). However, when the cells were cultured in DMEM, a medium containing 10% FCS and a physiological level of calcium (1.5 mM) with reduced amount of growth factors (see Appendix I), HEN stopped growing and died after 10 days. The two immortalized untransformed cell lines, HEN-16 and HEN-16-2, grew slowly and formed flattened, dendritic (having branched cytoplasm), and uneven sized cells, which were distributed heterogeneously in the tissue culture plates. On the other hand, the two CSC-transformed immortalized cells, HEN-16T and HEN-16-2T, grew well, had increased nucleus/cytoplasm ratio and displayed a morphology comparable to that in KGM (Figure 8).

Figure 6. Morphology of cervical cells grown in monolayers with KGM and DMEM. Each cell line is indicated to the left of the figure. Left panel represents cervical cells grown in KGM. Right panel represents cervical cells grown in DMEM. HEN could not grow in DMEM. HEN was used at passage 2. HEN-16, HEN-16-2, HEN-16T and HEN-16-2T were used at passage 75, 86, 80 and 91, respectively (Original magnifications: X100).


KGM
DMEM
HEN
HEN-16
HEN-16-2
HEN-16T
HEN-16-2T

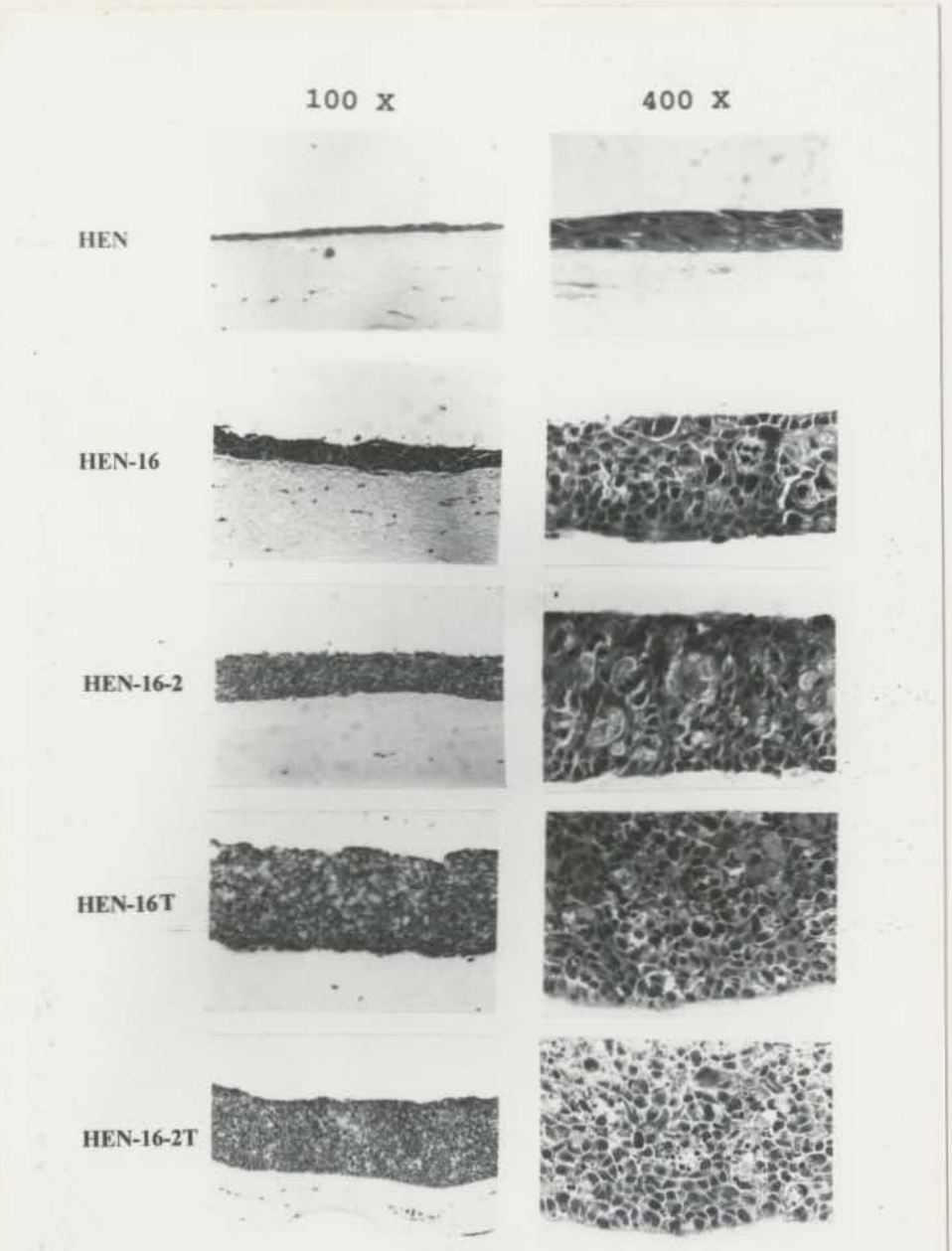
3.2.2 Raft culture

In the raft culture system, HEN cells formed thin well-differentiated stratified squamous epithelia that resembled normal endocervical epithelia *in vivo* with well-defined basal, suprabasal, and superficial layers (Figure 9). In contrast, both HEN-16 and HEN-16-2 proliferated extensively and formed thicker, aberrant, disorganized epithelia (Figure 9) resembling moderate to severe cervical intraepithelial neoplasia (CIN II-III) *in vivo*. HEN-16T and HEN-16-2T proliferated even faster than their respective immortalized cells and produced thicker, more undifferentiated layers of cells (Figure 9) similar to CIN III or carcinoma *in situ*.

3.3 Growth characteristics of primary cells and HPV16-immortalized and CSC-transformed cell lines

To characterize the growth potential of normal, immortalized and CSC-transformed cells, I examined their proliferation in two types of media, KGM and DMEM, and their

Figure 9. Morphology of endocervical cells grown in raft culture. Each cell line is indicated to the left of the figures. Left panels show X100 original magnification; Right panels show X400 original magnification. The cells differentiate away from the mouse fibroblasts, shown below. The passage number for each cell line was as described in Figure 8.


100 X
400 X
HEN
HEN-16
HEN-16-2
HEN-16T
HEN-16-2T

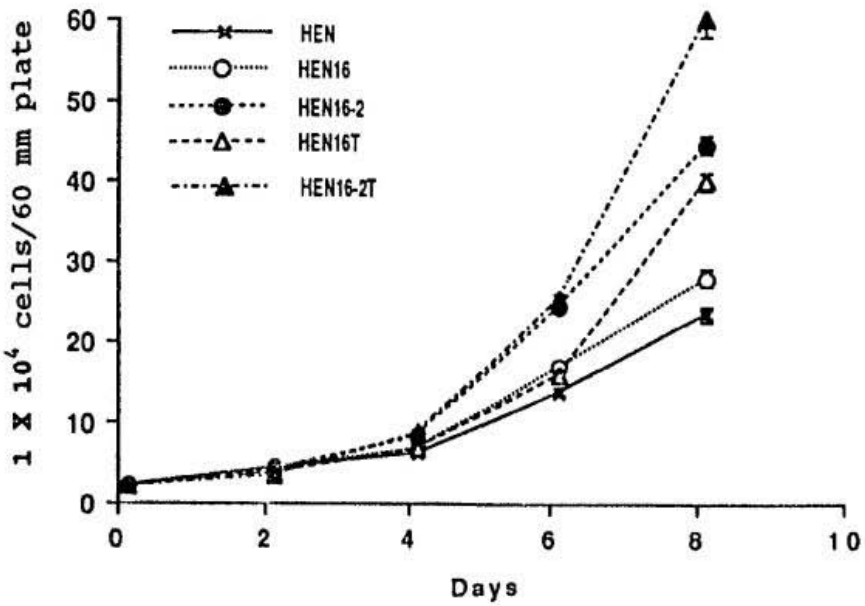
anchorage-independent growth in soft agar.

In KGM, HEN-16T and HEN-16-2T cells proliferated slightly higher than their untransformed immortalized counterparts, HEN-16 and HEN-16-2, respectively, but all four lines proliferated faster than HEN cells (Figure 10 A). The doubling times of HEN, HEN-16, HEN-16-2, HEN-16T, and HEN-16-2T were 43, 40, 36, 36, and 32 hr, respectively. However, in DMEM, HEN did not proliferate, and HEN-16 and HEN-16-2 proliferated more slowly than in KGM, whereas HEN-16T and HEN-16-2T proliferated much faster than HEN-16 and HEN-16-2 (Figure 10B). The doubling times of HEN-16, HEN-16-2, HEN-16T, and HEN-16-2T were 109, 43, 35, and 28 hr, respectively. In addition, CSC-transformed cells obtained higher saturation density than their immortalized counterparts. The saturation density of HEN, HEN-16, HEN-16-2, HEN-16T, and HEN-16-2T was 8.1 X 10^5, 1.1 X 10^6, 1.5 X 10^6, 1.6 X 10^5, and 2.1 X 10^6 cells/60 mm dish, respectively.

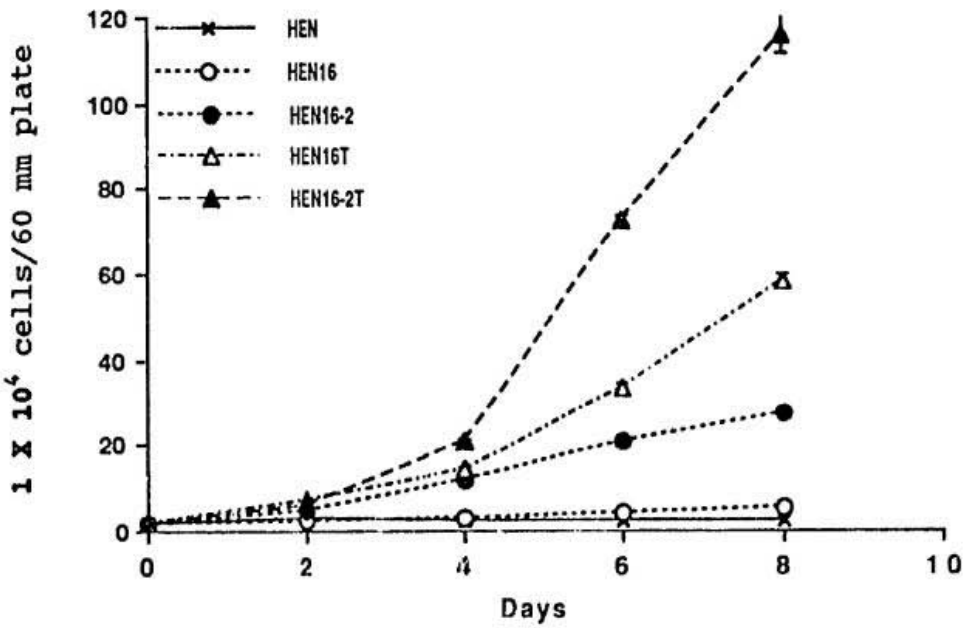
Oncogenicity is correlated with anchorage-independent growth of tumor cells. To characterize this oncogenic phenotype, soft agar assays were performed, in which all the

Figure 10. Proliferation of endocervical cells in KGM (A) and DMEM (B). Each value represents the mean and standard error (S.E) calculated from three independent experiments. Bars indicate S.E. See the text for details.

A


HEN
HEN16
HEN16-2
HEN16T
HEN16-2T
1 X 10⁴ cells/60 mm plate
Days
0
10
20
30
40
50
60
0
2
4
6
8
10

B

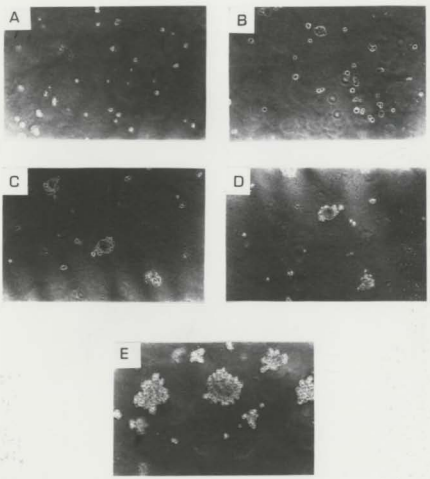

HEN
HEN16
HEN16-2
HEN16T
HEN16-2T
1 X 10⁴ cells/60 mm plate
Days
0
20
40
60
80
100
120
0
2
4
6
8
10

immortalized and CSC-transformed tumor cells and SiHa were allowed to grow in soft agar for 2-4 weeks. Generally, the formation of colonies was observable after one week of incubation, and the colonies were unequivocally identified after 2 weeks. HEN-16 (Figure 11A) and HEN-16-2 (Figure 11B) remained as single cells and did not form colonies in soft agar, whereas HEN-16T (Figure 11C) and HEN-16-2T (Figure 11D) formed colonies that were smaller than those formed by SiHa (Figure 11E).

3.4 Presence and expression of HPV16 DNA in immortalized and CSC-transformed cells

Increased viral expression or alteration of viral sequences was regarded as one of the mechanisms for tumorigenicity of HPV-immortalized cells (Cullen et al., 1991). To study the role of HPV in oncogenesis from primary cervical cells, the presence and expression of HPV16 were analyzed by Southern and Northern blot hybridization assays using whole length HPV16 DNA as probe.

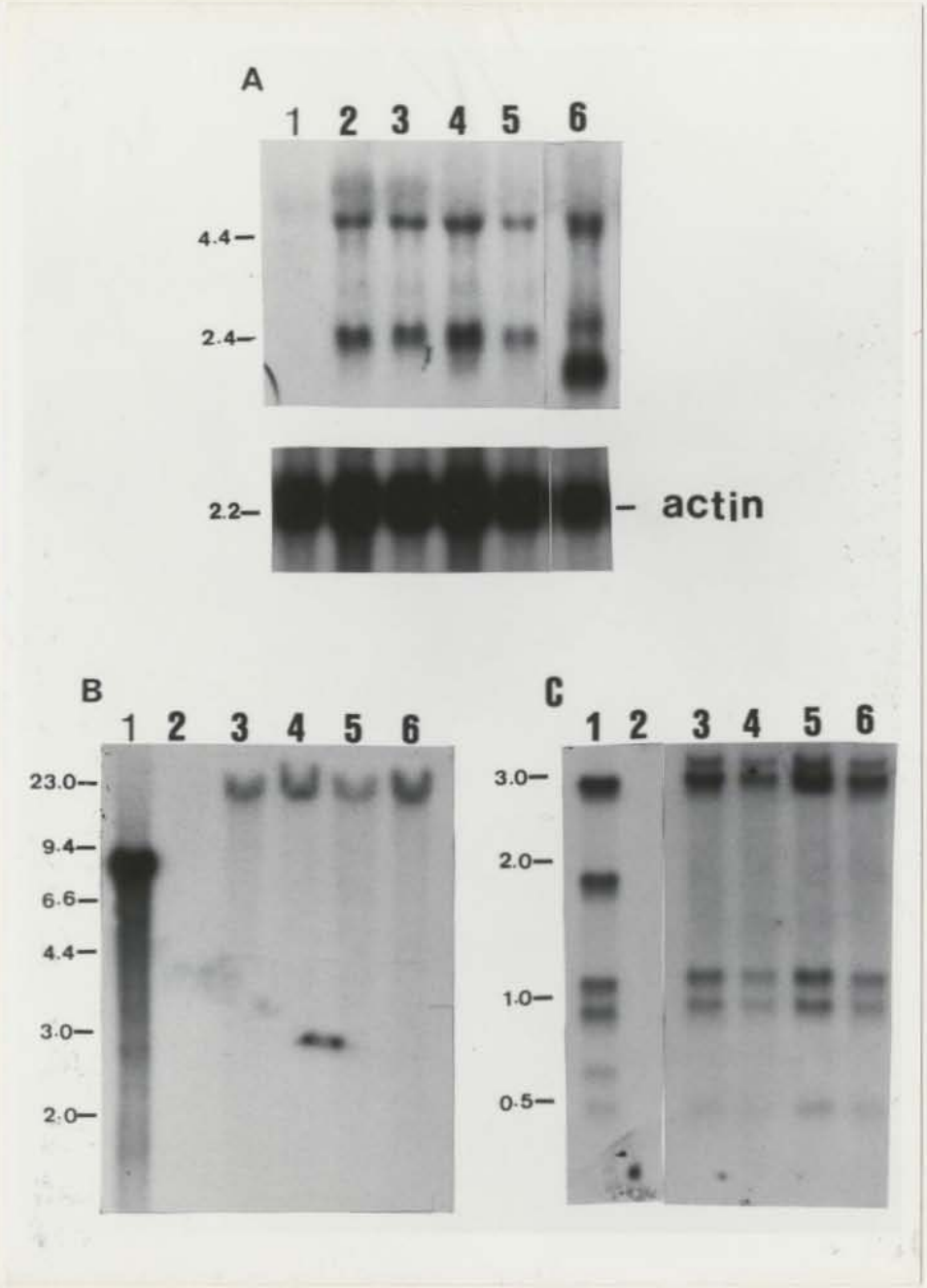
Figure 11. Anchorage-independent growth of CSC-transformed cells. A, HEN-16; B, HEN-16-2; C, HEN-16T; D, HEN-16-2T; E, SiHa. See the text for details. (Original magnification: X100.)


A
B
C
D
E

HEN-16T and HEN-16-2T high molecular weight DNA showed the same pattern of hybridization with the HPV16 probe as their immortalized counterparts, HEN-16 and HEN-16-2. After digestion with BamHI, which cleaves at a single site in the HPV16 genome, a single band about 20.0 kb, but not the 7.9 kb whole length HPV16 DNA, was detected (Figure 12B, lanes 1, 3-6). After digestion with BamHI/PstI, five bands of 3200 bp, 2817 bp, 1063 bp, 908 bp and 483 bp hybridized with the HPV16 probe, whereas two bands of 1776 bp and 641 bp failed to hybridize with the HPV16 probe, compared with the HPV16 DNA control (Figure 12C, lane 1, 3-6). These fragments contain sequences for the L1 ORF. In all cases, no HPV16 DNA was detected from HEN high molecular weight DNA (Figure 12B, C, lanes 2).

Northern analysis using HPV16 DNA as probe also showed that both immortalized and CSC-transformed cells expressed similar levels of the 2.3 kb and 4.5 kb mRNAs, which were different from those of the cervical carcinoma cell line, CaSki, in both pattern and level of mRNA expression (Figure 12A).

Figure 12. Expression and detection of HPV16 DNA in cervical cells. A) Northern blotting. Total RNA (20 μg) from HEN (lane 1), HEN-16 (lane 2), HEN-16-2 (lane 3), HEN-16T (lane 4), HEN-16-2T (lane 5), and CaSki HPV positive cell line (lane 6) was hybridized with the ^{32}P-labeled full-length HPV16 DNA. After the blot was exposed to X-ray film, the probe was stripped from the blot, and the blot was then hybridized for the internal gel loading and hybridization control, γ-actin (lower panel). B), C) Southern blot hybridization of 10 μg high-molecular weight, BamHI-digested (B) or BamHI/PstI-digested (C), cellular DNA with full-length ^{32}P-labeled HPV16 DNA. Lanes 1, HEN + HPV16 (100 pg); lanes 2, HEN; lanes 3, HEN-16; lanes 4, HEN-16-2; lanes 5, HEN-16T; lanes 6, HEN-16-2T. DNA or RNA molecular weight markers were used in separate lanes and are shown in kb on the left of each figure.


A
1 2 3 4 5 6
4.4
2.4
2.2
actin
B
1 2 3 4 5 6
23.0
9.4
6.6
4.4
3.0
2.0
C
1 2 3 4 5 6
3.0
2.0
1.0
0.5

3.5 Expression of cancer-related cellular genes

3.5.1 Oncogenes

Several studies have demonstrated that the activation of oncogenes has an important role in the pathways for the transformation of primary cervical cells (Durst et al., 1987; Crook et al., 1990). Therefore, the expression of two oncogenes, c-*myc* and H-*ras*, which had been shown to be amplified in cervical carcinomas, and B-*myb*, a newly identified oncogene that was transactivated by HPV E7 oncoprotein in cycling NIH3T3 cells *in vitro* (Lam et al., 1994), were examined in primary, immortalized, and CSC-transformed cells.

The levels of 2.4 kb c-*myc* and 1.3 kb H-*ras* mRNA transcripts were similar in all five cell types (Figure 13 and Table 7). While the expression of B-*myb* in was 17.8-27.0 times higher in immortalized cells than that in HEN cells, it was 1.9 times higher in HEN-16T CSC-transformed tumor cells than in HEN-16 immortalized cells (Figure 13 and Table 7).

3.5.2 Tumor suppressor genes

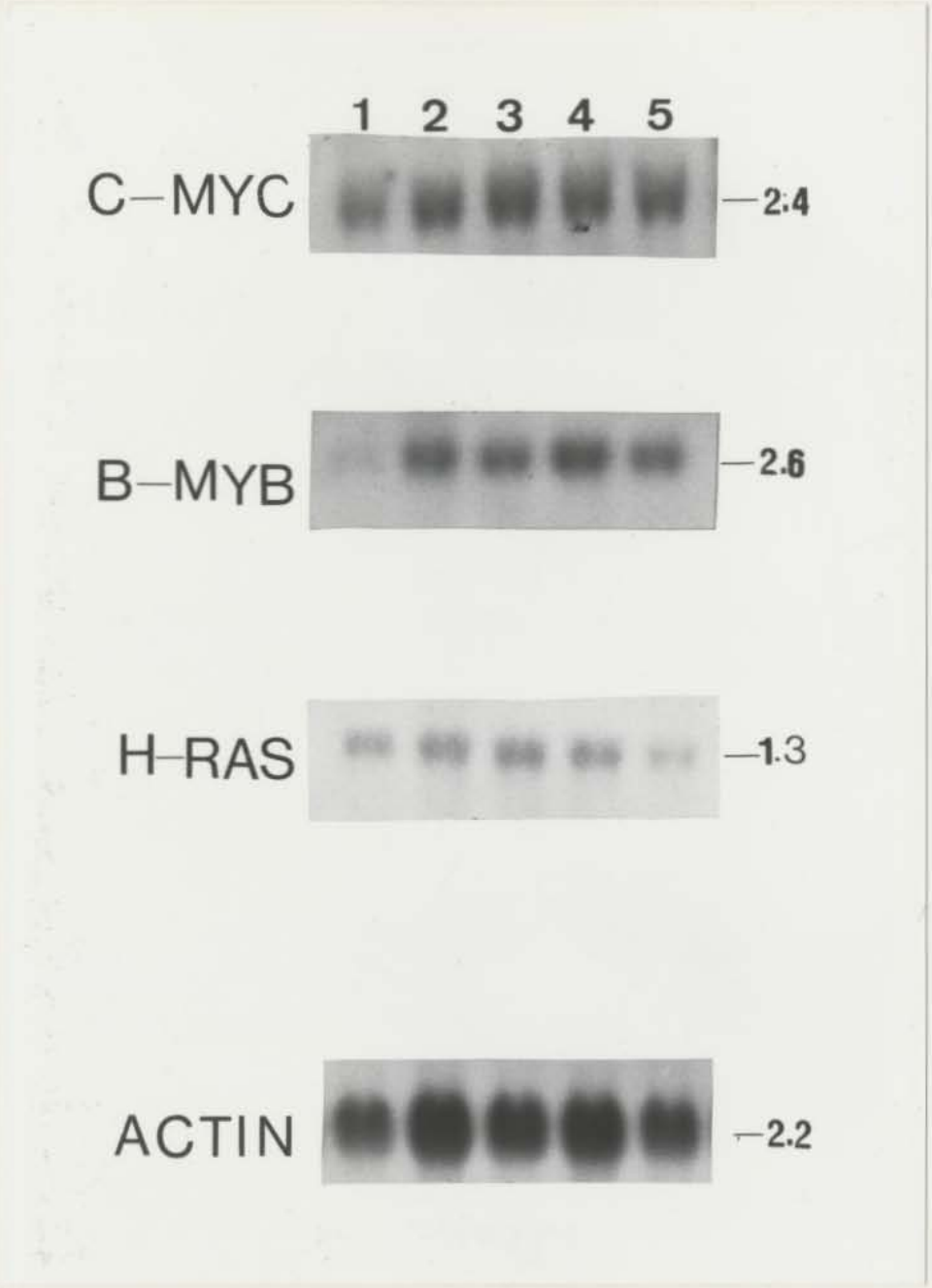
To study whether the inactivation of tumor suppressor

Table 7. Transcription of cellular genes in primary and immortalized, and CSC- transformed cells

Genes	Relative expression level*				
	HEN	HEN-16	HEN-16-2	HEN-16T	HEN-16-2T
c-*myc*	1	1.1	1.4	1.2	1.3
H-*ras*	1	1.1	1.3	1.1	0.8
B-*myb*	1	18.8	28.0	36.6	31.4
p53	1	8.7	5.3	8.5	4.8
Waf1	1	0.5	0.4	0.8	0.2
PCNA	1	2.1	3.2	7.1	5.0
GADD45	1	0.9	0.8	1.1	1.0
GADD153	1	0.4	2.8	1.9	1.0
Fibronectin	1	0.3	0.03	0.01	0.01

*The levels of transcription of each cellular gene mRNAs in HEN-16, HEN-16-2, HEN-16T, and HEN-16-2T were quantified relative to those of HEN after their normalization to the levels of actin mRNAs. RNAs were quantified by measuring the optical density of each message at medium exposure on X-ray film using densitometer.

Figure 13. Expression of mRNA for c-*myc*, B-*myb*, and H-*ras* in cervical cells. Lane 1,HEN; lane 2, HEN-16; lane 3, HEN-16-2; lane 4, HEN-16T; lane 5, HEN-16-2T. Northern blot hybridization of 20 μg total cellular RNA from the five indicated cell types with c-*myc* was carried out first, as described in Section 2.7.2. After the blot was exposed to X-ray film, the probe was stripped from the blot for sequential hybridizations for B-*myb*, H-*ras*, and γ-actin. The size of each message is shown in kb on the right of each panel and was determined from size markers in a separate lane.


1 2 3 4 5
C–MYC
2.4
B–MYB
2.6
H–RAS
1.3
ACTIN
2.2

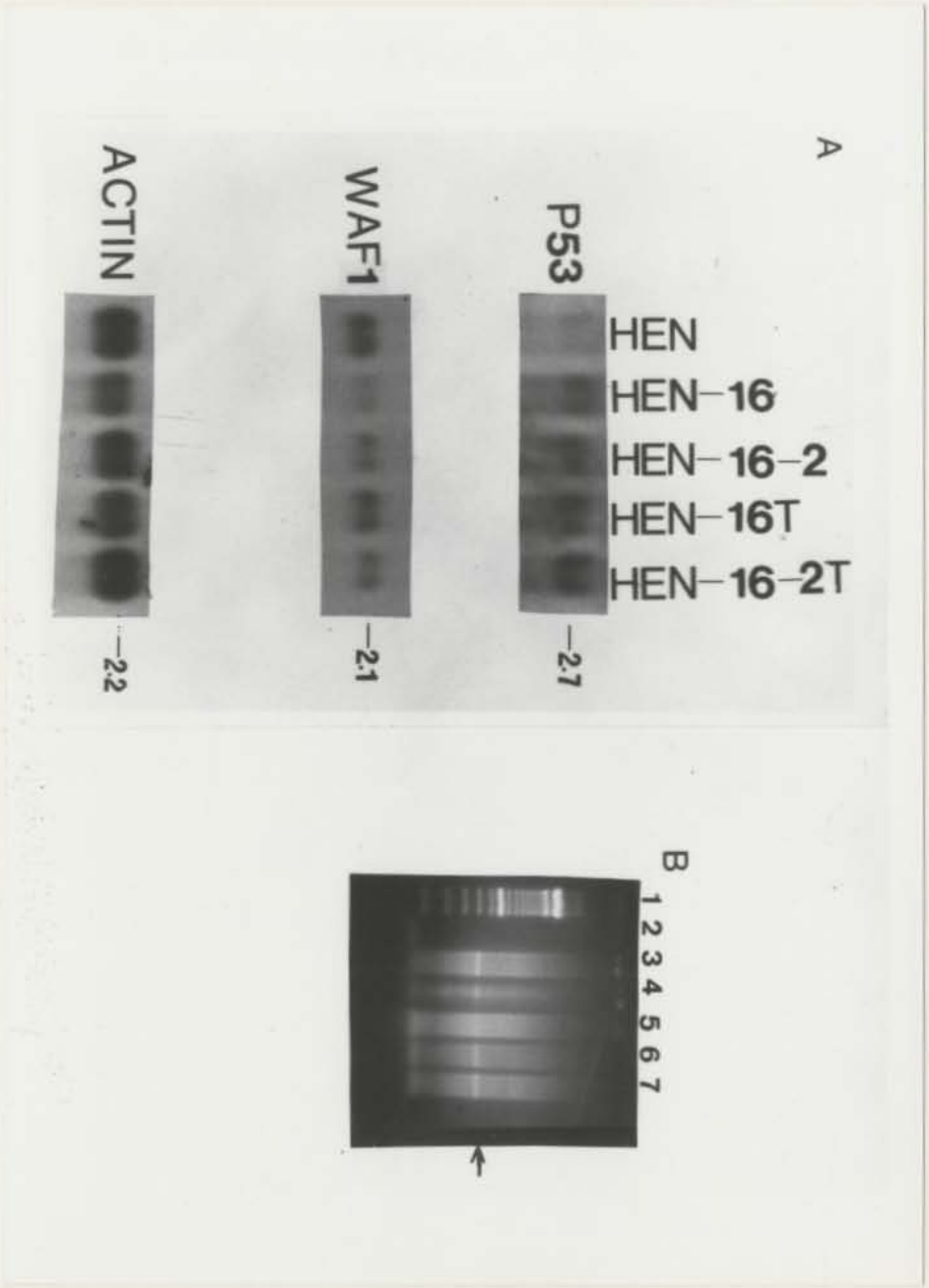
genes was involved in the *in vitro* oncogenesis of primary cervical cells, the expression of three tumor suppressor genes, p53, WAF1/Cip1/Sid1, and DCC, was examined in my unique *in vitro* model system using Northern blot and RT-PCR methods.

While the expression of p53 was 5 to 9 times enhanced in immortalized and CSC-transformed cells compared to primary endocervical cells, there was no consistent difference between immortalized cells and CSC-transformed cells (Figure 14 and Table 7). The level of the 2.1 kb Waf1 mRNA transcript was similar in immortalized and CSC-transformed cells, but all four were 20-80% lower than that in HEN cells (Figure 14A and Table 7).

Since the expression of DCC was very low, the RT-PCR method was employed to check if DCC was deleted in immortalized and CSC-transformed cells. The expected 400 bp fragment, which was frequently deleted in some carcinoma cells (Gao et al., 1993; Klingelhuz et al., 1993; Thompson et al., 1993), was detected in all the cell lines used and was negative in the no RNA, water control (Figure 14B).

3.5.3 DNA replication and repair genes

Figure 14. Expression of mRNA for p53, Waf1 and DCC in cervical cells. A) Northern blot hybridization of 3 mg mRNA to p53, Waf1, and actin probes was carried out as described in Section 2.7.2. Conditions and labels are described in Figure 13. B) RT-PCR of DCC. Lanes are: 1, 100 bp ladder; 2, No RNA control; 3, HEN; 4, HEN-16; 5, HEN-16-2; 6, HEN-16T; 7. HEN-16-2T. The 400 bp amplified DCC cDNA is indicated on the right of the figure (arrow).


A
P53
WAF1
ACTIN
HEN
HEN-16
HEN-16-2
HEN-16T
HEN-16-2T
-2.7
-2.1
-2.2
B
1 2 3 4 5 6 7

To determine if deregulation of mRNA expression of genes for DNA replication and repair was involved in the immortalization and tumorigenesis, the expression of three genes implicated in DNA replication and repair, PCNA, GADD45, and GADD153, was examined in primary, immortalized, and CSC-transformed cells.

The expression of PCNA was 1.1-2.2 times higher in HEN-16 and HEN-16-2 cells than in HEN cells. On the other hand, PCNA mRNA expression is 2.4 times higher in HEN-16T cells than in HEN-16 cells, and 56% higher in HEN-16-2T cells than in HEN-16-2 cells (Figure 15 and Table 7).

To examine the PCNA protein level, indirect immunofluorescence assays were employed in monolayer and raft culture. In monolayers, the level of PCNA protein in HEN cells was very low and heterogeneously expressed, whereas the expression of PCNA protein was more apparent and more homogeneously expressed in HEN-16 and HEN-16-2 cells. Relative enhancement of PCNA protein was observed in some HEN-16-2T cells, but the highest expression of PCNA protein in the nucleus was detected in HEN-16T cells (Figure 16, monolayer lane). These results was comparable to those of mRNA

Figure 15. Expression of mRNA for PCNA, GADD45 and GADD153 in cervical cells. A, PCNA; B, GADD45; C, GADD153. lanes 1, HEN; lanes 2, HEN-16; lanes 3, HEN-16-2; lanes 4, HEN-16T; lanes 5, HEN-16-2T. Conditions and labels were as in Figure 13.

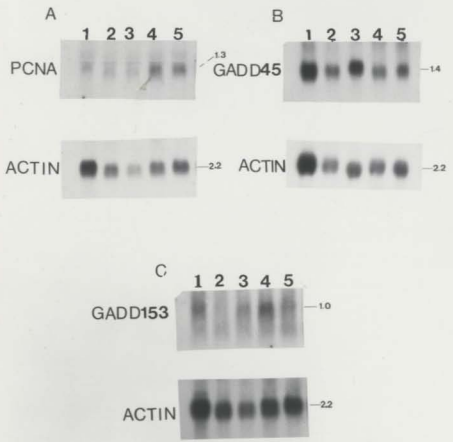
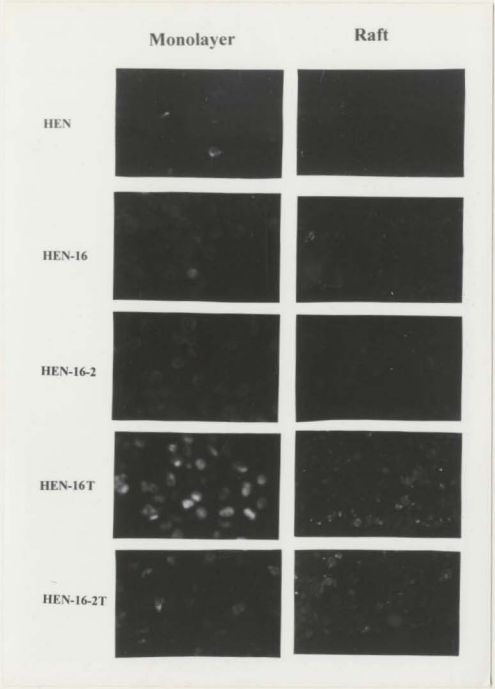

A
1 2 3 4 5
PCNA
1.3
ACTIN
2.2
B
1 2 3 4 5
GADD45
1.4
ACTIN
2.2
C
1 2 3 4 5
GADD153
1.0
ACTIN
2.2

Figure 16. Indirect immunofluorescence analysis of PCNA protein in cervical cells cultured in monolayer and raft system. The name of each cell type is indicated on the left. Left panels show the results for cells grown in monolayer. Right panels represent the results for cells grown in the raft system. Exposure time and printing conditions were identical for all photographs. (Original magnification: X400.)


Monolayer
Raft
HEN
HEN-16
HEN-16-2
HEN-16T
HEN-16-2T

expression studies (Figure 15C). In raft culture, the same trend of PCNA expression was observed as in monolayer culture: PCNA signal increased sequentially from primary endocervical cells to untransformed and to CSC-transformed cells (Figure 16, raft panels). Proliferation and PCNA confined to the basal layer of epithelia (Merrick et al., 1992). However, PCNA protein was present throughout the entire thickness of the epithelia for HEN-16, HEN-16-2, HEN-16T and HEN-16-2T cells. The signal was more pronounced in the tumor cell lines. The presence of PCNA in the basal layer in the raft was also observed by microscopy in HEN cells, but is not apparent in this picture.

No difference in GADD45 mRNA expression was observed in all cell lines (Figure 15B , Table 7). Relative to HEN, the expression of GADD153 was lower in HEN-16 and higher in HEN-16-2 cells. The expression of GADD153 mRNA increased 4.8-fold in HEN-16T and 2.8-fold in HEN-16-2T, compared with their immortalized counterparts (Figure 15C, Table 7).

3.5.4 Senescence-related fibronectin gene

Since primary endocervical cells usually die after 4-5 passages in tissue culture as a consequence of senescence, immortalization of these primary cells should bypass the senescence pathway. Thus, expression of the senescence-related gene, FN, was examined. As shown in Figure 17A and Table 7, the expression of FN was greatly diminished in both immortalized and CSC-transformed cells. Tumorigenesis led to a 30-fold decrease for HEN-16T and a 3-fold decrease for HEN-16-2T. *In situ* hybridization of monolayer tissue culture further confirmed that FN was expressed significantly, although heterogeneously, only in HEN cells but not in HEN-16, HEN-16-2, HEN-16T, and HEN-16-2T cells (Figure 17B). The FN signals were usually detected in the cytoplasm. In addition, indirect immunofluorescence of FN protein also showed that FN protein was abundant and heterogeneously expressed only in HEN cells but not in immortalized and CSC-transformed cells (Figure 18), which was consistent with the 10 times reduction of FN in HEN-16 after tumorigenesis (Figure 17).

Figure 17. Expression of FN mRNA in cervical cells.

A) Northern blot assays. Lane 1, HEN; lane 2, HEN-16; lane 3, HEN-16-2; lane 4, HEN-16T; lane 5, HEN-16-2T. Conditions and labels were as described in Figure 13. B) Detection of FN mRNA by *in situ* hybridization. HEN (a), HEN-16 (b), HEN-16-2 (c), HEN-16T (d), and HEN-16-2T (e) cells were grown in monolayer and then hybridized to biotin-labeled FN probe as described in Section 2.7.4. Exposure time and printing conditions were identical for all photographs. (Original magnification: X400.)

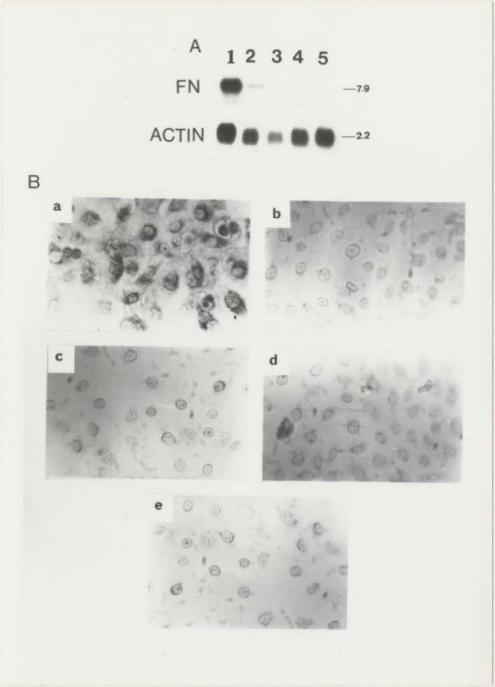
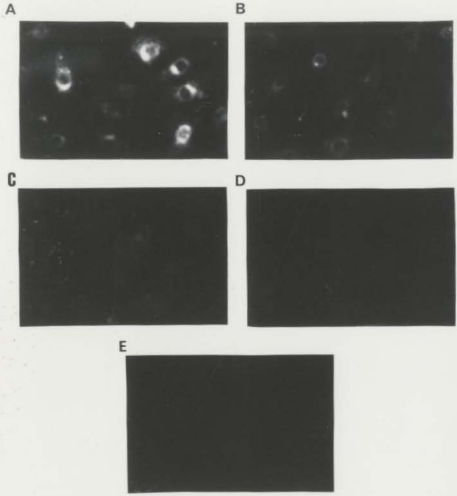

A
1 2 3 4 5
FN
—7.9
ACTIN
—2.2
B
a
b
c
d
e

Figure 18. Indirect immunofluorescence analysis of FN of cervical cells. Panels are: A, HEN; B, HEN-16; C, HEN-16; D, HEN-16T; and E, HEN-16-2T. Conditions were as described in Figure 16.


A
B
C
D
E

3.6 Response of primary, immortalized and tumor cells to TNF-α, TGF-β1, and RA

As discussed in the Introduction, loss of response to cytokines is regarded as a characteristic of some tumor cell lines. To understand the relationship between this process, immortalization and tumorigenicity, the effects of TNF-α, TGF-β1, and RA on cell proliferation, and the expression of HPV and some cellular genes were examined in primary, immortalized, CSC-transformed and CaSki cells.

3.6.1 TNF-α

Treatment of cervical cells with 250 U/ml of TNF-α inhibited, to a similar extent, proliferation of all cell lines including HEN, HEN-16, HEN-16-2, HEN-16T, HEN-16-2T, and CaSki (Figure 19) cells.

Treatment with TNF-α almost completely abolished the expression of HPV16, and greatly reduced that of c-*myc* in 3 of 5 cell types (Figure 20). In addition, no significant difference in the response of HPV and c-*myc* expression to TNF-α was found between immortalized cells and tumor cells (Figure

Figure 19. Effect of TNF-α on proliferation of cervical cells. The results represent the mean and S.E. of three experiments. Bars show S.E. See also the text for details.

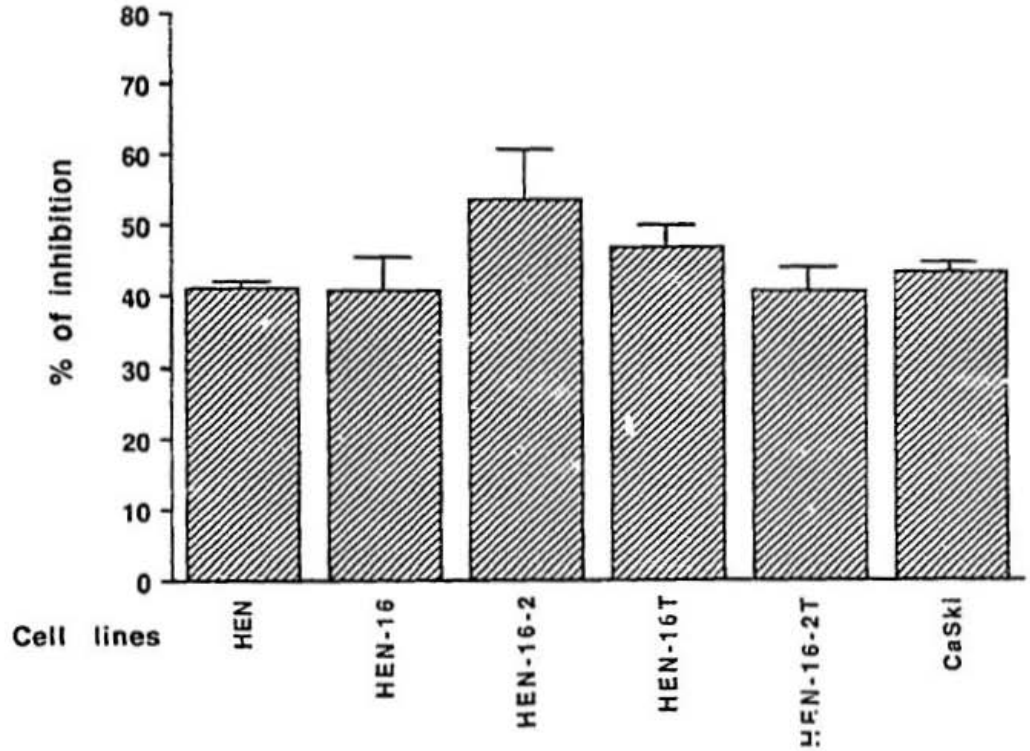
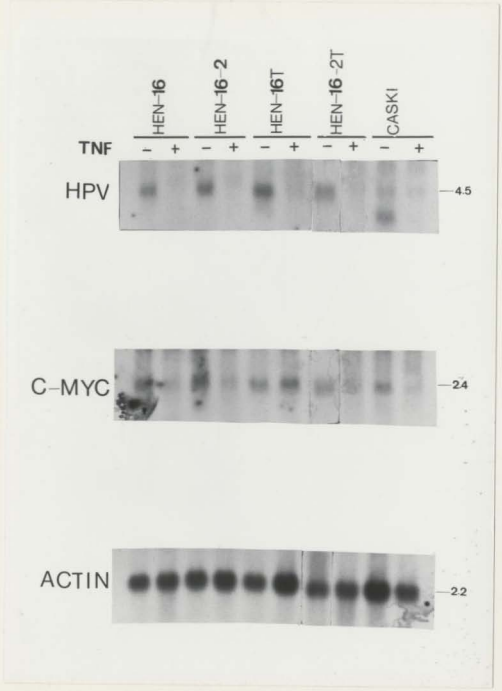

80
70
60
50
40
30
20
10
0
% of inhibition
Cell lines
HEN
HEN-16
HEN-16-2
HEN-16T
HEN-16-2T
CaSki

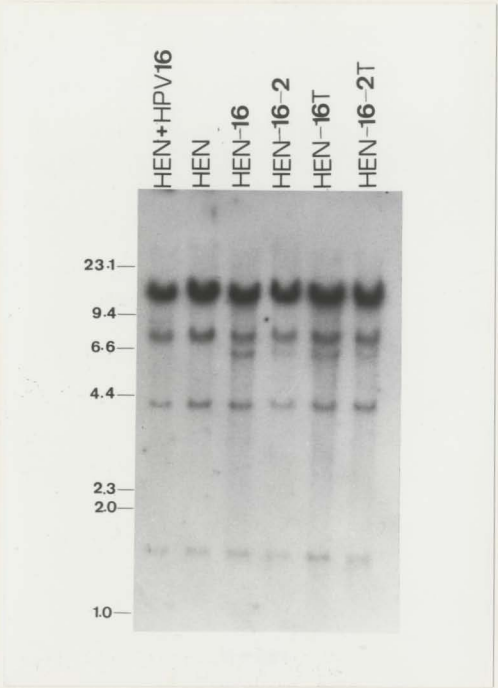
Figure 20. Effect of TNF-α on mRNA expression of HPV16 and c-*myc*. Northern blot hybridization of 20 μg RNA from TNF-α-untreated (-) and treated (+) cells with ^{32}P-labeled HPV16, c-*myc*, and γ-actin probes was carried out as described in Section 2.7.2. Conditions and labels were as described in Figure 13.


HEN-16
HEN-16-2
HEN-16T
HEN-16-2T
CASKI
TNF
- + - + - + - + - +
HPV
4.5
C-MYC
2.4
ACTIN
2.2

20).

Since it was suggested that the loss of JE or MCP1 gene expression and the induction of this expression by TNF-α is one of the important characteristics of tumor cells and that the JE/MCP1 gene played a pivotal role in intracellular communication by triggering an intracellular pathway which interferes with viral transcription in HPV-positive nontumorigenic cells (Rosl et al., 1994; zur Hausen and de Villiers, 1994b), the induction of JE/MCP1 mRNA after TNF-α treatment was examined. No induction of JE/MCP1 mRNA was observed after treatment of all immortalized and tumor cells with TNF-α (data not shown). In addition, Southern blot assays showed that JE gene genomic DNA could be detected easily in these cells (Figure 21). However, after digestion of high molecular weight DNA with BamHI, an extra band about 6.5 kb in size was detected only in immortalized and tumor cells (Figure 21).

Figure 21. Detection of JE gene in cervical cells. Southern blot hybridization of BamHI-digested high molecular weight DNA (10 μg) with labelled JE cDNA for the indicated cell lines was carried out as described in Section 2.8.2. Size markers are indicated on the left. See the text for details.


HEN+HPV16
HEN
HEN-16
HEN-16-2
HEN-16T
HEN-16-2T
23.1
9.4
6.6
4.4
2.3
2.0
1.0

3.6.2 TGF-β1

Treatment of cervical cells with 5 ng/ml TGF-β1 for 5 days inhibited proliferation by 78%, 79%, and 89% in HEN, HEN-16, and HEN-16-2 cells, respectively (Figure 22). The CSC-transformed cells were significantly less sensitive to TGF-β1 than primary and immortalized cells ($P < 0.05$). The growth inhibition of HEN-16T and HEN-16-2T was 58% and 56%, respectively (Figure 22). CaSki cells were resistant to the growth inhibitory effects of TGF-β1 (Figure 22). Therefore, the relative resistance to TGF-β1 growth inhibition was correlated with the tumorigenicity, but not immortalization, of the cells.

TGF-β1 treatment substantially inhibited the expression of HPV in all five HPV-containing cell lines: HEN-16, HEN-16-2, HEN-16T, HEN-16-2T, and CaSki (Figure 23). Densitometric analysis cnfirmed that the level of suppression of HPV expression was not related to the status of cells (data not shown). However, suppression of *c-myc* was most severe in HEN-16 and HEN-16-2, less severe in HEN-16T and HEN-16-2T, and not found in CaSki cells (Figure 23).

Figure 22. Effect of TGF-β1 on proliferation of cervical cells. The results represent the mean and S.E. of three experiments. *Percentage of growth inhibition was significantly lower ($p<0.05$), comparing HEN-16T and HEN-16-2T with HEN and with HEN-16 and HEN-16-2, respectively. Bars show S.E. See also the text for details.

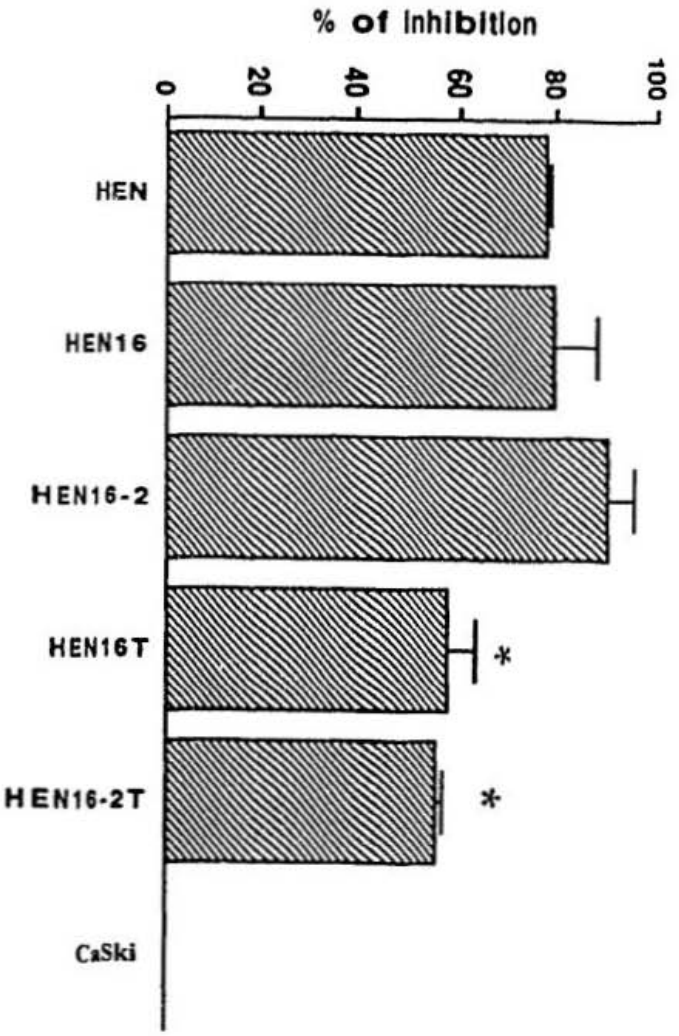
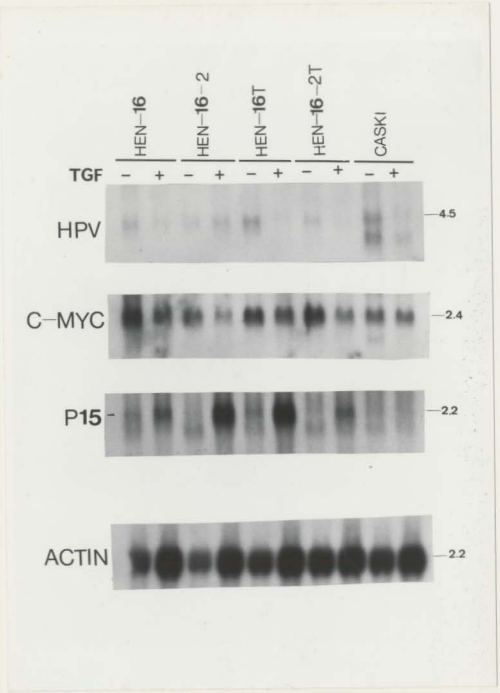

% of inhibition
0
20
40
60
80
100
HEN
HEN16
HEN16-2
HEN16T
HEN16-2T
CaSki
*
*

Figure 23. Effect of TGF-β1 on mRNA expression of HPV16, c-*myc* and p15. Northern blot hybridization of 20 μg RNA from TGF-β1-untreated (-) or -treated (+) cells with ^{32}P-labeled HPV16, c-*myc*, p15 and γ-actin was carried out as described in Section 2.7.2. Labels are as in Figure 20.


HEN-16
HEN-16-2
HEN-16T
HEN-16-2T
CASKI
TGF
– + – + – + – + – +
HPV
4.5
C-MYC
2.4
P15
2.2
ACTIN
2.2

Recently, a cyclin kinase inhibitor, p15 was cloned and identified as a potential effector of TGF-β1-induced growth arrest (Hannon and Beach, 1994). To study its possible role in differential inhibition of immortalized and tumor cells by TGF-β1, its induction was examined after treatment of immortalized and tumor cells with TGF-β1. Before treatment, p15 was not expressed in any of the cell lines examined. Treatment of the cells with TGF-β1 for 2 days caused the induction of p15 mRNA in both immortalized cells (HEN-16 and HEN-16-2) and CSC-transformed cells (HEN-16T and HEN-16-2T), whereas no induction of p15 was detected in CaSki cells (Figure 23).

3.6.3 RA

Treatment of cervical cells with 3 μM RA for 5 days resulted in growth inhibition of HEN, HEN-16, and HEN-16-2 67%, 60% and 63%, respectively (Figure 24). However, growth inhibition was very low (27%) in HEN-16T and undetectable (0%) in HEN-16-2T, and CaSki cells (Figure 24).

Interestingly, RA treatment did not have any significant

Figure 24. Effect of RA on proliferation of cervical cells.

Conditions and labels were as described in Figure 22.

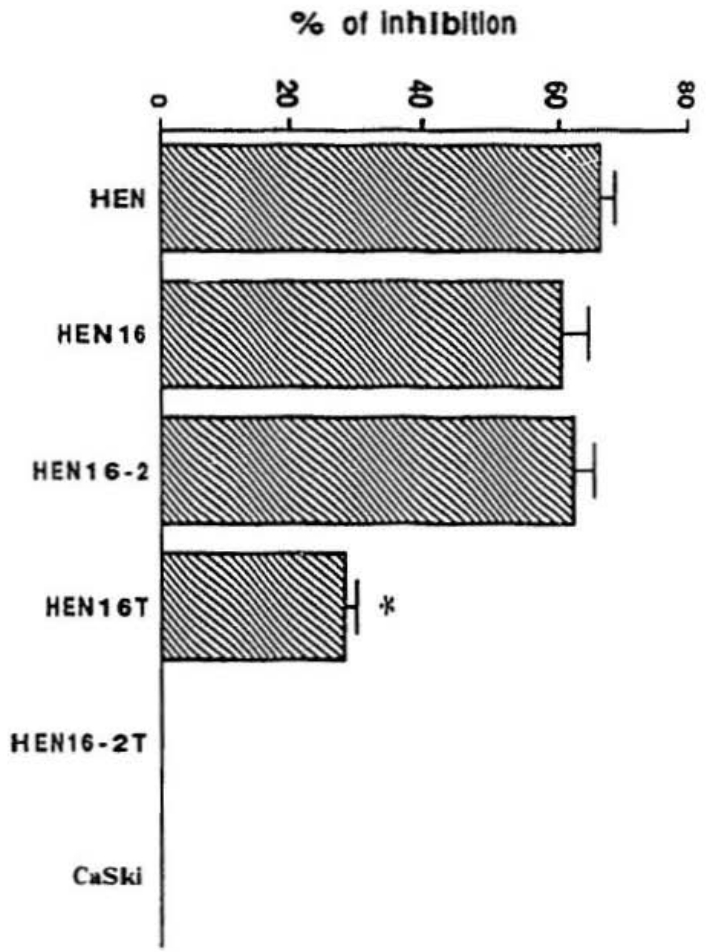
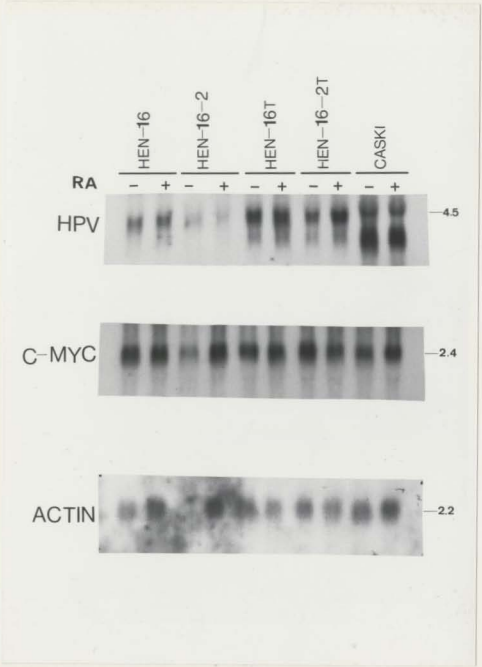

% of inhibition
0
20
40
60
80
HEN
HEN16
HEN16-2
HEN16T
*
HEN16-2T
CaSki

Figure 25. Effect of RA on mRNA expression of HPV16 and c-*myc*. Northern blot hybridization of 20 μg RNA from RA-untreated (-) and -treated (+) cells with ^{32}P-labeled HPV16, c-*myc*, and γ-actin probes was carried out as described in Section 2.7.2. Labels are as described in Figure 23.


HEN-16
HEN-16-2
HEN-16T
HEN-16-2T
CASKI
RA
– + – + – + – + – +
HPV
4.5
C-MYC
2.4
ACTIN
2.2

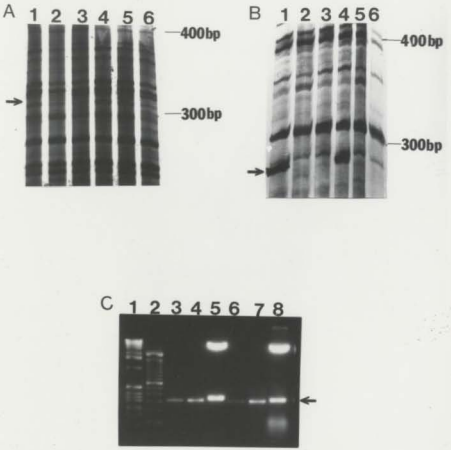
effect on the expression of HPV16 and c-*myc* in all of the immortalized and tumor cell lines (Figure 25)

3.7 Identification, isolation and characterization of novel genes differentially expressed in endocervical cells

3.7.1 Identification of differentially expressed genes using differential display assays

Three cell types, HEN, HEN-16, and HEN-16T, representing different stage of oncogenesis, were used in an attempt to isolate putative oncogenes or tumor suppressor genes responsible for multistage carcinogenesis of cervical cancer. For each cell line, 64 different combinations of primer sets were used for RT-PCR. Approximately 120 cDNA bands per lane were displayed on 6% sequencing gel after RT-PCR for each combination of primers. Of the 35 candidate cDNAs that were shown to be differentially displayed on sequencing gel, two cDNAs, designated PA4 and PA9, were confirmed to be differentially expressed by Northern blot assays. PA4 was specifically expressed in HEN-16 and HEN-16T cells but not in HEN cells (Figure 26A), whereas PA9 was uniquely expressed in HEN cells (Figure 26B). After reamplification, the cDNA

Figure 26. Identification of PA4 and PA9 by mRNA differential display. A), B) Autoradiogram of differential display of PA4 (A) and PA9 (B) (arrows) mRNA RT-PCR products from HEN (lane 1, 4), HEN-16 (lane 2, 5), and HEN-16T (lane 3, 6). In RT-PCR, $T_{11}CA$ was used as the 3' primer for both PA4 and PA9; AP4 and AP9 were used as the 5' primer for PA4 and PA9, respectively. The differentially displayed PA4 and PA9 cDNAs are indicated with an arrow. Size markers are shown on the right of each figure. C) Ethidium bromide-stained gel for reamplification and cloning of PA4 (lane 3, 4, 5) and PA9 (lane 6, 7, 9) cDNAs. After the first reamplification of PA4 (lane 3) and PA9 (lane 6), the cDNAs were amplified a second time (lane 4 for PA4 and lane 7 for PA9) to get enough cDNA for Northern blot (See section 2.11.1 for details). The PA4 and PA9 cDNAs were cloned into the PCRII vector (4 kb), and the plasmids were digested with EcoRI to check the size of the PA4 (lane 5) or PA9 (lane 8) inserts. Lane 1 and lane 2 are 1 kb and 100 bp size markers, respectively.

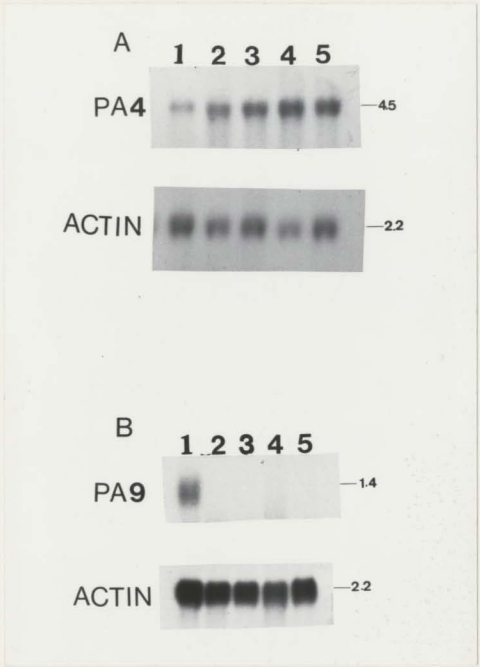

A
1 2 3 4 5 6
400bp
300bp
B
1 2 3 4 5 6
400bp
300bp
C
1 2 3 4 5 6 7 8

products for both PA4 and PA9 were consistent in size with the bands in the original display gel: about 310 bp for PA4 and 290 bp for PA9 (Figure 26A, B and C).

3.7.2 Isolation and Northern hybridization assays of differentially expressed mRNAs

Due to the heterogeneity of PCR fragments, sometimes nondifferentially expressed mRNA species can be detected together with differentially expressed candidates. Therefore, PA4 and PA9 were individually subcloned into the TA cloning vector. Several clones from each subcloning reaction were examined for the size of the insert and hybridization to RNA by Northern blot analysis. Of the 5 clones from the subcloning of PA4, all had an insert size slightly larger than the original cDNA because it also contained a 35 bp polylinker region of the vector. One clone hybridized in Northern blot assays with a single 4.5 kb mRNA transcript that w

Figure 27, Expression of PA4 (A) and PA9 (B) mRNA in cervical cells. Northern blot hybridization of 3 μg mRNA from HEN (lanes 1), HEN-16 (lanes 2), HEN-16-2 (lanes 3), HEN-16T (lanes 4), and HEN-16-2T (lanes 5) with ^{32}P-labeled PA4 , PA9, and γ-actin cDNAs was carried out as described in Section 2.7.2. Labels are as described in Figure 13.


A
1 2 3 4 5
PA4
4.5
ACTIN
2.2
B
1 2 3 4 5
PA9
1.4
ACTIN
2.2

transcript that was specifically expressed only in HEN cells. Other clones detected either no signal or nondifferentially expressed transcripts. Figure 26C and Figure 27 show an example of these results of the differentially expressed mRNAs.

3.7.3 DNA sequence analysis of two isolated clones

The PA4 and PA9 cDNA clones were analyzed by DNA sequencing, and the sequence data are shown in Figure 28. The size of PA4 and PA9 are 315 bp and 292 bp, respectively. Both PA4 and PA9 have flanking primer sequences identical to those used in differential display: AP4 and AP9 arbitrary primers sequence were identical to the 5' end of PA4 and PA9 (underlined), respectively, and the $T_{12}CA$ primer was complementary to the 3' end of both PA4 and PA9 cDNAs (Table 5 and Figure 28). No ORFs or polyadenylation signals were found in PA4 and PA9 cDNAs. A computer search in the GenBank.EMBL Genbank DNA sequence databases indicated that both PA4 and PA9 had no significant homology to any deposited DNA sequence.

Figure 28. Nucleotide sequence of PA4 and PA9 cDNA. A, PA4; B, PA9. Sequences of each primer sets originally used for differential display and polyadenylate tails are underlined.

A)

```
          10         20         30         40         50
GGTACTCCACGAATATGAGGAAGTATGAGTAGAGGGGAACTGGTACAGAA

          60         70         80         90        100
CAGAAGTTATTTTGGGTAAGATCAAGGGCAGGATGTAAGAAAACTAGTTT

         110        120        130        140        150
TCAGTTACAAGGGAATAACCATTGGGAATAGAGCTTGGCTTCAGTGAAGT

         160        170        180        190        200
ACTCAATGGTTTAATCCAGGGCTAAACCTCATGTTCTGGGCAGTCGTGCC

         210        220        230        240        250
CCCTGTAGTGCTTTAATTCACTATGGGAAAAGCATCCAATCTGTGTTTTA

         260        270        280        290        300
CAAAAAAGTACTTTTTGGAACCCAGTACCTACTAGCAGAGAGGACCTCCT

         300  315
GAAAAAAAAAAAAAA
```

B)

```
          10         20         30         40         50
GGGTAACGCCAAATGGTATATCACTGATTTTGTAGAGCTGCGGGAGAACC

          60         70         80         90        100
GGAAGAATAACATCCATTGTCAATACAGCTCCAAACAACTTCAGATGAAT

         110        120        130        140        150
TTTACAAGTTACACAGATTGATACTGTTTAGCTTACAATTGCCTATTACA

         160        170        180        190        200
ACTTGCTATAGAAAGTTGGTACAAGTGATCTGCACTGTCAAGTAAACTAC

         210        220        230        240        250
AGTTAGGAATCCTCAAAGATTGGTTTGTTTGATTTTTAACTGTAGTTCCA

         260        270        280          292
GTATTATATGATCACTATTGATTTCCTGAAAAAAAAAAAAAA
```

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

4.1 *In vitro* evidence of cigarette smoke as a co-factor in cervical cancer

It has been well recognized that cervical carcinogenesis is a multistage process in which HPV and other co-factors are necessary for the full malignant transformation of primary cervical cells (zur Hausen, 1990, 1994). As mentioned in the Introduction, smoking has long been regarded as a risk factor for cervical cancer (Winkelstein, 1986, 1990). Despite the extensive epidemiologic and other evidence in support of the hypothesis of an association between smoking and cervical cancer (Phillips and Smith, 1994), the interpretation of the association remains equivocal, and convincing, more direct, evidence is lacking. Therefore, neither the International Agency for Research on Cancer nor the US Public Health Service list cervical cancer as smoking related (Winkelstein, 1990).

In vitro system has been used widely in the studies on the role of HPV in oncogenesis of cervical cancer, and has provided most of the direct evidence that linked HPV to

immortalization and transformation of cervical cells (zur Hausen 1990, 1994). In addition, *in vitro* system has also been successfully used to study the roles of some chemical carcinogens such as NMU, benzo[a]pyrene, and MSEE, oncogenes such as v-fos, and herpes simplex virus in the malignant transformation of HPV-containing keratinocyte cells (DiPaolo et al., 1990; Li et al., 1992; Garret et al., 1993; Klingelhutz et al., 1993; Xu et al., 1993; Shin et al., 1994). However, few *in vitro* systems have been set up to mimic the multistep carcinogenesis of cervical cancer. Even though HPV-16 and HPV-18 immortalized foreskin and cervical cell lines have been widely established, tumor cell lines derived from HPV-immortalized cervical cells are rare. Most of tumor cell lines used for tumorigenicity studies, such as HeLa, SiHa, and CaSki, are derived from cervical carcinomas, have been kept in culture for decades and do not have isogenetic nontumorigenic counterparts. In addition, while the endocervix is the origin of over 95% of cervical tumors, HPV-immortalized cell lines and tumor cell lines that are derived from endocervical cells are lacking. An *in vitro* system mimicking the multistep carcinogenesis of cervical cells from the same original

primary endocervical cells will be very useful to study the molecular mechanism underlying this oncogenesis process.

In this study, it was found that endocervical cells immortalized by HPV16 were not tumorigenic and could not form tumors in nude mice (Table 6). However, when the immortalized cells were treated during culture with cigarette smoke condensate, the treated immortalized cells were malignantly transformed and formed squamous cell carcinomas in nude mice as confirmed by pathological analysis (Figure 7). The transformed tumor cell lines obtained the general growth characteristic of tumors: faster growth in serum-containing medium, anchorage-independent growth, and reconstruction of more undifferentiated epithelia in *in vitro* organotypic culture. These results provided the first *in vitro* evidence that cigarette smoke can induce cervical carcinoma. In addition, they supported the epidemiologic suggestion that cigarette smoking has a late stage or promotional effect in the oncogenesis of cervical cells (Daling et al., 1992). Neoplastic transformation of other cells, such as bronchial epithelial cells immortalized by adenovirus 12-SV40 hybrid virus, by CSC was also observed *in vitro* in other studies

(DeMarini, 1983; Klein-Szanto et al., 1992). However, because of the complexity of CSC components and the transformation process, the mechanism by which CSC causes the neoplastic transformation of human cells is not yet fully understood. CSC has been demonstrated to produce many genetic and epigenetic effects usually associated with carcinogens and promoters, such as alterations in cell growth and EGF binding, formation of single strand DNA breaks, inhibition of DNA repair, and induction of terminal squamous differentiation (DeMarini, 1983; Willey et al., 1987; Klein-Szanto et al., 1992; Simons et al., 1995).

Despite the significance of the present study, several points have to be considered carefully when discussing the above results. First, the CSC used in this study is composed of over 3,000 components. Therefore, it is difficult to determine which component in CSC is responsible for this tumorigenic transformation. Second, the lowest concentration of CSC that was used for the transformation of HPV16-immortalized endocervical cells was 75 μg/ml. Athough tumors could not be formed after treatment of HPV16-immortalized cells with low concentration of CSC, such as 25 and 50 μg/ml,

over 1 year (unpublished data), lower concentrations may also be effective. Since the CSC concentration used in the present *in vitro* study may be higher than that in cervical basal cells which most receive carcinogens via the epithelial layers from the mucus *in vivo*, it took only 6-12 months for the immortalized cells to be transformed into tumors by CSC *in vitro*. On the other hand, it usually takes decades for HPV-infected patients to develop cervical cancer (zur Hausen, 1994). In fact, it has been demonstrated that the risk for developing cervical cancer is enhanced with increased number of cigarettes smoked (Winkelstein, 1990), indicating that CSC concentration in cervix fluid may be critical in determining the interval between HPV infection and cervical cancer. Third, the *in vitro* monolayer cells and *in vivo* epithelia are markedly different. The status and expression of HPV, the cell-cell interactions, the concentrations of additional cofactors, such as hormones, and growth factors and the host immune response are some typical differences. Thus, how HPV and CSC interact to transform cervical cells *in vitro* and *in vivo* is still unclear.

Despite the above uncertainties, the *in vitro* system set

up in this study remains useful for us to understand the molecular mechanism of multistep carcinogenesis of cervical cancer.

4.2 Role of HPV and cellular genes in immortalization of primary cervical cells

Although immortalization of human cells *in vitro* is a key step in oncogenic progression, the molecular mechanisms underlying this event are still poorly understood (Shay et al., 1991).

4.2.1. HPV

Previous studies have demonstrated that HPV E6 and E7 products are the main proteins responsible for immortalization of human cervical cells (Munger et al., 1989a, 1992). Our two immortalized cell lines were established by transfection of HPV16 DNA into the human endocervical cells, and E6-E7 transcripts are the main transcripts in the HPV16-immortalized cells (Tsutsumi et al, 1992; Figure 12). In addition, HPV16 DNA was not episomal in immortalized cells, and at least 2.6 kb DNA coding for part of the L1 ORF have been deleted in

immortalized cells (Figure 12B, C). Therefore, expression of E6 and E7 proteins, integration of viral DNA into host genome and deletion of part of its DNA may be necessary for immortalization. This conclusion was also supported by our observation that HPV DNA was episomal in cervical cells before crisis and became integrated after crisis or immortalization by HPV18 (Yokoyama et al., 1995).

Even though continuous expression of HPV has been regarded as necessary for the establishment of immortalized cells, expression of HPV *per se* can not account for immortalization. For example, when two HPV18-immortalized cervical cells were fused, the hybrid cells became senescent but still expressed HPV, indicating that senescence of the hybrid cells was not due to loss of HPV18 mRNA expression (Chen et al., 1993). Therefore, alteration of cellular genes that either are regulated by HPV or function individually must contribute to or be directly responsible for immortalization.

4.2.2 Oncogenes

It has been shown that early passage rodent cells can be immortalized by oncogenes such as H-*ras*, c-*myc*, c-*jun* and *v*-

src (Cole and Kelekar, 1987; Linder and Marshall, 1990). Furthermore, amplification and activation of some oncogenes, most frequently c-*myc* and H-*ras*, has been observed in cervical carcinomas (Durst et al., 1987; Ocadiz et al., 1987; Sagae et al., 1990; Riou et al., 1990; Crook et al., 1990). Therefore, oncogenes have been postulated to be cofactors for immortalization. However, since conflicting results have been obtained by others (Ikeberg et al., 1987; Sagae et al., 1990; Pelisson et al., 1992), the role of oncogenes in transformation of cervical cells is still disputable.

Results in this study clearly showed that amplification of c-*myc* or H-*ras* was not found in HEN-16 and HEN-16-2 cells (Figure 13), indicating that neither oncogene is involved in the immortalization of primary endocervical cells by HPV16. On the other hand, B-*myb* expression was enhanced 19- to 37-fold in immortalized cells (Figure 13 and Table 7). B-*myb* has been recognized as an oncogene that regulates the proliferation of cells by interacting with cell-cycle regulating genes (Nomura et al., 1988; Sala and Calabretta, 1992; Lam and Watson, 1992, 1993). Recently, it has been demonstrated that B-*myb* can be transactivated by the HPV16 E7 oncoprotein (Lam et al., 1994).

Therefore, the activation of B-*myb* in immortalized cells may be caused by the transactivating effect of HPV16 E7. Furthermore, it was recently found that constitutive expression of B-*myb* could bypass p53-induced Waf1/Cip1-mediated G1 arrest (Lin et al., 1994). Thus, activation of B-*myb* in HPV-positive cells could allow these cells to bypass the normal cell cycle arrest mediated by some tumor suppressor genes, such as p53 and Rb, and lead to immortalization. This can also be complicated by the action of E6 and E7 on these tumor suppressor genes (Lin et al., 1994). This study provided the first evidence that deregulation of B-*myb* by HPV may be involved in one of the pathways leading to the immortalization of primary cervical cells.

4.2.3 Tumor suppressor genes

Inactivation of tumor suppressor genes, especially p53 and Rb, has been regarded as one of the most important pathways leading to immortalization of cells (Linder and Marshall, 1990; Shay et al., 1991). However, the pathways by which these tumor suppressor genes function are unclear. In addition, whether the inactivation of a specific tumor

suppressor gene is an early or late event in cervical cancer is also uncertain.

The p53 tumor-suppressor gene has been identified as a participant in cell cycle control, DNA synthesis and repair, maintenance of genomic stability, cellular differentiation, and programmed cell death (for review, see Donehower and Bradley, 1993). It was also recognized as one of the two important cellular genes known to be inactivated by HPV (Werness et al., 1989). Degradation of p53 by the HPV16 or HPV18 E6 protein has been shown to be necessary but not sufficient for the immortalization of primary cervical cells (Pecoraro et al., 1989; Jewers et al., 1992). Interestingly, in this study, the expression of p53 was greatly enhanced in all immortalized cells (Figure 14). Similar results were also found in HPV18-immortalized keratinocytes by others (Li et al., 1992; Shin et al., 1994). Although p53 protein levels were not checked in primary cervical cells and immortalized cells in this study, almost all the previous studies have demonstrated that the p53 protein levels are much lower in immortalized cells than in primary cells as a result of their degradation by the E6 protein (for reviews see Munger et al.,

1992 and Scheffner et al., 1994). The increase of p53 expression in immortalized cells may be a strategy of cells to compensate for the degradation of p53 proteins under this "stress" condition. This result provided additional information for the action of tumor suppressor genes in immortalization of cervical cells.

Waf1/Cip1/Sid1 is a recently identified universal cyclin-kinase inhibitor up-regulated by p53 (Xiong et al., 1993; Harper et al., 1993; El-Deiry et al., 1993; Noda et al., 1994). It participates in cell cycle regulation, DNA replication, DNA damage-induced G1 arrest, apoptosis, senescence and differentiation by a p53-dependent and p53-independent pathway (Noda et al., 1994; El-Deiry et al., 1994; Waga et al., 1994; Jiang et al., 1994; Steinman et al., 1994; Halevy et al., 1995; Parker et al., 1995). In this study, it was found that Waf1 was suppressed in all immortalized cells (Figure 14A). Since p53 proteins are usually degraded in HPV-immortalized cells, it is reasonable to conjecture that the transactivation of Waf1 by p53 was decreased as a result of decreased p53 proteins. This result indicated that deregulation of Waf1 may be involved in one of the pathways by

which p53 functions in the immortalization of cervical cells.

4.2.4 DNA replication- and repair-related genes

Human cells are usually continuously exposed to various agents, such as chemical carcinogens, irradiation or DNA tumor viruses, that can damage cellular DNA, RNA, and proteins. The damage can have two consequences: cell death or neoplastic conversion of the cells. Therefore, maintenance of the integrity of DNA in somatic cells over extended time is of importance in keeping the normal function of the cells. To overcome the genotoxicity of DNA-damaging agents, cells are equipped with several molecular defense mechanisms which can either prevent the induction of DNA damage or repair the damage (Kaufman and Kaufman, 1993). In the case of normal cells, cells usually stop division by transiently arresting cell cycle progression, called G1 arrest or G2 arrest, and permitting the repair of damaged DNA (Zhan et al., 1993). Failure of DNA repair allow for the inheritance of damaged genes and may result in the emergence of neoplastic cells. Recent study indicates that cervical and oral cells immortalized by 'high risk' HPV DNA fail to arrest cell cycle

progression when exposed to DNA damaging agents, such as UV-radiation or actinomycin D (Kuerbitz et al., 1992; Kessis et al., 1993; Gujuluva et al., 1994; Slebos et al., 1994). Therefore, deregulation of DNA replication and repair genes may be one of the pathways leading to immortalization of cervical cells.

PCNA, also called "cyclin", is an auxiliary protein of DNA polymerase þ, which is associated with DNA replication and repair (Almendral et al., 1987; Zeng et al., 1994). Recently, the amplification of PCNA expression was found in cervical intraepithelial lesions *in vivo* (Demeter et al., 1994; Karakitsos et al., 1994). Using Northern hybridization and indirect immunofluorescence assays, it was clearly demonstrated in this study that PCNA was also activated in immortalized cells *in vitro* (Figure 15A). Although the mechanism by which PCNA is involved in the immortalization process is still unclear, recent studies demonstrated that Waf1 may block DNA replication by interacting with PCNA (Waga et al., 1994). Thus, depression of Waf1, independent or dependent on p53, in immortalized cells may be part of one of the mechanisms for the relative increase of PCNA proteins in

immortalized cells.

GADD45 is an ubiquitously expressed mammalian gene that is induced by DNA damage (Papathanasiou et al., 1991; Kastan et al., 1992; Hollander et al., 1993; Carrier et al., 1994). It is associated with DNA damage-induced growth arrest (Zhan et al., 1994). Recently, it was found that GADD45 could interact with PCNA to repair damaged DNA (Smith et al., 1994). In this study, no significance difference in GADD45 expression was observed between HEN and HPV16-immortalized cells, indicating that GADD45 is not involved in immortalization. No change in GADD45 expression was found in HPV-immortalized oral keratinocytes (Gujuluva et al., 1994). Since GADD45 is also transactivated by p53, this result indicated that modulation of immortalization by p53 is not performed through the GADD45 pathway and the degradation of p53 by HPV16 E6 has no significant effect in this *in vitro* system.

GADD153 is another member of the GADD gene family, which is associated with growth arrest after DNA damage or stress, and differentiation, but is not regulated by p53 (Carlson et al., 1993; Barone et al., 1994; Luethy and Holbrook, 1994). In this study, although the experiments were repeated several

times, a decrease of GADD153 mRNA was observed in HEN-16 cells (Table 7 and Figure 15C), and a 1.9-fold increase of GADD153 mRNA was found in HEN-16-2. An opposite effect occurred during tumorigenesis. A decrease of GADD153 was found in HPV-immortalized oral keratinocyte cells (Gujuluva et al., 1994). Thus, the role of GADD153 in the immortalization of normal endocervical cells is still elusive.

4.2.5 Senescence-related genes

At the cellular level, three general mechanisms for tumor suppression have been documented: stabilizing the human genome, controlling proliferation, and terminally differentiating, which involves the irreversible loss of proliferative capacity. Senescence is a phenomenon in which cells can no longer respond to any proliferative stimulus. It has been regarded as the reversal of immortalization. Previous evidence has suggested that there are four dominant genes or complementation groups that are implicated in senescence, implying that the loss of these genes or their functions is essential for immortalization (Pereira-Smith and Smith, 1988).

FN is a well established marker of cellular senescence (Kumazaki et al., 1991; Khandjian et al., 1992). Previous studies indicated that FN mRNA and protein increased significantly during the process of cellular aging in tissue culture (Kumazaki et al., 1991). However, FN mRNA levels became low in SV40 transformed cells (Khandjian et al., 1992; Noda et al., 1994). In my study, FN mRNA was almost completely abolished in all the immortalized and tumor cells (Table 7 and Figure 17A). *In situ* hybridization and indirect immunofluorescence showed that FN mRNA and protein were expressed only in HEN cells (Figure 17B and Figure 18). Furthermore, expression of a recently identified senescence cell-derived inhibitor gene, Sid1, which later was proven to be Waf1 (Harper et al., 1993; Noda et al., 1994), was also enhanced in senescent cells (Noda et al., 1994) and suppressed in immortalized cells (Figure 14). These results confirmed that loss of senescence-related genes may be involved in one of the mechanisms for immortalization of primary cervical cells by HPV16.

4.3 Role of HPV and cellular genes involved in malignant

progression of immortalized cells

It has been generally accepted that immortalized human cell lines are not tumorigenic when inoculated into nude mice (zur Hausen, 1994 and Table 6), indicating that other changes are required for the full malignant transformation of the cells. However, our knowledge of the molecular mechanism of this malignant progression process is still fragmentary.

Previous studies have demonstrated that some chemical carcinogens, UV and low-dose X-ray irradiation, and some viruses, such as HSV and probably HIV, may cause the malignant conversion of immortalized cells (DiPaolo et al., 1990; Li et al., 1992; Garrett et al., 1993; zur Hausen and de Villiers, 1994). In addition, hormones have been shown to enhance the transformation of HPV-immortalized cell by an activated *ras* oncogene (Pater et al., 1988,1990,1994; Durst et al., 1989). In my study, cigarette smoke condensate was also shown *in vitro* to be a cofactor for this malignant progression. However, how this factor contributes to the malignant conversion, or which genes are affected to mediate this conversion, is still unclear.

Some studies suggested that further increases of HPV

expression or modification of HPV integration may be responsible for this malignant progression (Li et al., 1990; zur Hausen, 1991). However, my results and those of others (McDougall, 1994) clearly demonstrated that no change in the level of HPV expression was detected between HPV-immortalized cells and tumorigenic cells, indicating that HPV may be not involved in the malignant progression of HPV-immortalized cells.

Oncogenes have been suggested to be involved in the malignant transformation of HPV-immortalized cells for the following reasons. First, transfection of H-*ras* or v-*fos* into HPV immortalized cells can induce tumorigenicity of these cells (DiPaolo et al., 1989; Durst et al., 1989; Xu et al., 1993). Second, amplification of oncogenes such as c-*myc* and H-*ras*, *erbB2* has been detected in cervical carcinomas (Ocadiz et al., 1987; Riou et al., 1990; Sagae et al., 1989; Mitra et al., 1994). Third, enhanced expression of H-*ras* and c-*myc* has been found in transformed cell lines (Li et al., 1992; Shin et al., 1994; Iwasaka et al., 1993). However, unchanged oncogenes also were found frequently in transformed cell lines and cervical carcinomas (Ikenberg et al., 1987; Iwasaka et al.,

1993). In this study, no obvious amplification of c-*myc* and H-*ras* was observed, further suggesting that these oncogenes were not involved in the malignant transformation of HPV-immortalized cells by CSC. Therefore, the role of oncogenes in the malignant progression of immortalized cells needs further study.

Although tumor suppressor genes play a central role in the development of tumors and they can usually suppress the growth of tumor cells, the exact functions of these genes in inhibiting the multistep carcinogenesis of cancer remain not fully understood. In my study, the three tumor suppressor genes, p53, Waf1, and DCC did not show any changes of expression in CSC-transformed cells compared with immortalized cells (Figure 14A,B). However, the expression of p53 and Waf1 was substantially changed after immortalization, indicating that they may play a role in the early stage of endocervical cell oncogenesis-immortalization. This result clarifies the confounding role of p53 in immortalization and tumorigenicity of cervical cancer. In addition, as discussed in the Introduction, p53 was shown to be involved in the late stages of colon cancer, suggesting that the same tumor suppressor

gene may function at different stages of multistep carcinogenesis of different human cancers.

Several studies have shown that DCC was deleted in some chemical carcinogen-transformed HPV-immortalized keratinocytes (Klingelhutz et al., 1993 and Shin et al., 1994). Recently, wild-type full-length DCC but not the truncated form was shown to suppress the malignant phenotype of NMU-transformed HPV-immortalized epithelial cells (Klingelhutz et al., 1995). This indicated that DCC is a tumor suppressor gene, the loss of which may be involved in tumorigenesis of HPV-immortalized cells. However, no deletion of DCC was detected in CSC-transformed cells in this study (Figure 14B), suggesting that DCC deletions are not a common phenomenon in the chemical carcinogen-induced malignant progression. Alteration of DCC expression may also occur by point mutation and loss of heterozygosity, which needs to be further studied.

Overexpression of PCNA is observed in many types of malignancies including lymphomas, breast, and pancreatic tumors (Hall et al., 1990). In addition, PCNA amplification was observed more frequently in biopsies from patients with high grade lesions, CIN III and koilocytosis, than those with

low grade lesions, CIN I and CIN II (Karakitsos et al., 1994). The increase of PCNA mRNA in CSC-transformed cells compared with HPV-immortalized cells was consistent with the above clinical observations. Both results suggested that PCNA can be used as a molecular marker for the multistep carcinogenesis of cervical cancer.

Although the GADD gene family was originally known to represent DNA damage-induced genes, recent studies demonstrated that they can suppress the growth of tumor cells (Zhan et al., 1994). GADD45 and GADD153 were shown to suppress specifically the growth of an established cervical cell line, HeLa (Zhan et al., 1994), suggesting that the loss of their function may be associated with malignant conversion of cervical cells. However, in this study, no significant alterations were observed in the level of GADD45 and GADD153 expression in CSC-transformed cells compared with their untransformed counterparts. Recent studies suggested that the loss of the response of p53, Waf1, GADD45, and GADD153 to DNA damaging agents, such as UV light and actinomycin D, in HPV-immortalized cells may be one of the important pathways leading to tumorigenesis of HPV-immortalized cells (Baek et

al., 1994). Thus, the function of cellular genes involved in DNA repair may be impaired by inactivating their response to DNA damaging agents but not their basal transcription. This needs to be further studied.

4.4 Response of cervical cells at different stages of oncogenesis to cytokines and RA

4.4.1 Cytokines

Recently, increasing evidence suggests that the existence of an intracellular surveillance system is very important to protect host cells from the potentially deleterious effect caused by viral transforming genes and other factors. Loss of this autocrine self-regulating system is one of the most important pathways leading to the malignant transformation of cells (zur Hausen, 1994).

Cytokines such as TNF-þ, TGF-βl, and interferon-γ are one group of molecules released by immune effector cells and are thought to be involved in the intracellular surveillance system. Recent studies have shown that some cytokines, such as TNF-þ, TGF-þ1, INF-γ, and IL-6, can suppress proliferation and expression of HPV in HPV-positive cells, and the loss of

response to these cytokines is very common in established cervical carcinoma cell lines, such as CaSki and HeLa (Brau et al., 1990, 1992; Woodworth et al., 1992; Villa et al., 1992; Kyo et al., 1994; Rosl et al., 1994; Agarwal et al., 1994). Thus, it was suggested that the loss of sensitivity to inhibition by cytokines may be an important step in the development of cervical carcinomas. However, from the above studies, several questions could be asked. First, did the tumor cell lines lose sensitivity to all cytokines or did they lose sensitivity to only a specific type of cytokine? Second, in most of the above studies, only established cervical carcinoma cell lines or hybrid cell lines were used (Brau et al., 1990, 1992; Rosl et al., 1994). These established cell lines have undergone many genetic changes while being maintained in culture for several decades. So, are these cell lines reliable representatives of tumor cells? Regarding hybrid cells, the genetic complexity of these cells make the results difficult to interpret. Third, since cervical carcinoma cell lines were used in most of the studies, it remains unclear whether loss of sensitivity to cytokines is an early or late event. Fourth, the loss of sensitivity to

cytokines may mean the loss of suppression of proliferation or loss of suppression of HPV expression by cytokines. Were these two effects the same? Fifth, by what pathways did the cytokines suppress the proliferation and HPV expression? To address these issues, I performed several experiments using primary, HPV16-immortalized, CSC-transformed and CaSki cells. First, it was found that tumor cells may obtain insensitivity to certain kinds of cytokines but not to all cytokines. For example, CaSki were insensitive to TGF-β1 but not TNF-α (Figure 19 and Figure 22). Second, it was shown that the results obtained with established cervical carcinoma cell lines were sometimes not representative results for other tumor cells. For example, the loss of growth inhibition by TGF-β1 in tumor cells has been regarded as a common phenomenon for tumor cells (Brau et al., 1990, 1992). However, Figure 22 showed that, even though no suppression of proliferation by 5 ng/ml of TGF-β1 was found in CaSki cells, the proliferation of two CSC-transformed tumor cell lines, HEN-16T and HEN-16-2T, was suppressed for TGF-β1 by over 50%. Thus, the loss of response to cytokines may be not general for tumor cells. Third, since the loss of sensitivity to TNF-α and TGF-β1 was

not observed in HPV16-immortalized cells and CSC-transformed cells, it is difficult to say if it is an early or late event in this system. However, complete loss of response to TGF-β1 was found only in carcinoma cells that have undergone more extensive changes in women and in culture (Geest et al., 1994). Thus, it was suggested that loss of sensitivity to cytokines might be a late event. Fourth, it was demonstrated from my experiments that the inhibition of proliferation by cytokines may be different from the suppression of HPV expression by cytokines. As shown in Figures 22 and 23, although no suppression of proliferation by TGF-β1 was observed for CaSki cells, their HPV expression was significantly suppressed after treatment with TGF-β1 (Figure 22 and 23). This conclusion was consistent with that drawn by Agarwal et al. (1994), who studied the effect of IFN-γ on the proliferation and HPV expression of HPV16-immortalized cervical ectocervical cells.

Suppression of proliferation and/or HPV expression of cervical cells by cytokines has been observed by many researchers over many years (Brau et al., 1990, 1992; Villa et al., 1992; Woodworth et al., 1992; Malejczyk et al., 1994;

Geest et al., 1994). However, the exact mechanism underlying this suppression is still poorly understood. Until now, only one cellular gene, namely NF-IL6, has been shown unequivocally to mediate suppression of HPV expression by IL-6 through binding to the noncoding region (Kyo et al., 1993). It has been suggested that the suppression of HPV expression by cytokines is mediated by transcription factors binding to the LCR of HPV (Kyo et al., 1994). It has been proposed by others that c-*myc* is one of the cellular genes related to growth suppression by TGF-þ1, since c-*myc* expression was usually suppressed after TGF-þ1 treatment (Pietenpol et al., 1990). Based upon the above information, I checked the c-*myc* expression after TNF-þ and TGF-þ1 treatment. It was found that c-*myc* expression in all cell lines was suppressed after treatment by either (Figure 20 and 23). Since c-*myc* is a well-known oncogene that regulates cell proliferation, the suppression of this gene implies that it may mediate the suppression of proliferation by TNF-þ and TGF-þ1 treatment.

The JE gene, also called MCP-1, MCAF, and SMC-CF, encodes a secreted glycoprotein with cytokine-like properties, which stimulates human monocytes and induces monocyte-mediated

inhibition of tumor cell growth *in vivo* and *in vitro* (Rollins et al., 1989; Rollins and Sunday, 1991). Recently, it was shown by Rosl et al. (1994) that the JE gene was only expressed and induced by TNF-α in nontumorigenic HeLa-fibroblast hybrids but not in tumorigenic hybrids, and that a change of JE expression is accompanied by a similar change in HPV transcription. Thus, it was suggested that the loss of JE gene expression and the induction of this expression by TNF-α is one of the important characteristics of tumor cells and that the JE gene played a pivotal role in intracellular communication by triggering an intracellular pathway which interferes with viral transcription in HPV-positive nontumorigenic cells (Rosl et al., 1994; zur Hausen and de Villiers, 1994b). From above results, three questions were asked initially: 1) Is loss of JE expression or induction by TNF-α an early or late event? 2) Are either loss or induction specific for tumor cells or only for tumorigenic HeLa-fibroblast hybrids? 3) Is the correlation between suppression of HPV by TNF-α and that of JE by TNF-α a coincidence or a common phenomenon for nontumorigenic cells? In this study, it was found that 1) while JE gene DNA was detected in all the

cell lines examined, no JE mRNA was detected or induced by TNF-α in immortalized cells and tumor cells (data not shown), suggesting that loss of JE gene expression or induction by TNF-α was an early event but not specific for tumor cells. The loss of JE expression or induction by TNF-α may be related to the rearrangement of this gene (Figure 21). In addition, 2) since HPV16 was expressed but JE was not expressed in both immortalized cells, suppression of HPV by TNF-α may be unrelated to that of JE. These results challenge the role of JE in the suppression of HPV by TNF-α and provide new information on the role of JE in the multistage tumorigenesis of cervical cancer.

Some studies suggest that p53 and the Rb tumor suppressor genes products may interact with but not directly mediate the growth inhibition by TGF-β1, and this interaction was proposed to be associated with the regulation of cyclin-dependent kinase activity (Massague et al., 1992; Blaydes et al., 1995). To understand this process, I examined the induction of p15, a newly cloned cyclin-dependent kinase inhibitor induced by TGF-β1 in human keratinocytes (Hannon and Beach, 1994), in

immortalized and tumor cells after treatment with TGF-β1. Interestingly, p15 was induced in all immortalized and CSC-transformed cells after TGF-β1 treatment but not in CaSki cells (Figure 23). This result suggests that loss of inhibition of proliferation in CaSki cells by TGF-β1 may be caused by the loss of induction of p15 by TGF-β1, and that insensitivity to TGF-β1 only occurs in some tumor cell lines. In addition, since p15 and Waf1 are cyclin dependent kinase (cdk) inhibitors upregulated by p53, this result provides evidence for a new pathway leading to the inhibition of cell proliferation by TGF-β1 treatment.

Taken together, the results in this study add important new information on the response of cells to cytokines in the multistep carcinogenesis of cancer. Their exact roles in oncogenic progression is an important area for future study.

4.4.2 RA

RA is a vitamin A metabolite which is known to be an important regulator of epithelial cell growth and differentiation *in vivo* and *in vitro* (for review see Roberts and Sporn, 1984). More importantly, RA has been shown to have

an antineoplastic effect on many virally and chemically induced neoplasias, including cervical lesions and dysplasias (Creek et al., 1994). Recently, it has been found that RA can inhibit proliferation and HPV expression in HPV-immortalized cells and non-tumorigenic HeLa-fibroblast hybrids, but no suppression of HPV expression was observed in HeLa cells or tumorigenic HeLa-fibroblast hybrids (Pirisi et al., 1992; Bartsch et al., 1992; Creek et al., 1994). Thus, it has been suggested that loss of sensitivity to RA may be related to the tumorigenicity of cells (zur Hausen and de Villiers, 1994b). In my study, loss of suppression of proliferation induced by RA was detected, whereas no suppression of HPV expression was observed in both immortalized and both tumor cell lines (Figure 25). No significant suppression of HPV expression by RA was found by others in HPV16-immortalized cervical cells (Agarwal et al., 1994) or HPV18-immortalized cervical cells (Nakao, unpublished data).

Taken together, two conclusions can be drawn from the present experiments: 1) The loss of sensitivity to RA suppression of HPV expression is not specific for tumor cells. 2) Suppression of proliferation by RA might be different from

that of HPV expression by RA.

4.5 Significance of using mRNA differential display method to identify the genes involved in oncogenesis

As discussed above, multistep carcinogenesis of cancer is driven by a series of changes in gene expression. Thus, identifying the genes that are differentially expressed, either oncogenes or tumor suppressor genes, in the oncogenesis process is critical to understand the molecular mechanism of cancer.

A number of strategies have been devised to identify differentially expressed genes. The first approach is called differential hybridization, in which radioactive cDNA probes from two different sources (usually designated + and -) are separately hybridized with either + or - genomic DNA or a cDNA library and differentially hybridized + versus - radioactive genomic cDNA plaques or colonies are identified. This method has been used to identify genes induced by specific factors, such as growth factors (e.g. PDNF), oncogenes (e.g. *ras*), and serum (Oren and Levine, 1983, and references therein). However, only mRNA of above 0.1% abundance can be identified

with this method. Thus, a second method called subtractive hybridization, which can identify differentially expressed genes that are present in 0.001% to 0.1% abundance, was developed. In this method, most of the common sequences (mRNA or cDNA) in + and - sources are first removed by hybridization of complementary sequences of + and -. The resulting 'subtracted' mixture can either be used as a probe to screen the + or - library or make a 'subtraction library' which can be further screened by probes from either + or - source. The subtraction step usually enriches the differentially expressed genes by 10-fold or greater (Schweinfest and Papas, 1992). Subtractive hybridization has been used to isolate several genes involved in the carcinogenesis of cancer, including Waf1 (Lee et al., 1991; Schweinfest and Papas, 1992; El-Deiry et al., 1993).

Recently, a method called mRNA differential display has been developed and refined (Liang and Pardee, 1992; Liang et al., 1993; Bauer et al., 1993; Mou et al., 1994; Li et al., 1994). This method has several technical advantages over the existing methods mentioned above. The first advantage of mRNA differential display is its simplicity and speed. Technically,

only PCR and DNA sequencing gel electrophoresis are used in the experiment, and candidates can be identified, isolated and confirmed within 1-2 weeks. The other methods will take at least 3-6 months to identify differentially expressed genes. Another advantage of the mRNA differential display is its sensitivity. Only 2-20 μg of total RNA is enough to screen all the mRNA once. By contrast, more than 5-50 μg of mRNA from 500-5000 μg of total RNA are needed for two rounds of subtraction in the subtractive hybridization method (Schweinfest and Papas, 1992). Most importantly, because of its high sensitivity, the differential display method can be used directly to identify the mRNAs or sequence tags, which differentially expressed in cancer biopsies (Watson and Fleming, 1994). The last important advantage of the mRNA differential display method is that multiple cell lines can be compared in the same gel, and that over-expressed genes such as oncogenes and suppressed genes such as tumor suppressor genes can be identified simultaneously.

Using the differential display method, at least 15 genes have been identified so far to be specifically expressed in tumor cells, developing cells, glucose-treated cells, and *ras*-

transfected cells (Liang et al., 1992; Sager et al., 1993; Zhang and Medina, 1993; Nishio et al., 1994; Mou et al., 1994; Sun et al., 1994; Kumar and Haugen, 1994; Paul et al., 1994; Zimmermann and Schultz, 1994; Liang et al., 1994).

Using this mRNA differential display method in my experiments, mRNA from three cell types, HEN, HEN-16, and HEN-16T, representing normal cells and two stages of oncogenesis, was simultaneously reversely transcribed, PCR amplified, and displayed on the same sequencing gel. Since about 120 bands can be displayed in each lane, and since 64 combinations of PCR primer pairs were used, we screened about 8,000 mRNA species in total. Generally, there are about 15,000 genes expressed in any single type of mammalian cell. Therefore, about 53% of all expressed genes have been screened, of which 2 genes designated PA4 and PA9 were identified to be differentially expressed in these cell line. PA4 was activated in immortalized cells and CSC-transformed tumor cells compared with HEN cells. There was no difference in PA4 expression between immortalized and tumor cells, indicating that it may be an oncogene involved in the immortalization of primary cervical cells. PA9 was inactivated in immortalized

cells and tumor cells, indicating that it may be a tumor suppressor gene. Both PA4 and PA9 are novel genes because no homology with any other genes was found in the NIH Genbank database. Further characterization of these two genes will be very helpful for us to understand the molecular mechanism of immortalization of normal cervical cells.

Although the advantages of differential display are significant, a few problems were experienced using this method. One of these was the high incidence of false positives. Of the 35 positive cDNAs isolated from the display gel, only two of them were confirmed by Northern blot assays to be differentially expressed. The second problem encountered was that the cDNA cut from the sequencing gel contains several species. The cDNA of interest may be lost after cloning. Furthermore, since the differential display method provides clones of only 150-500 bps in length from the 3'-region of mRNA, it is frequently difficult to identify the coding region if the cDNA has a long 3'-untranslated region. Although PA4 and PA9 contained poly(A) sequences, they do not contain coding regions. Thus, 5'-end rapid amplification of cDNA ends (RACE) or library screening will be required to find the full

length transcript. Recently, a revised method using two arbitrary primers as primer pairs in the differential display has been developed to identify differentially expressed cDNAs of coding regions (Skolov and Prockop, 1994).

Since the cellular genes directly involved in the multistep carcinogenesis of cervical cancer are still unknown, differential display remains one of the most useful methods available to identify the novel genes involved in oncogenesis.

4.6 Future directions

This thesis has described in detail an *in vitro* study of the oncogenesis of primary cervical cells by HPV and cigarette smoke. This study provided a lot of useful information for us to further understand the molecular mechanism of cervical cancer. However, to fully understand the oncogenesis process in my *in vitro* system, several experiments could be done:

1) To further understand the mechanism of CSC-induced transformation of HPV-immortalized cell, the effect of different concentrations of CSC on growth, DNA damage, etc. can be measured. Furthermore, treatment of other immortalized cells with CSC should be carried out.

2) Since *ras* point mutations are very common in chemical carcinogen-induced tumors, *ras* point mutations should be checked in CSC-transformed cells.

3) Since overexpression of B-*myb* in immortalized and CSC-transformed cell was found, transfection of anti-sense B-*myb* plasmids into immortalized cells or CSC-transformed cells can be carried out to check if it plays a direct role in the process of oncogenesis *in vitro*.

4) To obtain a more complete picture, the effect of other cytokines, such as IFN-γ and IL-6, on proliferation, HPV expression and some cellular genes should be further characterized.

5) The discoveries of the role of the JE gene and p15 in the pathway of TNF-α- and TGF-β-mediated cell defense are interesting and they should be further studied.

6) 5'-RACE or cDNA library screens should be used to isolate the whole-length cDNAs for PA4 and PA9. The complete characterization of these cDNAs could then be carried out. The mRNA expression of PA4 and PA9 in different tissues and in biopsies of normal ecto- and endocervix, CIN I-III and cervical cancer could be compared.

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Appendix I. Composition of Media

Keratinocyte Growth Medium (KGM):

$[Ca^{2+}]$	0.15 mM
L-glutamine	500 mM
EGF, human, recombinant	2 µg/L
Bovine Pituitary Extract (BPE)	50 mg/L
Penicillin-Streptomycine	50,000 I.U /L

Dubecco's Modified Eagle Medium (DMEM):

$[Ca^{2+}]$	1.5 mM
D-glucose	4,500 mg/L
L-glutamine	584 mg/L
L-methionine	30 mg/L
L-leucine	105 mg/L
Pyruvate	110 mg/L
Phenol red	15 mg/L
Sodium phosphate	125 mg/L
Calcium chloride	200 mg/L
Pyridoxal.HCl	4.0 mg/L
FCS	10 %
Penicillin-Streptomycin	50,000 U.I./L
Hydrocortisone	400 mg/L