

Establishment of tumor cell lines by transient expression of immortalizing genes

Review Article

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Summary

Many basic and clinical studies on human cancers require establishing of tumor cell lines from fresh tumor tissues in a highly reproducible fashion. However, this goal has not been achieved since the first aneuploid epithelial cell line, HeLa, was established from cervix adenocarcinoma about 5 decades ago. A widely accepted concept is that malignant tumor cells are almost immortal so the difficulty in establishing new tumor cell lines is believed to arise from problems of cell culture techniques. Evidence from our experiments demonstrated that this difficulty has a genetic origin: many primary cancer cells have not completely lost their 'tumor suppressor' pathways. In this review the immortalizing genes, transient expression systems of foreign genes in mammalian cells, and the potential applications of transient expression of immortalizing genes are summarized.

I. Introduction

Tumor cell lines have profound significance for the development of cancer cell biology and have made a great contribution to a variety of cancer researches (Stoker, 1996). Most stable human cell lines are established from cancer tissues and are widely used in the study of all fields of cancer. Some clinical trials such as gene-modified cancer vaccines demand growing a number of proliferative autologous tumor cells (Blankenstein, 1996). However, despite improved cell culture conditions it is still difficult to establish permanent cell lines from human primary tumors in a reproducible fashion. Growing human cancer cells in primary culture is hard work that requires patience, intuition, care and experience. The reason for the difficulty in growing human primary cancer cells is not completely understood.

The advances in DNA recombinant technology and gene transfer have made it possible to develop new methods for establishing permanent tumor cell lines from clinical tumor samples by the introduction of immortalizing genes into primary tumor cells (Pantel, 1995; Li, 1997). This review focuses on the background

and difficulty of the establishment of tumor-derived cell lines and new ways to overcome this difficulty as well as the future prospects of these new technologies.

II. Historical Background

A. Old and new problem: Difficulties for establishing long-term tumor cell lines

Since the first human tumor cell line HeLa was established from a cervical adenocarcinoma (Gery, 1951), a variety of permanent ('immortalized') tumor cell lines have been established from different human tumor tissues (primary, invasive, metastatic or recurring) of most types of tissues (Fogh, 1975). However, the establishment of long-term tumor cell lines is hard, time-consuming, and unpredictable work with a very low success rate.

The success rates of establishing permanent tumor cell lines vary notably from one tumor cell type to another, ranging from 50% or more of malignant melanoma to 1% or less of breast cancers (Stamps, 1992) and is related to tumor type and clinical stage.

Among human cancers, malignant melanoma cells are easiest to establish from primary lesions, metastases and effusions (Moor, 1996). Melanoma cells, in a malignant effusion (chest or abdomen), are the easiest to establish, over 50% success; then cells from metastases, 20-50%; and lastly primary cancers, 10-25%. Human breast carcinomas are one of tumors that are the most difficult to culture. Only 10% breast cancer cells proliferate in vitro and less than 1% of them are able to be established as long-term cell lines (O'Hare, 1991).

A lot of evidence showed that most tumor cell lines were established from the later stage cancer tissues. The early stage cancer cells are much more difficult to grow in vitro. Of the 136 analyzed tumor cell lines at tumor cell bank of American Type Culture Collection (ATCC), we found that most cell lines (76/136, 56%) were established from metastatic or invasive lesions. In primary tumors one third of the cell lines (23/60) derived from blastoma and sarcoma, which are often more malignant. Edington, et al. (68) compared cultures from different stages of head and neck squamous carcinoma and found that the immortal cultures were not obtained from normal (0/4) and pre-malignant (0/4) tissue cultures but rather from T1/T2 carcinoma (2/7), T4 carcinoma (6/10), and metastases (2/3). All recurrent tumors were immortal.

Wilson (1996) summarized the literature concerning at least 70 ovarian tumor cell lines. The majority of these cell lines derived from ascitic fluids (n=59) rather than solid tumors (n=14) and were also most frequently established from patients with a poor prognosis. There was a lack of cell lines from well-differentiated or benign tumors. Review of the literature indicates that there are no particular growth factors or media that result in cell line development.

B. Malignant tumors are often more difficult to culture than normal cells

Earlier observations showed that some cancer-derived epithelial cells such as breast carcinoma, nasopharyngeal carcinoma (Li, 1994) and prostate cancer, etc., were even more difficult to grow in vitro than the normal epithelial cells from same tissue type. Human breast carcinomas are the representative examples. The normal epithelial cell of breast could be passaged 3-4 times while those derived from cancer only survived one passage (Smith, 1984). Using improved media normal epithelial cells can be grown up to 10-20 passages but carcinoma-derived cells only proliferated 3-5 passages (our unpublished data).

It is not clear so far why most of so called 'immortal' primary tumor cells are difficult to grow in vitro and even more difficult than normal epithelial cells. The failure to culture some tumor cell types was considered due to

technical problem because primary tumor cells may be more dependent on specific growth factors than normal cells.

III. Improvement for establishing new tumor cell lines

A. Modified cell culture methods

The traditional method for establishment of tumor cell lines is tissue culture. Basic procedure is that tumor biopsies are dissociated with mechanical and enzymatic treatment (Speirs, 1996) and isolated tumor cells are in 5-20% FBS medium. The improvement of conditions of tissue treatment and culture medium can enhance the success rate of establishing tumor cell lines.

Modification of preparations of tumor samples for primary culture: the preparation of tumor samples with mechanical disaggregation or enzymatic digestion is the first and important step to establish successful primary culture. The tumor tissues lack an integrate basement and are sensitive to enzymatic digestion. It has been confirmed that complete digestion of tumor tissues into single cells can not produce successful primary culture. Use of partial enzymatic dissociation can improve the viability of tumor cells (Dairkee, 1997). In colon carcinoma the mechanical disaggregation may result in higher rates of primary cultures (Dillman, 1993).

1. Modification of growth media

We have analyzed the primary culture growth media for human tumor tissues in 136 tumor cell lines of tumor cell bank at ATCC. The most common used media are RPMI 1640, DME, Eagle's EME, and L-15 supplemented with 10-20% fetal bovine serum. Similar results were obtained from reviewing the literature of the last decade. The most successful primary culture and long-term cultures of human tumor cells are malignant melanomas, which grow well in RPMI 1640 media supplemented with 10-20% fetal bovine serum (Semple, 1982; Marincola, 1996). The most commonly used supplement for in vitro culture to most of established cell lines is bovine serum, which provides hormones, nutrients and promotes cell growth. However, in serum-containing medium, contaminating fibroblasts impose a big obstacle and often grow over tumor cells. Fibroblasts are easy to grow in serum-containing media but normal and neoplastic epithelial cells are not. The culture of epithelial cells in serum-supplemented media is prone to terminal differentiation and always poses the problem of fibroblast overgrowth in cultures; indeed, serum does not contain enough basic growth factors and growth-inhibitory or differentiation-inducing factors for particular epithelial cells (Miyazaki, 1989). Masui, et al. (1986) found that

type beta transforming growth factor is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells.

2. Serum-free and low serum medium for human epithelial cells

The development of defined serum-free media greatly improved the in vitro culture of a variety of different types of human epithelial cells. Many specific media are designed for particular epithelial cell types, such as keratinocyte growth media MCB153 (Boyce and Ham, 1985; Boisseu, 1992), mammary epithelial cell growth media MCB170 (Hammond, 1984; Weaver, 1995) or DFCI-1 (Band, 1989), and bronchial epithelial cell growth media LHC9 (Lechner, 1989). Serum-free media have advantages in culture over serum-supplemented media by providing defined reproducible systems without inhibitory factors and permitting selective growth of tumor cells from fresh clinical specimens. For instance, the rate of successful primary cultures from tumor-derived tissues of colon (van der Bosch, et al. 1981) and lung (Carney, et al. 1981) was higher in serum-free media than in serum-containing media (Collodi, et al. 1991).

However, using serum-free media to culture human tumor cells has not given the expected success as normal epithelial cells, from which most human malignant tumors originate. The serum-free media do not support the long-term growth of human tumor cells from primary cultures. For instance the primary and early-passage culture of human mammary carcinoma using defined serum-free or low serum media (MCDB 170, DFCI-1 and CDM3) results in outgrowth of normal epithelial cells or fibroblasts (Band, 1995). The establishment of permanent tumor cell lines has not been significantly improved with serum-free or low serum media.

B. Xenograft

The discovery of immunodeficient nu (nude) (Pantelouris, 1968) and scid (Bosma, 1983) mutant mice has provided new methods to establish tumor cell lines by xenogeneic transplantations. Many tumor cell lines have been established using xenograft in these immunodeficient mice. The take rates are dependent on the type of tumor tissues (Arnold, 1996). Establishment of human tumor cell lines by xenograft of tumor biopsies into immunodeficient mice is time-consuming and expensive and is not sufficient for the construction of autologous tumor vaccines.

Because of the common difficulty of establishing long-term or permanent cultures of human tumor cells from fresh surgical tumor samples new techniques are needed to solve these problems. We have been trying to

use the immortalizing techniques of mammalian cells to establish new tumor cell lines.

IV. In vitro immortalization of human cells

A. Immortalization methods

A wide variety of human tissues can be cultured and maintained in vitro just for a limited period time. All of these cells finally enter a 'crisis' phase, that is, cell division ceases and the culture becomes senescent except that they obtain mutations and become immortalized. With the exception of fibroblast most primary human cells such as epithelial cells have a short lifespan in vitro. A formal definition of an immortal cell line is that growing indefinitely in vitro. A normal fetal human fibroblast typically reaches senescence at 60 population doublings (PD) so an operational definition is that a fetal human fibroblast exceeds 100 generations or population doublings in culture, or shortly after a colony derived from single-cell cloning can be propagated to 10^6 to 10^7 cells (MacDonald, 1990). In order to obtain permanent cell lines a number of immortalization methods have been developed.

- (i). Treatment with carcinogenic chemicals.
- (ii). Exposure to a DNA tumor virus such as SV40 virus, EBV, and papilloma virus.
- (iii). Cell fusion between the cell with a limited lifespan and a permanent cell line.
- (iv). Viral and cellular immortalizing genes / oncogene transfer.

With the development of recombinant DNA techniques and the advent of gene transfer immortalization of mammalian cells by the introduction of immortalizing genes became an important method for developing new cell lines from normal cells for animal cell biotechnology. A variety of cell lines from different species and types have been established via infection or transfection of viral and cellular oncogenes (MacDonald, 1990).

B. Immortalizing genes

There are many genes involving in vitro cellular immortalization (Duncan, 1993). Two main types of immortalizing genes are summarized based on the targeting genes. 1) Anti-tumor suppressor immortalizing genes: this kind of immortalizing genes involves the inactivation of tumor suppressors such as pRB and p53 and includes SV40 large T antigen; HPV16 E6 and E7; Adenovirus E1A and E1B; Polyoma large T; and Mutant p53, etc. 2) Oncogene-relating immortalizing genes: c-

myc, *bmi-1*, *c-Ha-ras* etc. 3) Other genes: telomerase gene.

1. Anti-tumor suppressor immortalizing genes: SV40 large T antigen

SV40 large T antigen gene is one of the most successful immortalizing genes. This gene encodes a nuclear phosphoprotein containing 708 amino acids. T antigen has a variety of biochemical activities, including viral DNA replication and transformation functions. Large T can be divided into many different functional regions of: specific DNA binding, ATPase activity, nuclear location signal, etc. (for review, Fanning, 1992; Bryan, 1994; Manfredi, 1994). To date, several transformation regions of SV 40 large T antigen have been identified. a) Amino-terminal transforming domain (amino acids 1-82) binds to DNA polymerase and the p300 protein. b) Rb-binding region (amino acids 101-118), binding to pRb and p107. c) Nuclear location signal resides in amino acid 126-132. d) p53-binding domain (amino acids 351-450 and 533-626): A lot of mutation research suggest that p53 binding domain is located in a region between residues 272 and 626. Amino acids 351-450 are important for direct contact with p53 and amino

acids 533-626 are important for regulating the interaction. e). Region involved in conformation: a. Zinc finger (amino acid 302-318) The mutations of this region alter the overall conformation of the protein and result in the decrease of foci formation in primary mouse embryo fibroblasts and of viral DNA replication. b. Hydrophobic region (amino acids 571-589) this region maintains the general stability of protein and is indirectly important for transformation.

Normal human cells immortalized by SV40 large T antigen: A variety of human primary cell types have been immortalized with SV40 virus, SV40 genomic DNA, and plasmid DNA or retrovirus expressing SV40 large T antigen (**Table 1**). For a long time SV 40 large T antigen has been introduced to a variety of cells which are difficult to culture in vitro in order to obtain cell line with long-term growth. SV40 large T transformed human fibroblasts routinely have an extension of the lifespan of the normal parental cell but still eventually cease to proliferate. Approximately 25% of cloned transformants yielded immortalized cell lines with frequencies ranging from one per 10³ to 10⁷ cells. Keratinocytes and epithelial cells may generate immortalized derivatives at a higher frequency.

Table 1. Examples of normal human cells immortalized by SV40

Cell Type	Method	Ref.
Fibroblast	Ori+ plasmid	Shay and Wright, 1989
	Ori- plasmid	Banerjee et al., 1992
Keratinocytes	Virus	Taylor-Papadimitriou et al., 1982
Bronchial epithelium	Virus	Reddel et al., 1988, 1995
	ori- plasmid	Cozens et al., 1992
Breast epithelium	SV40 Virus,	Chang et al., 1982
	Retrovirus	Bartek et al.,1990, 1991
	Ori-plasmid	Berthon et al., 1992
Prostatic epithelium	Ori-plasmid	Hayward, et al., 1995
Esophageal epithelium	Ori-plasmid	Stoner et al., 1991
Uroepithelium	Virus	Christian et al., 1987
Liver epithelium	SV 40 T-plasmid	Miyazaki, 1993; Schippers, et al., 1997
Retinal epithelium	Ori+plasmid	Dutt et al., 1994
Bone marrow stromal cells	SV40-adenovirus	Aizawa, et al., 1994
Human osteoblastic cells	tsA58	Hariss, et al., 1995
Monocyte/macrophage	Ori-DNA	Nagata et al., 1983; Kreuzburg, 1994
Microglial cells		Janabi, et al., 1995
Granulosa-lutein cells	SV40 T-plasmid	Lie, et al., 1996
Endothelial cells	SV40 T-plasmid	Fitzgerald, et al.,1994
Thymic nurse cells	SV40 virus	Pezzano, 1991
Dendritic cells	Large T gene	Volkman, 1996
Endometrial cells		Merviel, et al., 1995

Tumor cells immortalized by SV40 large T: Pantel, et al. (1995) immortalized the micrometastatic tumor cells of prostate cancer, renal cell cancer, lung cancer, breast cancer, and colorectal cancer with microinjection of SV 40 large T plasmid DNA. After microinjection of the propagated cells with T-antigen DNA, permanent cell lines were obtained and showed no notable changes in the pattern of expressed epithelial antigens and were able to disseminate into bone marrow in SCID mice.

2. Human papilloma virus E6 and E7 genes

HPV E7 proteins bind and inactivate the retinoblastoma protein RB (Pei, 1998), while E6 increases the degradation of p53 (Van Dyke, 1994). The introduction of both HPV-16 or -18 E6 and E7 can efficiently immortalize human keratinocytes from the cervix or foreskin (Hawley-Nelson, 1989), and mammary epithelial cells (Shay, 1991). High expression of HPV-16 E7 alone can immortalize human keratinocytes at low frequency compared with a similar virus containing HPV-16 E6 and E7 (Halbert, 1991).

3. Adenovirus E1A and E1B

A human kidney epithelial cell lines was established from the adenovirus DNA transfection (Graham, 1977). E1A proteins bind pRB (Whyte, 1988) and other cellular proteins p107, p130 and p300 (van Dyke, 1994), while E1B inactivates p53 protein (Sarnow, 1982).

4. Polyoma virus large T

A lot of mammalian cells have been immortalized with polyoma virus large and middle T antigen such as human fetal sinusoidal liver cells (Hering, 1991), human fibroblast (Jat, 1986; Strauss, 1990), mice astroglial cells (Galiana, 1990), etc. The large T antigen of polyoma virus can bind to tumor suppressor pRb protein (Dilworth, 1990).

5. Mutant p53

P53 is one of most important tumor suppressors and a transcription factor, which functions as a cell cycle G1 checkpoint molecule. P53 protein transmits damaging cellular stress signals (such as DNA damage) to cell cycle and induces cell growth arrest or apoptosis. Thus, cells that lose p53 protein are prone to be immortal. A germ line mutation of p53 gene exists in the breast cancer-prone Li-Fraumeni syndrome (Shay, 1995). Normal epithelial cells from this syndrome are spontaneously immortalized in vitro. Gao, et al. (1996) reported that a single-amino acid deletion mutant (del239) of p53 is able to immortalize primary human mammary epithelial cells. Further studies showed that

p53 mutants R175H and R249s immortalized human mammary epithelial cells in a reproducibly way, whereas R248W and R273H mutants induced an extended life span but not immortalization (Cao, 1997).

B. Oncogene-relating immortalizing genes

1. C-myc

Myc protein plays a critical role in the normal control of proliferation and differentiation. Ectopic expression of Myc is enough to drive many cells into the cell cycle in the absence of external mitogens (Bouchard, 1998). C-myc or v-myc gene can immortalize primary rodent fibroblasts (Kelekar, 1987) and human mammary epithelial cells (Valverius, 1990). Myc and raf oncogenes cooperate with absence of p53 to immortalize hematopoietic cells (Metz, 1995).

2. Bmi-1

The *bmi-1* is a transcriptional repressor belonging to the mouse Polycomb group and cooperates with c-myc in the generation of mouse lymphoma. Overexpression of *bmi-1* allows fibroblast immortalization immediately or after a slow growth period, downregulates expression of p16 and p19, and, in combination with H-ras, leads to neoplastic transformation (Jacobs, 1999).

3. ras and raf

The ras and raf oncogenes can not immortalize mammalian cells alone. On the contrary, they induce premature senescence (Serrano, 1997). But these oncogenes cooperate with other oncogenes such as myc and HPV E6 to immortalize some types of cells such as fibroblasts, microglial cells (Blasi, 1990), epithelial cells (Comper, 1989).

4. EBV genes

Epstein-Barr virus can efficiently immortalize human B lymphocytes. 10%-100% of infected B cells become immortal (Sugden, 1989). The mechanism of EBV-induced immortalization is not completely to be understood. Several viral genes and the altered expression of cellular genes are required for the efficient immortalization of resting B cells. EBV-encoded EBNA-1, EBNA-2, LMP1 and LMP2 proteins are involved in the B cell immortalization.

C. Telomerase

It has been reported that telomere shortening is a key mechanism for cellular senescence (Shay, 1991, Bodnar, 1998, Smith, 1996). The causal relation

between senescence and gradual loss of chromosome ends was proposed about 25 years ago. The chromosome ends consist of telomeres, which are noncoding G-rich repeats and maintain the chromosomal stability. The incomplete replication of 5' ends of linear DNA molecules leads gradual chromosome shortening. Telomerase can restore telomere length through adding telomeric repeats (Kim, 1994). Bodnar, et al. (1998) found that the introduction of telomerase into normal fibroblasts resulted in the extension of life-span. Telomerase-expressing clones had elongated telomeres, divided vigorously, and showed reduced staining for β -galactosidase, a biomarker for senescence.

However, a number of evidences showed that telomerase is not enough to immortalize cells, although telomere maintenance is essential for immortalization (Carman, 1998). Some telomerase-positive cells also senesce and some senescent cells expressed telomerase activity (Carman, 1998). Recently, Kiyono, et al. (1998) reported that telomerase activity is not sufficient for immortalization of human keratinocyte or mammary epithelial cells. However, inactivation of the Rb/p16 pathway in combination with telomerase activity, is able to immortalize epithelial cells efficiently.

V. Mechanism of immortalization

A. Immortalization induced by SV40 large T antigen

The SV40 large T antigen-induced immortalization is a two step process (Wright, et al., 1989; Shay, et al., 1991), which was first found in human fibroblasts (Girardi, et al., 1965) and later confirmed in breast epithelial cells (van der haegen, 1993). Wright, et al. (1989) immortalized IMR normal human diploid fibroblasts with a steroid inducible mouse mammary tumor virus-derived SV40 large T antigen and found that after dexamethasone removal the cells stop growing during the precrisis extension of life-span and after immortalization. According to these data a two-stage model for cellular senescence have been put forward. Mortality stage 1(M1) is responsible for the precrisis cell growth arrest and mortality stage 2(M2) for the failure of cell division during crisis. For cellular immortalization the first step is an extension of culture lifespan (about 20 PD after the senescence of the normal cells), which is dependent on the binding of SV40 large T antigen to the tumor suppressor p53 and Rb. And then the cells enter the second step and undertake 'crisis', in which cell death increases. A few cells escape the crisis and become colonies of immortalized cells. This step involves in an entirely independent mechanism. Recent studies showed that telomerase is an important enzyme

which let cells escape M2 (Holt, 1996; Bodnar, et al. 1998).

B. Yin-yang model of cellular proliferation, senescence, and immortalization

Cellular immortalization is defined as a process that cells can 'indefinitely' proliferate in vitro. The regulation of cell proliferation is dominated by two groups of genes (Dyson, 1999): proliferation-stimulating genes (proto-oncogenes) such as ras, myc, and bcl-2, etc and proliferation-inhibiting genes (tumor suppressor genes) such as p53, Rb, and p16, etc. The cell proliferation is delicately regulated through the complicate interaction among the products from these two groups of genes. The positive and negative signals form a large feedback loop and maintain a dynamic balance. This dynamic balance of positive and negative signals determines whether cells proliferate or are arrested. Deregulation of these positive and negative regulator results in abnormal apoptosis, senescence, immortalization, or uncontrolled cell proliferation (cancer). We proposed that cellular immortalization not only involves in the inactivation of tumor suppressors (Shay, 1995) but also in the changes of oncogene expression (Fig. 1) (Li, 1998). Zinky, 1998 reported that myc signaling regulates immortalization through ARF tumor suppressor.

VI. Transient expression system of foreign genes

The continuous presence of large T or other immortalizing genes may change the antigenicity of the cells or alter gene expression or cause mutation not specific for the tumor. It is necessary to develop controllable expression system for immortalizing gene, such as SV40 large T antigen. The transient expression system of foreign genes can be obtained with several methods such as inducible promoter system, temperature-sensitive mutants, and site-specific recombination system, etc. The former two systems pose the risk of 'leakiness' or not total absence of the protein and can not remove the targeting gene from genome. The site-specific recombination system can precisely cut out the gene from the genome (Dale, 1991; Bergeman, 1995).

A. Inducible promoter system

Early inducible expression vectors based on the inducible promoter and its control agents, e.g. heat shock protein and heat control, metallothionine promoters and heavy metal ion control or steroid regulatory promoters

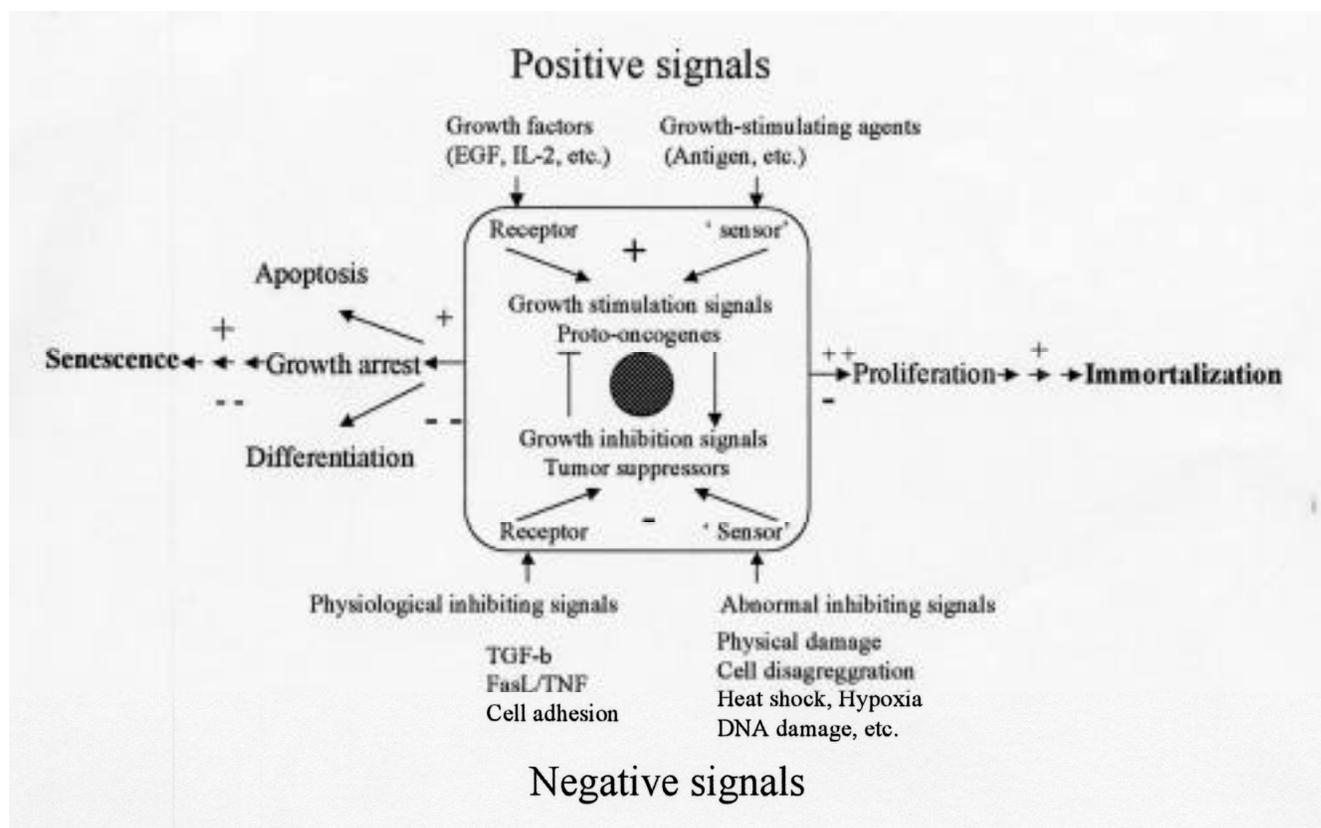


Fig. 1 Dynamic dual-signal (Yin-Yang) feedback / interaction model of cellular proliferation: The cell proliferation is controlled by positive and negative signals. Most of positive signals for cell proliferation are specific growth factors or growth-stimulating agents from the outside of cells, and the negative include growth-inhibiting factors, differentiation-induced factors, or abnormal stresses, etc.

and steroid control (Yarranton, 1992). These traditional inducible systems can regulate some level of controlled gene expression (10-20 fold induction) but often have higher background expression ('leaky expression').

Use of bacterial transcriptional control system in mammalian cells can obtain higher specificity and induction ratios because the bacterial operator is unlikely to bind the promoter sequence of a mammalian genes. The well-characterized inducible systems that based on bacterial control elements are E.coli lac repressor-operator and tetracycline responsive expression system. Extremely high induction ratio up to 10^5 -fold was achieved in the tet-on and tet-off system (Hofman, 1996).

B. Human progesterone receptor mutant (hPR891) and RU486 system

The human progesterone receptor mutant hPR891 loses 42 amino acid in its C-terminal and ability to bind progesterone or other endogenous hormones, but still can bind the synthetic antagonist RU486 (Vegeto, 1992). This

characteristics of hPR891 can be used as a switch and regulatory system of protein function (Wang, 1994). The fusing gene between the C-terminal hormone binding domain and targeting gene expresses a protein, whose activity is hormone-dependent.

C. Temperature-sensitive mutants

Earlier studies found a temperature-sensitive (ts) mutant of SV40, which can transform primary cells at the permissive temperature (33°C) but is inactive at the nonpermissive temperature (39°C) (Tegtmeyer, 1975; Petit, 1983). This mutant SV40 virus encode a thermolabile large T antigen tsA 58 which loss about 500 nucleotides at its C-terminal (Jat, 1989).

D. Site-specific recombination system

The site-specific recombination is different in mechanism and efficiency from homologous recombination, which occurs at any homologous sequence with a low rate and is dependent on the endogenous

recombination mechanism of the cell (Sauer, 1993, 1994). In contrast, site-specific DNA recombination that relies on exogenous recombinase is conservative and occurs at specific sites on the DNA molecule with high rate. The well-characterized site-specific systems are Cre recombinase of bacteriophage P1 and FLP recombinase from *Saccharomyces cerevisiae*. The former is more common used.

E. Cre/LoxP site-specific recombination system

Cre/Lox system is one of well-characterized site-specific recombination systems and consists of two basic components: Cre recombinase and LoxP site (**Figure 2**). These two components are sufficient to carry out site-specific recombination in vitro and in vivo (Gu, 1993).

Cre recombinase is a 38.5 KD protein of bacteriophage P1 and catalyzes conservative site-specific recombination between two LoxP sites. LoxP site is a 34

bp dyad-symmetric DNA sequence, which is composed of two 13 bp inverted repeats, separated by an 8 bp spacer molecule. Cre recombinase binds to the two 13 bp inverted repeats of LoxP sites and catalyzes precise recombination between the asymmetric 8-bp core region of two LoxP sites. The result of recombination is dependent on the direction of core region of two LoxP sites in one DNA sequence. Recombination between two directly repeated sites on the same DNA molecule results in deletion of the DNA fragment lying between two loxP sites. For a molecule with two inverted LoxP sites Cre recombinase catalyzes the inversion of the intervening DNA fragment.

Inducible Cre/LoxP system: the inducible expression system and Cre/LoxP site-specific deletion system have been combined to obtain controlled expression of Cre recombinase. Kühn et al. (1995) developed a method for inducible gene targeting in mice using interferon-responsive promoter to control the expression of Cre recombinase.

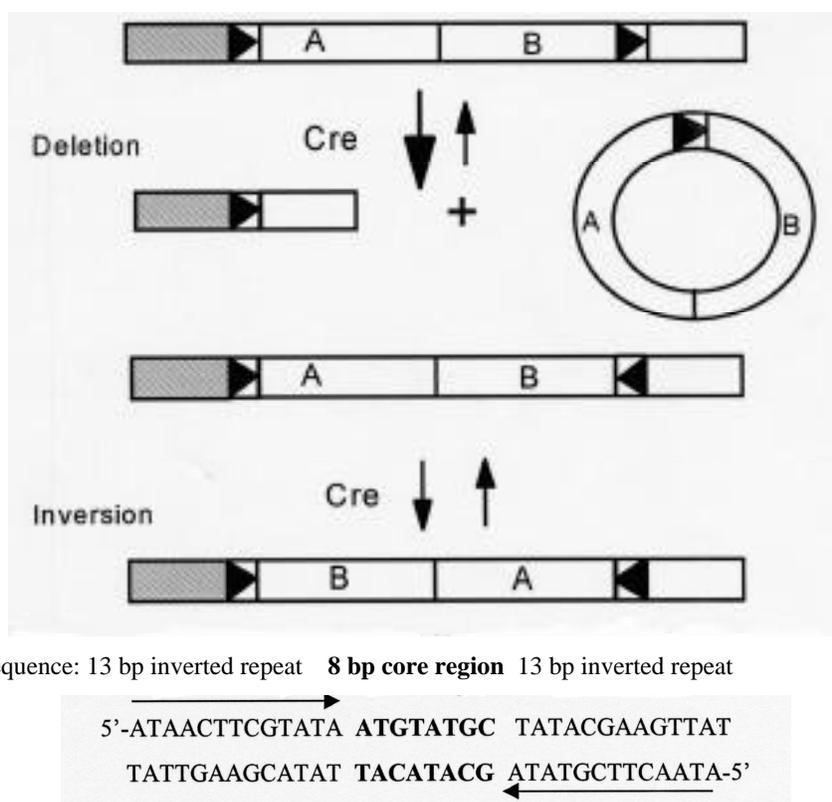


Fig. 2 Cre/Lox site-specific recombination system (A) DNA deletion: On a molecule with two same directional LoxP sites the Cre recombinase catalyzed the site-specific deletion of DNA sequence between LoxP sites. (B) DNA inversion: Cre/LoxP site-specific recombination results in the inversion of the intervening DNA fragment between two inverted LoxP sites. Black triangle, loxP sites; A, gene A; B, gene B.

Another inducible Cre/LoxP system uses a fusion protein of Cre recombinase and human steroid receptor such as progesterone (Kellendonk, 1996) or estrogen (Metzger, 1995) receptor. Normal progesterone or estrogen receptor has been fused with Cre recombinase, which are controlled by progesterone and estrogen. However, endogenous (in vivo) or media-containing (in vitro) steroids result in some background expression of Cre recombinase. Use of human progesterone receptor mutant hPR891 can overcome this problem. Kellendonk et al. (1996) fused Cre recombinase and hPR891 gene and showed that the recombination activity of fusion Cre recombinase was highly dependent on RU486. The recombinant rate was up to 50% to 80%. The uninduced recombination (background expression of Cre recombinase) varied from 2% to 5.5%.

Self-deleting retrovirus vector: Russ et al. (1996) constructed a complicated self-deleting retrovirus vectors with Cre/LoxP site-specific recombination. This system based on the natural life cycle of retrovirus, involving duplication of terminal control region U5 and U3 to generate long terminal repeats (LTRs) and Cre site-specific recombination. But insertion of foreign gene into the U3 region of retrovirus results in low virus titer.

Cre adenovirus: Adenovirus vector was used to express Cre recombinase (Anton, 1995). High titer of adenovirus let it to infect in vivo and in vitro cells efficiently.

VII. Establishment of tumor cell line by transient expression of SV40 large T antigen mediated with Cre/LoxP deletion recombination

In order to overcome the difficulty of establishing tumor cell lines from fresh tumor tissues, we chose SV40 large T antigen as stimulator agent of cell proliferation to obtain long-term proliferating cell line and Cre/LoxP-mediated site-specific deletion recombination as controlled expression system of SV40 gene. Using this transient expression system of SV40 large T antigen, we found that retroviral large T gene transfer allowed rapid expansion of some primary tumor cells without significant cell crisis and that subsequent elimination of T antigen resulted in cell growth arrest in a breast cancer line grown for more than a year. Remarkably, these cells changed morphology and stopped proliferation comparable to the cells obtained from biopsy, demonstrating the requirement of large T for growth (Li, 1997).

Later we confirmed these observations in other 10 cases of mammary carcinoma (Manuscript in preparation). Comparing in vitro growth and SV40 large T-induced immortalization of normal epithelial cells and cancer cells

from same patient we found that all epithelial cells from normal and tumor tissues lose proliferation in vitro after 5-10 passages but could be immortalized by SV40 large T antigen in a similar way after a period of 'selection'. These data showed that primary breast carcinoma cells maintain functional tumor suppressor pathway (Rb or p53) and that SV40 large T inactivate these tumor suppressor pathway to induce the immortalization of primary breast cancer cells. These evidences suggest that the difficulty to establish new tumor cell lines from some clinical cancer samples is not just a technique problem but has genetic reason and that the transfer of immortalizing genes into primary tumor cells should be a new feasible method to establish long-term cancer cell lines.

VIII. Potential application of transient expression of immortalizing genes

A. Use of SV40 LT amplified autologous tumor cells as vaccine

The motivation for attempting immortalization of many types of normal and tumor cells was to obtain a permanent line in order to study the characteristics of the original normal tissue or to use them as a vaccine for the original tumor.

Immortalization of primary normal cells: For normal tissues it is desirable that the immortalized cell line keep the differentiated properties of the primary cell type. The maintenance of differentiated properties has been showed to varying degrees. For instance three murine peritoneal macrophage-like cell lines have been established by transfecting primary cells with SV40 origin-deleted DNA (Kreuzburg, et al., 1994). These cell lines show to express many macrophage-specific properties, e.g. Fc receptor and staining for non-specific esterase. The cell lines phagocytosed IgG-coated particles, they were positive for murine macrophage-specific marker F4/80 and they showed antigen-presentation function.

On the other hand, most SV40 LT-positive cell lines lose many of their differentiated properties and take on a more transformed phenotype. During the immortalization of keratinocytes, the capacity of the cells to differentiation progressively decreased (Taylor-Papadimitriou, et al. 1982).

Immortalization of primary tumor cells: The immortalization of primary tumor cells is different from that of primary normal cells. (i) The aim to immortalize tumor cells is to use them as a source of tumor vaccine. The most important things for this purpose are that SV 40 large T immortalized tumor cells keep tumor antigen(s) and retain an ability to present tumor antigens to immune cells (Boon, 1994). (ii) Tumor cells have no function in

vivo and possess a lot of antigenic or non-antigenic mutations (Hepper, 1998).

For cell-based tumor cell vaccine it is necessary that transduced tumor cells must maintain tumor antigens and are able to present them to immune response cells. Previous studies showed that SV40 large T antigen immortalized normal cells express cell surface major histocompatibility complex class I molecules. The SV40 large T transformed intestinal epithelial cells were able to respond to the treatment of interferon- γ and express a high level of class II molecules (Vidal, 1993). These cells had an ability to process and present native protein antigens to specific CD4⁺ T cell hybridomas, via functional class II molecule. Pantel's (1995) studies showed that the expression of molecules relevant to an efficient immune response, such as MHC class I molecules and intercellular adhesion molecule-1 (ICAM-1) is not down-regulated in the genome of micrometastatic cells expressing SV40 large T antigen. So far there are no direct experimental data that show whether or not SV 40 large T antigen influence the expression of tumor antigens and their immunity.

B. Reversible transformation of human cells

The transient amplification and immortalization of primary normal and tumor cells by controlled expression of immortalizing gene or oncogenes such as SV40 large T has a lot of potential applications for basic and clinical studies (Westerman, 1996).

The Cre/LoxP-mediated controllable SV40 transformation and immortalization system may facilitate investigations of cell differentiation, oncogenesis, cell cycle, and senescence by allowing controlled cell proliferation and accurate on/off studies of various oncogenes in primary cells. This system also can be used to compare the phenotype of primary normal and tumor cells. From our observations the primary cells of normal and tumor cells transformed by SV40 large T showed some different phenotype and morphology. Because the stringent positive and negative selection yielded highly pure cell populations having permanently excised the transferred oncogene, this method may also prove especially adapted for the safety concerns of cell and gene therapies. Taking advantage of the cell expansion phase to perform gene targeting by homologous recombination may make genetic disease amenable to correction rather than gene addition.

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