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TRANSFORMATION OF NORMAL HUMAN BREAST EPITHELIAL CELLS BY IONIZING RADIATION AND THE EXPRESSION OF THE CELL CYCLE REGULATING GENES

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By

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ABSTRACT

TRANSFORMATION OF NORMAL HUMAN BREAST EPITHELIAL CELLS BY IONIZING RADIATION AND THE EXPRESSION OF THE CELL CYCLE REGULATING GENES

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Based on substantial evidence that ionizing radiation is a breast carcinogen and that certain type of human breast epithelial cells (HBEC) is more susceptible to neoplastic transformation, experiments were conducted on two types of normal HBEC (Type I and Type II) with low doses of X-rays. The results show that 24 clones with extended lifespan (E. L.) were obtained from Type II cells from two of six HBEC cultures whereas no E. L. clones were isolated from Type I HBEC. These E. L. clones did not become immortal after prolonged growth or additional X-ray irradiation. The p53 and p21 were frequently and concomitantly elevated in these E. L. clones. These E. L. clones, however, appear to contain wild type p53 since the cells showed radiation - induced G1 arrest. While normal and immortal HBEC expressed only the phosphorylated form of Rb. All except one of the E. L. clones tested, expressed both phosphorylated and unphosphorylated forms of Rb. Unlike the other cell cultures used in this study, the cell culture which yielded most E. L. clones was found to be deficient in p16 expression. The expression of p16 is, therefore, suspected to be correlated with susceptibility of HBEC to X-ray induced transformation. The results suggest a profile of cells with extended lifespan (i.e. high Rb and p53 expression and low p16 expression), a reasonable doubt about Type II HBEC as target cells for neoplastic transformation, and a new strategy to transform Type I HBEC by X-rays.

TO
MY PARENTS
MY WIFE--CHIU-MEI
AND
MY DAUGHTER--AMY
FOR THEIR LOVE AND SUPPORT

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LISTS OF ABBREVIATIONS

HBEC.....	Human Breast Epithelial Cell
AT.....	Ataxia Telangiectasia
BPE.....	Bovine Pituitary Extract
BSA.....	Bovine Serum Albumin
cpdl.....	cumulative population doubling level
E. L.	Extended Lifespan
EGF.....	Epidermal Growth Factor
FBS.....	Fetal Bovine Serum
HME.....	Human Mammary Epithelium
HPV.....	Human PapillomaVirus
PCNA.....	Proliferating Cell Nuclear Antigen
RPA.....	Replication Protein A

Introduction

Although advances in breast cancer research have been made in recent years, including the cloning of the hereditary breast cancer genes for BRCA1 and BRCA2 (Miki et al., 1994,Wooster et al., 1995), the etiology of breast cancer is not fully understood. For the endogenous breast cancer risk factors reported, early menarche (Bouchardy et al., 1990), late menopause (Ewertz et al., 1988), and obesity (Gail et al., 1989), are linked to the cumulative exposure to estrogen (Henderson et al., 1993). Many environmental agents (e.g. organochlorine compounds such as DDE, PCBs, chlordane, and polycyclic aromatic hydrocarbons) suspected to cause breast cancer are similarly linked by functioning as xenoestrogens or estrogen potentiating agents (Davis et al., 1993). The evidence for this is not yet conclusive. However, ionizing radiation is a well-established environmental agent known to cause breast cancer. The evidence came from excess risk of breast cancer associated with Japanese women exposed to atomic-bomb radiation in Hiroshima and Nagasaki (Land et al., 1991). Women treated by radiation therapy for malignant (Pollner et al., 1993) and non-malignant conditions (Hildreth et al., 1989; Shore et al., 1977) also have increased risk. The mechanism by which X-rays induce breast cancer is not clear. Presumably, X-rays are capable of eliminating tumor suppressor genes through mutation. In *in vitro* studies, X-ray irradiation has been found to induce tumorgenicity in an immortal tumorigenic cell line (Kang et al., in press) and to immortalize normal human breast epithelial cells (HBEC)(Wazer et al., 1994). Several possible mechanisms may mediate this transformation. Alternations in the expression of

several cell cycle regulating genes have been demonstrated to be plausible mechanisms in a variety of carcinogenesis models including X-ray induced (Wazer et al., 1994). The factors of interest include, but are not limited to, p53, Rb, p21, p16 and cyclin D1. Recently, a cell culture method has been developed in which two types of normal HBEC, derived from reduction mammoplasty, can be grown (Kao et al., 1995). Type II HBEC is capable of gap junctional intercellular communication (GJIC) and expresses basal epithelial cell phenotypes. Type I HBEC is deficient in GJIC and has luminal and stem cell characteristics. These two types of cells differ substantially in their response to oncogenic stimulus (SV40 Large T antigen), i.e. Type I cells have the ability to establish anchorage independent growth and easily become immortal (Kao et al., 1995). Studies were conducted to determine if ionizing radiation is an effective initiator of neoplastic transformation in these two types of HBEC. Additional experiments were conducted to determine if phenotypic changes caused by X-ray is associated with alteration in expression and function of cell cycle regulating genes.

Literature review

Risk factors for breast cancer

Breast cancer accounts for 39% of all cancers in women and is the second leading cause of cancer death among women in the USA. Approximately 180,000 new cases and 40,000 deaths due to this disease were estimated to occur in the USA in 1992 (Boring et al., 1992). The chance of developing breast cancer in women by the age of 85 is one in nine (Marshall et al., 1993).

Many risk factors for breast cancer in females have been documented (Marshall et al., 1993). The most significant ones (relative risk more than 2) include (high vs. low): age (old vs. young), country of birth (North American, northern Europe vs. Asia, Africa), age at first full term pregnancy (>30 vs. <20), oophorectomy (no vs. yes), body build after menopause (obese vs. thin), family history of premenopausal bilateral breast cancer (yes vs. no), history of fibrocystic disease (yes vs. no) and history of primary cancer in ovary or endometrium (yes vs. no). Many environmental agents have been associated with breast cancer by functioning as xenoestrogens (e.g. o p′-DDT, PCBs, heptachlor and other pesticides) or as estrogenic potentiating factors (e.g. alcohol, low fiber, fat/total calories)(Davis et al., 1993).

Ionizing radiation and breast cancer

Among the few established environmental agents that have been associated with breast cancer, ionizing radiation is perhaps the best established exogenous carcinogen of breast cancer. This is supported in studies with atomic-bomb survivors in Japan and in women treated with radiation therapy (Land et al., 1991; Pollner et al., 1993; Hildreth et

al., 1989; Shore et al., 1977). Ionizing radiation could induce chromosomal alterations such as deletions and mutations (Renan, 1992). X-rays, as inducer of deletion mutations, could eliminate either tumor suppressor genes or cell cycle regulating genes. Thus, cells may have chances to escape proliferation control and/or cellular senescence to become immortal. Cells have at least two ways of responding to irradiation. First, cells are arrested in G1 and induced to repair their DNA (Lane, 1992). Alternatively, cells could be induced to apoptosis to avoid passing the defective DNA to progeny cells (Levine, 1997).

Genes and breast cancer

It has been proposed that tumors are the result of a progressive accumulation of mutations and epigenetic alterations of oncogenes and tumor suppressor genes (Weinberg, 1996). Several genes have been associated with the development of breast cancers.

Oncogenes:

C-erbB2

The c-erbB2 (neu/Her-2) gene, which is located on chromosome 17q21, encodes a 185 Kda protein tyrosine kinase which, together with EGF receptor (erbB1), erbB3, and erbB4, is a member of the Type I receptor tyrosine kinases (Carraway et al., 1994). The ligand heregulin has been shown to bind with high and low affinity with erbB4 and erbB3 respectively and induce heterodimerization with erbB2, thereby activating both signaling pathways (Peles et al., 1993; Carraway et al., 1994). The gene is amplified in approximately 30% of primary human breast carcinomas (Slamon et al., 1987; Iglehart et

al., 1990) and other 10 % over express c-erbB2 without amplification of the gene (Kraus et al., 1987; Slamon et al., 1989; Hollywood et al., 1993). The amplification of the gene indicates poor prognosis and shorter time to relapse (Borg et al., 1990).

C-myc

C-myc is a nuclear phosphoprotein, which play a role in both cell proliferation and apoptosis. The c-myc gene is located in chromosome 8q24. C-myc has a dual function: stimulating proliferation and inducing apoptosis (Harrington et al, 1994). Abnormalities in the c-myc gene were reported in about 6-32% of breast cancer and most of them are resulted from gene amplification or rearrangement (Van der Vijver and Nusse 1991). C-myc expression was found to be induced by estrogen in estrogen responsive human breast cancer cells (Dubik et al., 1992) and is necessary for estrogen induction of proliferation of breast cancer (Watson et al., 1991). Data from transgenic mice confirmed the abnormalities of c-myc are a direct cause of breast cancer (Stewart et al., 1984). Several studies have shown that amplification of the c-myc gene to be associated with inflammatory carcinoma, poor prognosis and post menopause disease (Van Der Vijver and Nusse 1991; Berns et al.,1992; Escot et al.,1986).

Int-2

The int-2 gene encodes a member of the fibroblast growth factor family (Dickson et al., 1987). The gene is amplified in up to 23 % of human breast carcinomas (Van Der ViJver and Nusse 1991). Amplification of int-2 has been associated with local recurrence, age more than 50 years and the presence of lymph node metastases (Van Der ViJver and Nusse 1991). In most of the tumors with int-2 amplification, a whole cluster

of adjacent genes, i.e. hst, bcl-1, is co-amplified (Yoshida et al., 1988) leading to the speculation of the presence of another gene which can confer a selective growth advantage when amplified.

Tumor suppressor genes

Besides the three oncogenes, many tumor suppressor genes have been associated with breast cancer. First, there are several hereditary breast cancer genes which contribute to an estimated 5% of all breast cancer cases, and up to 36% of diagnosed causes before age 30 (Claus et al., 1991). These genes are briefly described below.

BRCA1

This gene was mapped to chromosome 17q21 in 1990 (Hall et al., 1990) and cloned in 1994 (Miki et al., 1994). Mutations in BRCA1 alone account for about 45% of the families with high incidence of breast cancer and 80% of families with high incidence of both breast and ovarian cancer (Easton et al., 1993). The gene, however, appears to play no role in sporadic, nonhereditary form of breast cancer (Futreal et al., 1994). The function of the gene is not clear. The gene may act as a transcription activator (Monteiro et al., 1996), or may interact with RAD 51 to participate in nuclear processes that lead to normal chromosome recombination and genome integrity control (Scully et al., 1997)

BRCA2

This gene was localized to chromosome 13q12-13 and cloned in 1995(Wooster et al., 1995). Germline mutations of BRCA2 predispose both men and women to breast cancer. Studies with BRCA2 knock out mice indicate that the gene may play a role in DNA repair and cell proliferation by interacting with the DNA-repair protein RAD51 (Sharan

et al., 1997). In multiple fetal and adult tissues, the spatial and temporal pattern of BRCA2 mRNA expression is virtually indistinguishable from that of BRCA1 (Rajan et al., 1997)

p53

The p53 gene located on chromosome 17q13 is the most commonly mutated tumor suppressor gene in human cancer (Hollstein et al., 1991). Li-Fraumeni syndrome which predisposes the patient to breast cancer, sarcomas and other neoplasms was found to carry a germline p53 gene mutation (Malkin et al., 1990). As a tumor suppressor gene, the normal function of the gene may be inactivated by several means in tumors:

1). Mutation

This is a common way by which p53 is inactivated. About half of all human tumors have a missense mutation or deletion of the p53 gene, and 95% of these mutations occur in the central region (Levine, 1994). There are several hot spots for point mutation, which are mostly located in five highly conserved blocks. The codons 175, 248, 249, 273 and 282 are the most frequently mutated sites in all tumors (Levine, 1994). Different cancers also have different hot spots, for example, the 249 codon in hepatocarcinoma and the 175, 248, 273 codons in breast cancer (Levine, 1994).

2). Inactivation by viral /cellular oncogenes

At least 17 viral and cellular proteins have been reported to interact with the p53 protein (Soussi, 1995). Viral oncogenes can inactivate p53 by several mechanisms. The SV40 large T antigen binds to the p53 protein and inactivates its function. Human papilloma virus (HPV)16 E6 Protein down regulates the level of p53 protein by enhancing its degradation through ubiquitination with the help of E6AP (Maki et al., 1996). The most

interesting cellular protein is MDM2 which has a p53 binding site on intron I of the gene. P53 transactivates MDM2. Yet, MDM2 binds to p53 (both wild type and mutant) and inhibits the transcriptional activation activity of p53 (Momand et al., 1992).

3). Localization

Cellular localization is also important for p53 to be functional. It is believed that p53 functions only in the nucleus. There is a report that one third of breast cancer with wild type p53 have p53 localized in the cytoplasm where it is not functional (Moll et al., 1992). Another paper has shown changes in the localization of p53 during differentiation. P53 is in the nucleus during differentiation, whereas it appears in the cytoplasm after differentiation (Elizenberg et al., 1996).

While p53 may negatively regulated by MDM2, the function of the gene may be positively activated by protein phosphorylation (Mayr et al., 1995). The p53 gene, containing 11 exons (Benchimoll et al., 1985), is expressed in most cells. The product of the human p53 gene is a 393 amino acid protein which is divided into three domains: the N-terminal transactivation domain, the core DNA binding domain and the c-terminal domain which contains the oligomerization, nuclear localization signal, regulatory phosphorylation sites and non-specific DNA/RNA binding activity. In normal tissue, the wild type p53 has a short half-life from 30 minutes to 3 hours depending on cell type (Delmolino et al., 1993). The mutant p53 is more stable and has a longer half-life than the wild type (Delmolino et al., 1993).

As a transcription factor, p53 transactivates the expression of p21 (El-Deiry et al., 1993), GADD45 (Carrier et al., 1994), which plays important roles in cell cycle regulation and maintains genome integrity, and bax (Miyashita et al., 1995), which

induces apoptosis in addition to MDM2 which regulates p53. The major functions of p53 are briefly described in the following:

1). Regulation of cell cycle progression and maintaining genome integrity

When cells encounter a stressor such as DNA damage, hypoxia or infection by a virus, the cell cycle may be arrested at G1 and G2. The G1 arrest is mediated through the p53 induction of p21 which inhibits G1 cyclins and cdk complexes, whereas G2 arrest is mediated through p53 induction of GADD45 which inhibits cdc2/cyclin B1 (De-Toledo et al., 1995). The cell cycle arrest allows cells to repair damage before resumption of cell cycle progression and thus reduces gene or chromosomal mutations. Therefore, p53 is considered as the guardian of the genome (Lane, 1992)

2). Induction of apoptosis

p53 induces apoptosis in response to DNA damage in some cells. The mechanisms of apoptosis could either be transcriptionally dependent or independent (White, 1996). In transcriptionally dependent apoptosis, the activity of p53 as a transcription factor is required (White, 1996). In this case, p53 either directly induces the death signal by activating the transcription of Bax, and/or suppresses the expression of bcl-2 (Miyashita et al., 1995). The functions of p53 in inducing apoptosis and in arresting the cell cycle are independent of each other (White, 1996).

3). Enhancing fidelity of DNA replication

There is some evidence suggesting that p53 may participate in DNA repair directly. First, p53 was found to co-localize with proliferating cell nuclear antigen (PCNA), DNA polymerase α, DNA ligase and replication protein A (RPA)(Wilcock et al., 1991). Second, aside from specific binding to DNA, p53 also nonspecifically binds to single

stranded and double stranded DNA (Kern et al., 1991). Third, the p53 protein was reported to have 3' to 5' exonuclease activity (Mummennbrauer et al., 1996) and to enhance DNA replication fidelity of DNA polymerase α (Huang et al., 1997).

4). Induction of cell differentiation

Several reports have shown that transfection of p53 into many different cell lines induced cell differentiation (Kokunai et al., 1997: Moretti et al., 1997). Although p21 was reported to induce cell differentiation (Jiang et al., 1995), it is possible that there are other mechanisms involved in p53 induced differentiation. The initial level of p53 may determine if the fate of a cell is towards apoptosis or differentiation. In HL-60 cells, high levels of p53 induce apoptosis while lower levels of p53 induce G1 arrest and cell differentiation (Ronen et al., 1996).

Besides the three hereditary breast cancer genes described above, the ataxia-telangiectasia (AT) gene may be also considered as a hereditary breast cancer gene, since the carriers of the gene had greatly enhanced the risk for breast cancer (Swift et al., 1991). The gene, ATM (11q22-23), was found to be mutated in AT patient and may account for 7 % or more of breast cancer incidence (Easton et al., 1993).

Cell cycle regulating genes

The time interval that one cell duplicates and divides into two is defined as the cell cycle. One cell cycle contains four phases: G1 (gap 1), S (DNA synthesis), G2 (gap2) and M (mitosis). Those cells that remain quiescent and do not proliferate are in G0 (resting stage). It is believed that the cell cycle is designed to regulate faithful duplication of gene and accurate partitioning of chromosomes. The major components that control the cell cycle are cyclin dependent kinase (cdks), cyclins and cyclin dependent kinase inhibitors

(CKIs). There are at least two checkpoints for cell cycle: one is in the G1-S transition (G1 checkpoint), and the other is in G2/M transition (G2 checkpoint). The purpose of these checkpoints are believed to serve as brakes, to block cell cycle when something goes wrong or when cells are not ready. The main controllers of the checkpoints are cdks, which in the G1 checkpoint are cdk2, cdk4 and cdk6. The activity of cdks depends on cyclins association and phosphorylation. The negative control of cdks is accomplished by cdk inhibitors, which can be divided into two families, the p21 and the p16 family. Loss of control of the transition G1/S may cause cells to enter S phase precociously.

G1 cyclins

The D-type cyclins are synthesized in G1 phase and are induced in response to agents that promote re-entry into the cell cycle (Motokura et al.,1991). The main function of cyclin D1 is to bind to cdk4/cdk6 and activates these cdks which then phosphorylate the Rb, resulting in the release of the transcription factor E2F which transactivates several critical genes required for S phase entry including thymidine kinase, c-myb, cyclin E and E2F itself (Weinberg, 1996). In cultured cells, a cDNA of cyclin D1 can contribute to cell transformation by complementing a defective adenovirus E1A oncogene, indicating cyclin D1 as an oncogene (Hinds et al., 1994). Cyclin D1 has been found to be over-expressed in various human tumors, including breast carcinomas (Lammie et al., 1991). The proximity of the over-expressed cyclin D1 to 11q13 translocation breakpoints in B cell tumor strongly suggests its identity as the putative "bcl-1" oncogene (Rosenberg et al., 1991). Another cyclin which regulates the G1/S transition, cyclin E. has been shown to be over-expressed or aberrantly expressed in breast cancers (Keyomarsi et al., 1993).

p21/waf1

P21 plays an important role in cell cycle regulation. It can modulate growth arrest in response to a variety of conditions such as DNA damage (El-Deiry et al., 1994), cell differentiation and growth factor stimulation (Datto et al., 1995; Elbendary et al., 1994). p21 is a universal cdk inhibitor which binds to cdk/cyclin complexes and inhibits the activity of cdks including cyclin D/cdk4, cyclin E/cdk2 and cyclin A/cdk2 (Harper et al., 1995). The inhibition requires multiple molecules of p21 (Zhang et al., 1994). p21 is a down stream effector of p53 and is responsible for p53 -induced G1 arrest in response to DNA damage (Deng et al., 1995). p21 also binds to proliferating cell nuclear antigen (PCNA a protein required for DNA replication and repair) and inhibits the replication but not the repair function of PCNA (Li et al., 1994). Additionally, P21 has been reported to associate with cellular senescence and differentiation. p21 was found to be elevated in senescent cells (Noda et al., 1994), and over-expression of p21 in cells induces senescence and differentiation (Steinman et al., 1994). Recently, it has been shown that inactivation of p21 in human fibroblasts can bypass senescence and extend the lifespan (Brown et al., 1997). On the other hand, oncogenic ras provokes premature cell senescence with concomitant elevation in p53, p21 and p16 (Serrano et al., 1997). The induction of p21 can result from different mechanisms (Macleod et al., 1995). In response to DNA damage, the p53 protein is required for p21 induction. By binding to the promoter of p21, p53 transcriptionally activates p21 expression. On the other hand, p21 can be induced in the absence of p53 by some growth factors, chemicals or cell differentiation (Macleod et al., 1995; Liu et al., 1996).

p16/ink4a

p16/ink4a is a small protein product of the CDKN2/MTS1 gene located on chromosome 9p21. p16 binds to the cdk4 part of cdk4/cyclin D complex and inhibits the activity of cdk4 (Serrano et al., 1993). By preventing phosphorylation of pRb , p16 can arrest cells in the G1 phase. Unlike p21, p16 is differentially expressed in various tissues. For breast epithelial cells, p16 levels are lower than that in other tissue (Tam et al., 1994). The level of p16 protein oscillates during cell cycle. It reaches the highest at the peak of DNA synthesis (Tam et al., 1994). At first, CDKN2 was found frequently deleted in many tumors (Kamb et al., 1994). Later, several reports have shown that deletion of CDKN2 only occurred in breast cancer cell lines, suggesting that p16 deletion is only a result from selection in cell culture (Liu et al., 1994; Quesnel et al., 1995; Musgrove et al., 1995; Brenner et al., 1995). There is only one instance that aberrant expression of p16 in primary breast cancer (Geradts et al., 1996). Highly expression of p16 was correlated with inactivated pRb. This led to the hypothesis that p16 acts as a negative feedback loop for pRb (Serrano et al., 1993). There are several mechanisms involved in p16 inactivation: deletion, mutation, gene rearrangement and hyper-methylation (Larsen et al., 1996). P16 is reported to be deleted in the early event of immortalization (Reznikoff et al., 1996; Noble et al., 1996).

Rb

Rb is a tumor suppressor gene discovered in retinoblastoma. Rb plays a critical role in cell cycle regulation. Unphosphorylated Rb binds to and sequesters the E2F family of transcription factors to prevent transcription of critical genes that are essential for the cell cycle. To enter S phase, Rb should be inactivated through phosphorylation by cyclin/cdk.

Once phosphorylated, Rb releases E2F that transactivates several critical genes required for entry into S phase (Weinberg et al., 1995). The target genes of E2F include cdc2, thymidine kinase, myb, dihydrofolate reductase, cyclin E and E2F itself (Weinberg et al., 1995). Mutations, deletions and / or introduction of viral oncoproteins can inactivate Rb. Rb mutations are detected in many tumors including small cell lung carcinoma and breast carcinoma. Inactivation of Rb is believed to play an important role in the formation of carcinogenesis.

In addition to the tumor suppressor genes described above, some unidentified tumor suppressor genes on specific chromosome arms are known to be frequently lost in breast cancer (loss of hetrerozygosity). These include chromosome 1p, 1q, 3p, 11p, 16q and 18q (Callahan et al., 1990; Sato et al., 1990)

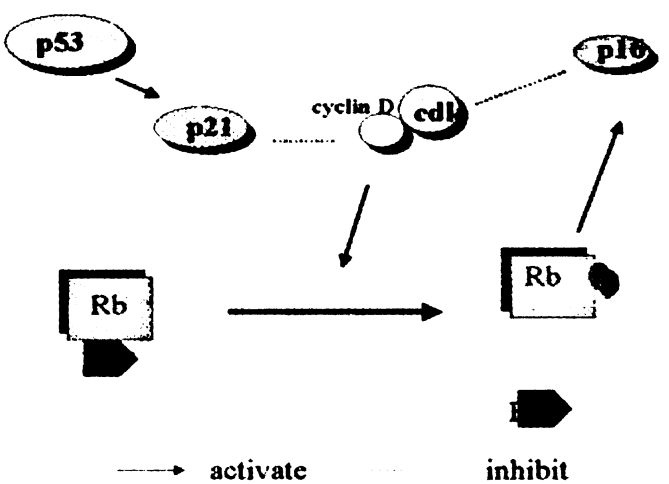


Figure 1. The cell cycle control in G1 phase

In vitro neoplastic transformation of human breast epithelial cells (HBEC)

The importance of developing an in vitro transformation model

Carcinogenesis is a complex multi-step (Vogelstein et al., 1988), multi-mechanism (Weinstein et al., 1984), and multi-pathway (Callahan et al., 1990) process involving genetic and possibly epigenetic alterations of specific sets of oncogenes and tumor suppressors (Knudson et al., 1993). Furthermore, tumors are believed to be derived from stem cells or early precursor cells and have a phenotype similar to normal undifferentiated cells at that stage (stem cell theory of cancer and oncogeny as blocked or partially blocked ontogeny theory)(Potter,1978; Trosko and Chang 1989). The mechanisms of carcinogenesis may be understood by comparative study of normal cells and tumor cells or preneoplastic cells *in vivo* as shown by the colorectal cancer model (Vogelstein et al., 1988). The duplication of this model for breast cancer, however, has been hampered by the relative lack of human tissue available for study, since the tiny samples of atypical hyperplasia or in situ carcinoma have of necessity gone to the surgical pathologist for diagnosis (Bartow et al., 1993). On the other hand, there are advantages for developing the *in vitro* transformation model. By stepwise transformation of normal cells to tumor cells, the genetic alterations and their resulting phenotypes at different stages of neoplastic transformation can be revealed. Furthermore, by using different types of normal cells for transformation, the target cells for neoplastic transformation may be identified.

In vitro neoplastic transformation of HBEC

Immortalization is generally preceded by extended lifespan as shown by viral or chemical transformation of HBEC (Stampfer et al., 1988; Kao et al., 1995). It is commonly accepted that immortalization is a critical step for tumorigenesis. Several methods have been used to immortalize HBEC *in vitro*: chemical, viral transfection, oncogene transfection and irradiation. Among these, viral transfection is the most efficient. The commonly used viruses are Simian virus 40 (SV40) and human papillomavirus (HPV). Generally, the mechanism by which viruses immortalize cells is inactivating p53 and /or pRb by expression of viral proteins. With SV40, the large T antigen can bind to and inactive p53 and pRb; with HPV16 or HPV18, E6 and E7 proteins bind to p53 and pRb respectively. These observations suggest that inactivation of p53 and pRb are critical in immortalization. Inactivation of HBEC by these viral agents has been reported for milk cells (Bartek et al., 1990, 1991; Garcia et al., 1991) and for organoids or cells derived from reduction mammoplasty (Berthon et al., 1992; Van Der Haegen et al., 1992; Shay et al., 1993). There are fewer reports on successfully immortalized human cell by ionizing irradiation (Fushimi et al., 1997; Tsutsui et al., 1997; Wazer et al., 1994), and only one (Wazer et al., 1994) was able to immortalize human breast epithelial cells by X-ray irradiation.

Methods

The objectives of this study are 1). To determine if X-rays are effective in inducing the initiation of neoplastic transformation of a specific type of normal HBEC, i.e. lifespan extension and immortalization, and 2). to determine if cell cycle regulating genes are frequently altered in these initiated cells. The specific procedures and methods are described as follows.

1. Cell culture

First passage normal HBEC, derived from reduction mammoplasty, were thawed at 37 ^{0} C from liquid nitrogen storage. To remove the freezing solution, the cells were transferred to a 15 ml centrifuge tube, mixed with 5 ml MSU-1 medium and pelleted at 1000 rpm for 8 minutes. The pelleted cells were added to 8 ml of MSU-1 medium supplemented with 5% FBS (Type I medium), dispersed by pipetting several times and then transferred to a 100 mm dish (plate A). After incubation (37 ^{0}C 5% CO_2 and humidified air) for 2 hours to allow the residual fibroblasts to attach on the plate, the unattached cells in medium were transferred and centrifuged. The pelleted cells were suspended in 8 ml MSU-1 medium with 4% bovine pituitary extract (Type II medium) and inoculated in a new 100 mm dish (plate B). The plate B was incubated over night for Type II HBEC to attach. The next day, the remaining unattached cells in medium were transferred and pelleted by centrifugation and then suspended in Type I MSU-1 medium and plated in a third dish (plate C). As reported previously (Kao et al., 1995), Type I HBEC developed in Type I medium in plate C whereas Type II HBEC developed in Type II medium in plate B. Subculture of HBEC was accomplished by using a phosphate buffered saline (PBS) with 0.01 % trypsin and 0.01% EDTA. The trypsin is inactivated

by 5% FBS after trypsinization.

2. X-ray irradiation of HBEC

The first passage Type II HBEC developed in a plate (plate B) for 3-4 days were subcultured into three 100 mm dishes. After incubation for 4-5 days, each plate contained about 1-2x10 ^{6} cells (50-60% confluence). These cells were irradiated by low dose X-ray at a dose rate of 2 Gy/m for 1 minute using a Torrex 150 Kv cabinet X-ray machine operated at 150 Kv and 5 mA. After irradiation, the medium was changed immediately and the cells returned to an incubator. Three days after the irradiation, the cells were irradiated again. After repeating the treatment for three times, the cells were subcultured with a split ratio of 1:3 to allow room for the cells to proliferate. These plates after incubation for 7 days were exposed to the same X-ray treatment for additional 2-3 times.

3. Selection of clones with extended lifespan

After the last X-ray irradiation, the cells were incubated, and subcultured when necessary, for colony development. The colonies were allowed to grow to 3-10 mm in diameter. Large actively proliferating colonies were marked on dishes. Cells were rinsed with PBS once. Glass cylinders were tapped on cello-seal grease, then carefully and firmly put on the colonies. Trypsin solution (50-100 μl) was added to each cylinder and warmed on a 37 °C plate for 8 minutes. The cells were pipetted several time and removed from the cylinders by Pasteur pipettes .The isolated cells were plated in T25 flasks filled with 10 ml Type II medium. Once approaching confluence, the cells were subcultured to T75 flasks. After becoming confluent, these cells were trypsinized and counted by a hemacytometer. 2x10^{5} cells were inoculated into a new flask for continuous culture and cell counting, the remaining cells were frozen for storage in liquid nitrogen.

The cells were propagated to determine the cumulative population doubling level (cpdl) that each cell line can achieve. The process of one cell dividing into two cells is defined as one cpdl. A normal human breast epithelial cell can be propagated to no more than four million cells, i.e. 22 cpdl. We define a cell clone with extended lifespan when a clone has achieved more than 24 cpdl. That means one cell proliferated to at least sixteen million cells. For an immortalized cell line, the cpdl should be more than 100.

The cpdl is calculated as:

$$cpdl = \ln(\text{final cell number}/\text{initial cell number})/\ln 2$$

4. Western blot analysis

a. Protein extraction

1). Total protein

Cells were cultured on 100 mm dishes until confluence and lysed with 0.5 ml 20% SDS plus 1 nM PMSF. Cells were scraped off the dishes and transferred into micro tubes on ice. The protein lysis were sonicated 30 seconds to break genomic DNA, then dispensed into 100 μl aliquots for storage at -20°C.

2). Separation of nuclear and cytoplasmic fractions

Cells were harvested in 3 ml PBS by a rubber policeman, transferred into a centrifuge tube and pelleted at 1000 rpm at 4 ^{0}C for 5 minutes. After decanting the PBS, the pelleted cells were washed with 2 ml Buffer I (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 0.1 mM EGTA and 0.3M sucrose, pH7.9), resuspended in 1 ml Buffer I and then transferred into Dounce A . The cells were dounced, transferred to a microcentrifuge tube and centrifuged at 10,000 rpm at 4^{0}C for 30 minutes to separate the cytoplasms and nuclei (Dignam et al., 1983). The supernatant

(cytoplasm fraction) was transferred to a new tube. Both fractions were lysed with 20% SDS plus 1 nM PMSF for western blot analysis.

b. Lowry protein assay (Bio-Rad DC protein Assay)

Six protein standards (BSA) from 0 mg/ml to 1 mg/ml were prepared in 20 % SDS solution. The samples were five time diluted with 20 % SDS. 10 µl of samples /standards were added to each microtube, followed by 50 µl of solution A and 400 µl of solution B. After mixing and reacting for 15 minutes at room temperature, the absorbance is measured at 750 nm using a Beckman DU7400 spectrophotometer.

c. SDS -Polyacrylamide Gel Electrophoresis of Protein

A discontinuous gel system was used in this study (Molecular Cloning, Maniatis). The apparatus used was a Bio-Rad mini gel system. The glass plates were assembled according to the instructions of the manufacturer. Ten ml of the resolving gel solution was prepared containing the desired concentration of acrylamide and other components (for 10% gel, 4 ml H_2O, 3.3 ml 30% acrylamide mix, 2.5 ml 1.5 M Tris pH 8.8, 100 µl 10% SDS, 4 µl TEMED and 100 µl 10% ammonium persulfate). These ingredients were added into a disposable plastic tube, mixed by vortex and poured into the gap between the glass immediately. A layer of double distilled H_2O (0.5ml) was added carefully to prevent air from diffusing into gel. The gel was placed at room temperature for 30 minutes to polymerize. After polymerization, the gel was washed with double distilled H_2O two or three times. The 5 % stacking gel (4 ml for two) was prepared in a plastic tube as follow: 2.7 ml double distilled H_2O, 660 µl 30 % acrylamide, 0.5 ml 1M Tris pH6.8, 40 µl 10% SDS, 4 µl TEMED and 40 µl 10 % ammonium persulfate. The solution

was poured into the gap. The combs were inserted immediately and carefully to avoid air bubbles. After gel polymerization, the combs were removed and the wells were washed with ddH_2O several times. The gels were mounted in electrophoresis apparatus. Tris-glycine electrophoresis buffer [25 mM Tris, 250 mM glycine and 0.1% SDS] was filled into reservoirs. Samples (30 μg /10 μl) were added to an equal volume of 2x loading buffer [4% SDS, 20% glycerol, 0.2% bromophenol blue and 2% β-mercaptoethanol], then mixed and loaded to gels (10μl each). Gels were run at 158 V for 40 - 90 minutes.

d. Protein transfer

Whatman 3MM paper and PVDF membrane were cut (wearing gloves) into sheets of 75mm x50mm (exact size of gels). The PVDF membrane was wetted with methanol, rinsed with distilled H_2O, then soaked in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% methanol). After electrophoresis, the gels were removed from glass plates and directly soaked in transfer buffer for 10 minutes. Blotting was done using a BIO RAD mini trans -blot transfer cell. Gel holder cassettes were laid on a tray. One wet fiber pad was placed on the cathode (black) side, covered with 3 sheets of 3 MM papers. A gel was put on the paper carefully. The PVDF membrane was placed on top of the gel while the gel covered with buffer. Three sheets of 3 MM paper were put on the membrane. The paper was rolled over several time to squeeze out the air bubbles using a pipette. The cassette was assembled after putting on another fiber pad and inserted into electrode unit. The tank was filled with transfer buffer and inserted into a cooling ice basket. The transfer was run at 23 volts at room temperature for overnight.

e. Staining the membranes

After transfer, PVDF membranes were removed from cassettes, rinsed with water, and then soaked in Ponceaus stain for 5 minutes. The membranes were placed between clear polypropylene for scanning into computer to confirm that equal amounts of total protein were loaded.

f. Immunodetection

The membranes were incubated with 10 ml blocking solution (5% non-fat milk, 0.1 % Tween 20 in PBS) on a rocker at room temperature (RT) for one hr. After blocking, the membranes were washed three times with PBS containing 0.1% Tween 20 (PBST) for 10 minutes on a rocker. The membranes were incubated with 5 ml primary antibodies, diluted 1:1000 in blocking solution for 1-2 hr on a rocker at RT. Then, the membranes were washed with PBST three times as above. After the final wash, the membranes were incubated with 6 ml secondary antibodies conjugated with horseradish peroxidase in 1:1500 dilution on a rocker at room temperature for 1 hr and then washed extensively with PBST. Chemiluminescent detection (ECL, Amersham) was used to reveal the specific immuno-conjugated protein for Western blot. The membrane was covered with 2 ml ECL detection reagent for 1 minute at room temperature (1 ml of substrate and 1 ml of buffer were mixed in a centrifuge tube). Membranes were than placed in a film cassette and exposured for 30 seconds to 3 minutes.

5. Flow cytometry

The expression of p53 in clones with extended lifespan was studied by Western blot for quantitative analysis at the protein level and by flow cytometry for functional analysis of p53.

a. X-ray irradiation and fixation of cells

Cells were cultured in three 100 mm dishes until 70 % confluent. One dish was used as control, the others were irradiated with X-rays for 4 Gy and 8 Gy at dose rate of 2 Gy /min. The medium was replaced immediately after irradiation. After 24 hr incubation, cells were rinsed with PBS once and removed by trypsinization for subculture. Cells were counted and aliquoted $2x10^6$ cell per centrifuge tube. To remove trypsin, cells were pelleted, washed with PBS once and pelleted again. Then 2 ml of cold 70% ethanol was added to fix cells for 1-3 hr at 4°C. Cells were stored at -20 °C until staining.

b. DNA staining and DNA content analysis

The ethanol -fixed cells were centrifuged at 1000 rpm at 4°C for 5 minutes. The ethanol was removed as much as possible by blotting on tissue papers. The cells were re-suspended in 3 ml PBS, transferred into a 12x75 mm tube and centrifuged as above. After decanting the PBS, the cells were re-suspended in 0.5 ml DNA staining reagent (0.1% triton X-100, 0.1 mM EDTA, 0.05 mg/ml RNaseA and 50 μg/ml propidium iodide in PBS, pH7.4) and incubated overnight in dark at 4°C. The cells were analyzed with a fluorescence-activated cell sorter (FACS) (Becton Dickinson) available in the Dept. of Biochemistry, Mich. State Univ.

Materials

The sources of chemicals, antibodies, cell culture supplies and reagents are listed as follows.

Chemicals:

Sodium Dodecyl Sulfate(SDS) Glycine	Boehringer Mannheim, Indianapolis IN
PMSF Bovine Serum albumin, EDTA Propidium iodide, Ponceaus reagent β-mercaptoethanol, Bromophenol blue	Sigma, St Louis MS
30 % acrylamide, TRIS, TEMED Ammonium persulfate, Tween 20 DC Protein assay, Triton X-100	BIO-RAD, Hercules CA
Cello-seal grease	Fisher, Fair Lawn NJ
PVDF membrane	Millipore, Bedford MA
Methanol	J.T. Baker, Phillipsburg NJ
ECL detection reagent	Amersham Life Science, Arlington Heights, IL

Cell culture

Normal human breast epithelial cells (HBEC) derived from reduction mammoplasty of different women were used. The cells used in this study were Human Mammary Epithelium (HME): HME 5, HME7, HME12, HME14, HME15, HME17 and HME20. The extended lifespan clones were derived from two HBEC cultures after X-ray irradiation (A designated initial plate, B designated second plate)..

M15XA6, M15XA12------ HME15A (20 Gy)

M15XB5, M15XB6, M15XB7, M15XB11, M15XB12------ HME15B (12Gy)

M15XA25L1, M15XA25L4, M15XA25L7 ------ HME15A (8Gy)

M15XB23L2, M15XB23L3, M15XB23L4, M15XB25L1------ HME15B (10Gy)

M20LBX1, M20LB6-3------HME20 (10Gy)

M12B4 ------ an extended life clone derived from HME12 after 5-bromodeoxyuridine (BrdU) treatment was also used in the study.

The E.L. clones used for experiments were at 25-35 cpdl. Except three clones from M15XB23 which were recovered from the same plate, all others are independent clones from different dishes.

Some immortal and cancer cell lines were also used :

M13SV22---- HME13 Type I HBEC immortalized by SV40

M15SV30---- HME15 Type II HBEC immortalized by SV40

M13SV1R2N1---- HME13 Type I HBEC immortal and tumorigenic after SV40,X-ray and neu transformation

MCF-7---- breast cancer cell line

T47D---- breast cancer cell line

Medium /growth factors/antibodies

Bovine pituitary whole	Pel-Freez, Rogers AR
Fetal bovine serum Trypsin Gentamicin	Life Technologies, Gaithersburg MD
MSU-1 medium, 17-β estradiol Insulin, Human transferrin Hydrocortisone, Epithelium Growth Factor	Sigma, St Louis MS
P53 (Ab-2) pAb1801 Rb (Ab-5) LM95.1	Calbiochem-Novabiochem International, Cambridge MA
P16 (C20) a.a.137-156 P21 (C19) a.a.146-164 Cyclin D1 (HD11)	Santa Cruz Biotechnology, Santa Cruz, CA
Anti rabbit IgG horseradish peroxidase linked whole antibody	Amersham Life Science, Arlington Heights, IL
Anti mouse IgG horseradish peroxidase linked whole antibody	Amersham Life Science, Arlington Heights, IL

RESULTS

1. Induction and isolation of extended lifespan (E. L.) clones from Type II HBEC after X-ray irradiation

Ten experiments were carried out to induce extended lifespan of Type II normal HBEC by X-ray irradiation as listed in Table 1. In seven of these experiments, duplicate plates were used (designated as A and B plate in Table1). In these experiments, Type II HBEC derived from reduction mammoplasty of six different women were used (i.e. HME5, 7, 12, 14, 15, 20). The cells were exposed to multiple (3-6 times) low dose (2 or 4 Gy) X-rays for a cumulative total dose of 6-20 Gy. These experiments yielded twenty-six putative E. L. clones from two different cell subjects (HME15 and HME20) in three successful experiments. To confirm if the isolated putative E. L. clones are indeed having extended lifespan according to our definition (i.e. 24 cpdl), the cpdl of each of these clones was determined. The results as listed in Table 2 shows that twenty-four of these clones are indeed E. L. clones. Normal Type II HBEC (Fig 2.)were highly proliferative in early stage and became senescent (Fig 3.) within two months. In these experiments, most cells were proliferating actively in early stage until they became senescent when they changed cell morphology (enlarged) and stopped proliferating. At this stage, a small fraction of cells may continue to proliferate and form E. L. colonies (Fig 4, 5.). The morphologies of early passage E. L. clones are indistinguishable from early passage HBEC (Fig 6.). In late passage, these E. L. clones also became senescent showing some elongated fibroblast -like cells or giant multinuclear cells (Fig.7).

Wazer et al. (1994) have reported the isolation of an immortal HBEC clone after irradiation of cells with a relatively high cumulative dose of X-rays (30 Gy).

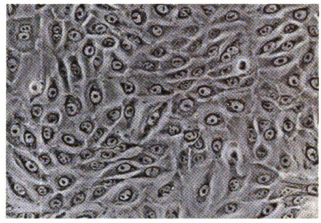


Fig 2. The morphology of normal Type II HBEC (HME20) cells.
(phase contrast,100X)

Fig 3. The morphology of senescent Type II HBEC (HME15).
The small and pack cells are healthy cells, the large cells are senescent cells.
(phase contrast 100X)

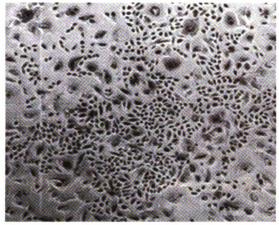


Fig 4. A colony proliferating Type II HBEC after 6 Gy X-ray exposure. (phase contrast, 40X)

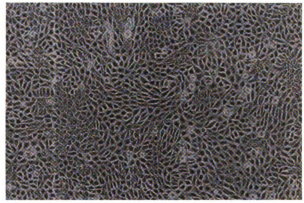
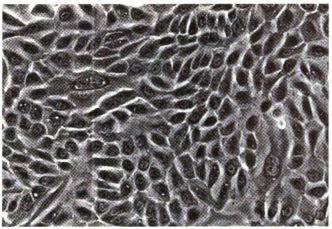
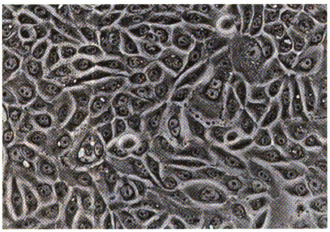


Fig 5. An extended lifespan colony proliferating after X-ray irradiation. (M15XA25) (phase contrast 40X)

A.



B.



C.

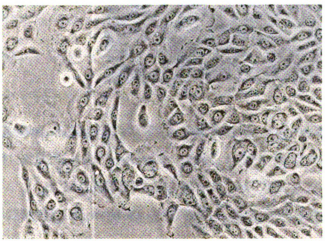


Figure 6. Morphology of the E. L. cells in early passage.
A. M15XA25L1 B. M15XB23L3 C. M20LBX1
Cells were at 30 cpdl (phase contrast 100X)

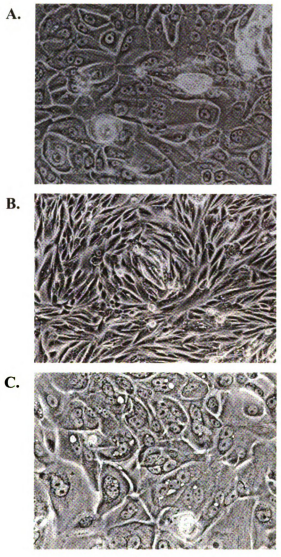


Figure 7. Morphology of the E. L. cells in late passage.(phase contrast 100 X)

A. M15XB23L4 showed enlarged and multinuclear cells at about 45cpdl.

B. M15XB25L1 appeared fibroblast-like at 50 cpdl.

C. M20LBX1 showed the giant and multinuclear cells in about 36 cpdl.

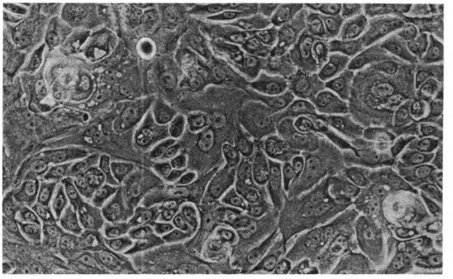


Figure 8. The E. L. clone after 20 Gy X-ray irradiation

M15XB25L1 showed multinuclear and large cell morphology (phase contrast 100X)

To determine if continual exposure of our E. L. clones could convert them into immortal clones, eight highly proliferative E. L. clones (cpdl 25-35) were irradiated again with X-rays (2 or 4 Gy) each time for an additional total dose of 8 or 10 Gy (Table 3.). The total cumulative doses for these clones range from 20-28 Gy. All of these cultures, however, eventually became senescent (Fig 8.) and no immortal clone emerged.

Table 1. Induction and isolation of extended lifespan (E. L.) clones from Type II HBEC after X-ray irradiation

Exp.	Date	cells	Treatment (dose x times)	Total dose (Gy)	Extended life clone obtained
1	7/10/95	HME15A	4 x 5	20	2 clones
		HME15B	2 x 6	12	6 clones
2	12/6/95	HME15A	2 x 4	8	11 clones
		HME15B	2 x 5	10	5 clones
3	2/29/96	HME14A	2 x 3	6	None
		HME14B	2 x 3	6	None
4	4/20/96	HME14B	2 x 3	6	None
5	5/20/96	HME5A	2 x 4	8	None
		HME5B	2 x 4	8	None
6	5/20/96	HME7A	2 x 5	10	None
		HME7B	2 x 5	10	None
7	7/31/96	HME12A	2 x 4	8	None
		HME12B	2 x 4	8	None
8	7/31/96	HME14A	4+2x3	10	None
		HME14B	4+2x3	10	None
9	8/20/96	HME20R	2 x 3	6	None
10	8/20/96	HME20L	2 x 5	10	2 clones

Table 2-1. The proliferation potential of putative E. L. clones isolated from Type II HBEC (HME15) after X-ray irradiation

The cpdl of E.L. clones. Cells were trypsinized and counted on the indicated date .

DATE	9/8/95	9/18/95	9/29/95	10/12/95	
M15XA6	21.9	26.5	29.8	32.9	
DATE	8/22/95	9/5/95			
M15XA12	23	26.8			
DATE	8/30/95	9/11/95	9/25/95		
M15XB3	21.8	24.6	27.8		
DATE	8/27/95	9/8/95			
M15XB5	22.3	26.7			
DATE	9/13/95	9/25/95	10/18/95		
M15XB6	22.1	27	30.3		
DATE	8/25/95	9/8/95			
M15XB7	21.5	24.7			
DATE	9/5/95	9/17/95	9/29/95	10/10/95	11/6/95
M15XB11	22.3	27.2	31.2	33.5	36.8
DATE	9/6/95	9/17/95	9/29/95	10/12/95	
M15XB12	22.5	25.6	30.3	32.1	

Table 2-2. The proliferation potential of putative E. L. clones isolated from Type II HBEC (HME15) after X-ray irradiation

DATE	3/15/96	4/11/96						
XA23L1	21.3	25.6						
DATE	3/12/96							
XA23L7	22.3							
DATE	2/5/96	3/6/96	3/25/96					
XA25L1	23.1	25.8	30.7					
DATE	2/5/96	3/12/96	3/29/96					
XA25L2	24.4	27.5	33					
DATE	2/5/96	3/12/96	4/9/96					
XA25L3	23.4	26.9	29.7					
DATE	2/5/96	3/6/96	4/3/96					
XA25L4	22.5	26.4	30.2					
DATE	3/25/96							
XA25L5	20							
DATE	2/5/96	3/13/96	4/9/96					
XA25L6	23.4	26.9	30.8					
DATE	2/5/96	3/6/96	3/25/96					
XA25L7	23.1	26.6	31.5					
DATE	2/5/96	3/13/96	4/3/96					
XA25L8	23.3	27.2	31.3					
DATE	2/5/96	3/13/96	4/30/96					
XA25L9	22.7	26.8	29.9					
DATE	3/22/96	4/5/96	4/17/96	4/29/96	5/13/96	5/31/96		
XB23L2	22.1	26	30.8	34.6	39	42.5		
DATE	3/21/96	4/2/96	4/17/96	4/29/96	6/8/96	7/5/96		
XB23L3	23.7	29.2	35.2	39.9	43.7	47.2		
DATE	3/15/96	3/29/96	4/16/96	5/2/96	5/21/96	6/8/96	7/5/96	
XB23L4	21.8	27.2	33	37.8	42.5	46.6	48.5	
DATE	3/15/96	3/26/96	4/11/96					
XB23L5	22.3	27.2	33					
DATE	3/12/96	3/25/96	4/8/96	4/19/96	4/29/96	5/8/96	5/20/96	6/8/97
XB25L1	23.4	29.3	35.1	38.1	40.6	44.7	49.1	52.2

Table 2-3. The proliferation potential of putative E. L. clones isolated from Type II HBEC (HME20) after X-ray irradiation

DATE	12/6/96	12/23/96	1/2/97	1/13/97	2/3/97
M20LBX1	22.4	25.5	30.6	34.9	38.6
DATE	12/16/96	2/10/97			
M20LB6-3	23.4	27.8			

Table 3. Results of additional X-ray irradiation of E. L. clones

E. L. cells at cpdl 25-35 were exposed to X-ray at 4Gy /2Gy the addtional exposure, total accumulated dose and result are listed.

CELL	ADDITIONAL EXPOSURE	TOTAL EXPOSURE	Final outcome of cell culture
M15XA6	+8 Gy	28Gy	Senescence
M15XB11	+10 Gy	22Gy (XB11 22 Gy)	Senescence
M15XB23L2	+10 Gy	20Gy (XB23L2 20Gy)	Senescence
M15XB23L3	+10 Gy	20Gy (XB23L3 20Gy)	Senescence
M15XB23L4	+10 Gy	20Gy (XB23L4 20Gy)	Senescence
M15XB25L1	+10Gy	20Gy (XB25L2 20Gy)	Senescence
M20LBX1	+10 Gy	20Gy (LBX1 20Gy)	Senescence
M20L6-3	+10 Gy	20Gy (20LBX2)	Senescence

2. Failure to obtain E. L. clones from Type I HBEC after X-ray irradiation

Both Type I and Type II HBEC were obtained from first passage of each cell culture. Type II HBEC were used in experiments described previously. The number of Type I cells is usually considerably less than Type II cells. The colony- forming cells derived from each vial are estimated to be about 500 and 50,000, respectively for Type I and Type II HBEC. After culturing 4-5 days for Type II HBEC and 10 days for Type I HBEC, these Type II and Type I cells attaining a total of about 2×10^6 and 2×10^5 cells respectively were irradiated to initiate the experiments. The experiments carried out to induce E. L. clones from Type I HBEC by X-rays are listed in Table 4. In initial experiments, it was noticed that Type I HBEC were more sensitive to low-dose X-ray treatment (1-2 Gy). Very few colonies were formed after the subculture of these irradiated cells. Therefore, the doses of X-rays in each treatment were decreased in latter experiments (0.1-0.6 Gy). The reduction in dose allowed the cells to have prolonged proliferative activity. After repeated treatment, the cells eventually became senescent. No E. L. clone was found from these experiments (Table 4.).

Table 4. Results of the effects of repeated X-ray irradiation of Type I HBEC on induction of E. L. clones

Cells derived from different subjects and the treatments are listed

	Cells	Treatment (Gy)	E. L. clone obtained
1	HME15	2,2,2 (2Gy/min 1min)	None
2	HME15	1,1,1 (2Gy/min 30 sec)	None
3	HME15	1,1	None
4	HME15	.5,.5,.5 (2Gy/min 15sec)	None
5	HME14	.5,.5	None
6	HME15	.5,.5	None
7	HME17	.5,.5	None
8	HME15	2,2,2 1,1,1 1,1,1 (0.5Gy/min 2 min)	None None None
9	HME14	.1,.3 (0.2Gy/min 30sec)	None
10	HME5,7	.1,.3,.6,.5,.5	None
11	HME12	.1,.3,.6,1,2	None
12	HME14	.1,1,1,1,1	None
13	HME15	.1, 1,1,2,2,2,2	None
14	HME15	.3,.3,.3,.3,.3,.3,2,4,4,	None

3.Expression of genes related to cell cycle regulation

The genetic and molecular basis for lifespan extension in HBEC induced by X-ray irradiation is not clear. To gain insight into the mechanism of the first significant change related to transformation (i.e. lifespan extension), the E. L. clones isolated were characterized for the expression of genes related to cell cycle regulation.

A.p53

Western blot analysis

The p53 protein expression was studied by Western blot analysis. Cells defective in p53 may show decreased or enhanced amounts of p53 protein. The latter is attributed to the longer half-life of some mutant p53 protein compared to the wild-type p53. In experiments to determine the p53 protein expression in E. L. clones (Fig.9-10), two breast cancer cell lines, MCF-7 and T47D, which express wild-type and mutant p53, respectively, were included in the experiments as shown in Fig. 9. T47D cells contain considerably more p53 proteins than MCF-7. Also included in the experiments are two SV40 immortalized Type II HBEC (M13SV22 and M15SV30) and one tumorigenic SV 40 immortalized Type I HBEC (M13SV1R2N1). The high level of p53 proteins expressed in these cells are presumably inactivated and stabilized by complexing with the SV40 large T antigen. Thirteen E. L. clones isolated from HME15 were examined in the Western blot analysis. In two of these clones, cells at different stages of X-ray irradiation were studied (i.e. XB11, XB11 22 Gy; XB25L1, XB25L1 20 Gy). The results (Fig. 9) show that seven of the thirteen clones appear to contain elevated level of p53 protein (XA25L7, XB11, XB12, XB23L2, XB23L3, XB23L4, XB25L1) compared to the parental Type II HBEC (HME15).

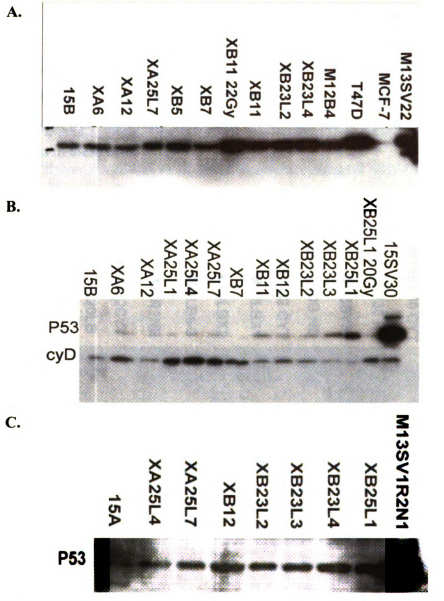


Figure 9. p53 expression in E. L. clones by Western blot

Clones are listed on top. Normal Type II HBEC (15B, 15A), positive controls (T47D, MCF-7, M13SV22, M15SV30, and M13SV1R2N1). In between of normal and positive control are E.L. clones induced by X-ray irradiation or by BrdU treatment (M12B4). CYD: cyclin D1

E. L. clones at different stage of X-ray irradiation may exhibit different level of p53 protein as shown by XB25L1. An E. L. Type II HBEC clone transformed by 5-bromodeoxyurindine (BrdU) in a previous experiment (M12B4) also contain higher amount of p53 protein. All the E. L. clones derived from HME20 also contain higher level of p53 protein (Fig.10). In this experiment, the nuclear and cytoplasmic fractions of cell lysate in two clones were separately assayed. It is clear that the p53 proteins are located in the nucleus in these cells.

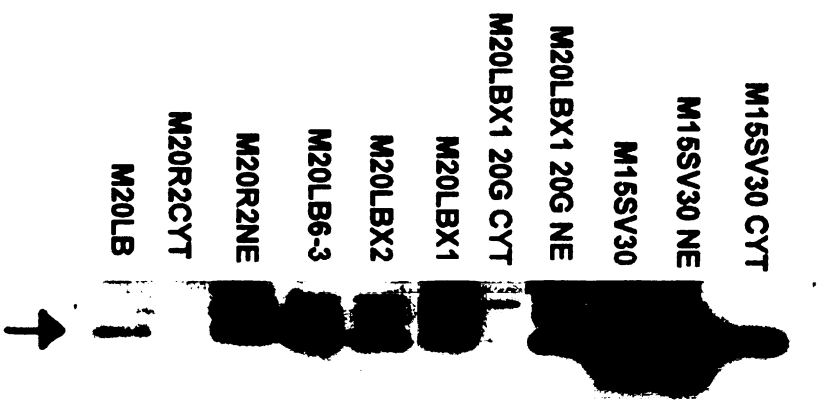


Figure 10. Expression and localization of p53 in HME20 normal and E. L. cells studied by western blot.
Experiments using different fractions of cell extracts: Normal Type II HBEC total protein (M20LB), cytoplasmic fraction (M20R2CYT), and nuclear fraction (M20R2NE); the positive control total protein (M15SV30), cytoplasmic fraction (M15SV30 CYT), and nuclear fraction (M15SV30NE). Position of p53 proteins are indicated by arrow.

Flow cytometry analysis

To determine whether the increase in p53 protein is due to the presence of mutant p53 or not, a functional p53 assay was done for six of these E. L. clones. Cells with wild type p53 normally show G1 arrest after exposure to ionizing radiation. Cells with mutant p53 show only G2 arrest instead of G1 arrest. The status of cell cycle is reflected by cells with different DNA content which can be detected by a flow cytometer. The data of FACS were shown by charts with cell number (Y-axis) vs. DNA content (X-axis). The first peak is G1 phase and the second peak at twice the DNA content is G2 phase, and the broad distribution between the two peaks is the S phase. The results of these studies are summarized in Table 5. Overall, these results did not provide evidence that the E. L. clones examined are defective in p53 function since no X-ray-induced G1 arrest was evident.

MCF-7, a breast cancer cell line which expresses wild type p53, was chosen as a positive control. After 4 Gy exposure, the cells in G1 increased to 91.1% of total cells from 71.4% in non-irradiated cells (Fig 11). The results of MCF-7 show a clear G1 arrest after X-ray irradiation. The two negative controls are T47D and M15SV30. The former is a breast cancer cell line with mutant p53 and the latter is a Type II HBEC (HME15) immortalized by SV40. These cells show a dramatic decrease cells in G1; a decrease from 61.7% to 40.8% for M15SV30 and 66.8% to 33.6% for T47D (Fig.12, 13). In the meantime, cells were arrested in G2. The percentage of cells in G2 increased from 5.3 to 28.6 for T47D and from 9.0 to 44.6 for M15SV30. After 8 Gy irradiation, G2 peaks are higher than G1 peaks in T47D and M15SV30 (data not shown). For parental Type II HBEC 53% of cells remain in G1 after exposure to 4 Gy of X-ray irradiation (Fig. 14)

compared to 58.6% of non-irradiated cell, indicating radiation induced G1 arrest. The percentage of G1 cells in XA25 decreased to 47.1 from 63.3 after 4 Gy X-ray irradiation (Fig.15). Similar results were shown in the XB25L1 with 56.9% in control and 49% for 4 Gy X-ray irradiated cells (Fig. 16). Although with a lower p53 and p21 expression (results to be shown), the XB25L1 20 Gy clone also showed a similar effect of G1 arrest. (45.8% in G1 after irradiation compared to 54.6 % for non-irradiated cells) (Fig.17). The HME20, the normal Type II cells showed a significant G1 arrest after irradiation. The percentage of G1 cell increased to 87 after 4 Gy X-rays exposure, while the control only has 71.3 % of cells in G1 (Fig.18). The extended lifespan clone from HME20, 20LBX1, showed a pattern similar to HME15 and HME15 E. L. clones and slightly different from its parental cells, i.e. the percentage of G1 in irradiated cells slightly lower than the non-irradiated cells (59.2% vs.64.6%) (Fig.19).

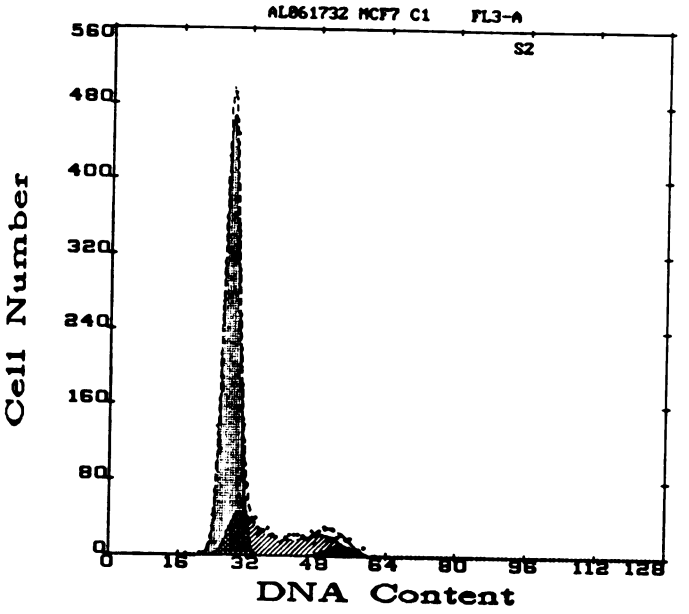
Table 5. Summary of results of cell-cycle analysis for control and X-ray irradiated E. L. clones

Cell cycle date were analyzed with a flow cytometer. Cells and treatments are listed in the first column. The percentage of cell in different cell cycle stage are shown .

CELLS	G1	S	G2
15B	58.6	32	9.5
15B-2	65.1	26	9
15B+ 4Gy	48.6	28.7	22.7
15B+ 4Gy#2	53	14.7	32.3
15B+ 8Gy	40.6	18.4	41
15XA25pool	63.3	16.6	20.1
15XA25pool+4Gy	47.1	18.8	34
15XA25pool+8Gy	47.7	11.9	40.4
15XB23L2	58.8	16.9	24.3
15XB23L2 +4Gy	57.3	11.9	30.7
15XB23L2 +8Gy	60	10.8	29
15XB23L4	64.7	20.6	14.7
15XB23L4+4Gy	50.7	23	26.3
15XB23L4+8Gy	44.5	31.3	24.2
15XB25L1	56.9	18.4	24.7
15XB25L1+4Gy	49	23.6	27.4
15XB25L1+8Gy	44.5	13.7	41.8
15XB25L1 20Gy	54.6	8.1	37.2
15XB25L1 20Gy+4Gy	45.8	27.3	26.9
15XB25L1 20Gy+8Gy	50.3	13.3	36.5

Table 5 (Cont'd)

Cell	G1	S	G2
20LB	71.3	26.3	2.5
20LB +4Gy	87	2.6	10.4
20LB +8Gy	75.9	20.2	3.8
20LBX1 80% confluence	81.1	6.5	12.4
20LBX1 density arrest	88.4	1.9	9.7
20LBX1	64.6	17.5	17.9
20LBX1 +4Gy	59.2	12.1	28.7
20LBX1 +8Gy	47.7	17.1	35.2
M15SV30	61.7	29.3	9
M15SV30 +4Gy	40.8	14.6	44.6
M15SV30 +8Gy	17.4	10.4	72.2
T47D	66.8	27.8	5.3
T47D +4Gy	33.8	37.6	28.6
T47D+ 8Gy	29	28.7	42.2
MCF-7	71.4	25.6	3
MCF7 +4Gy	91.1	8.6	0.3
MCF7+ 8Gy	92.8	2	5.2



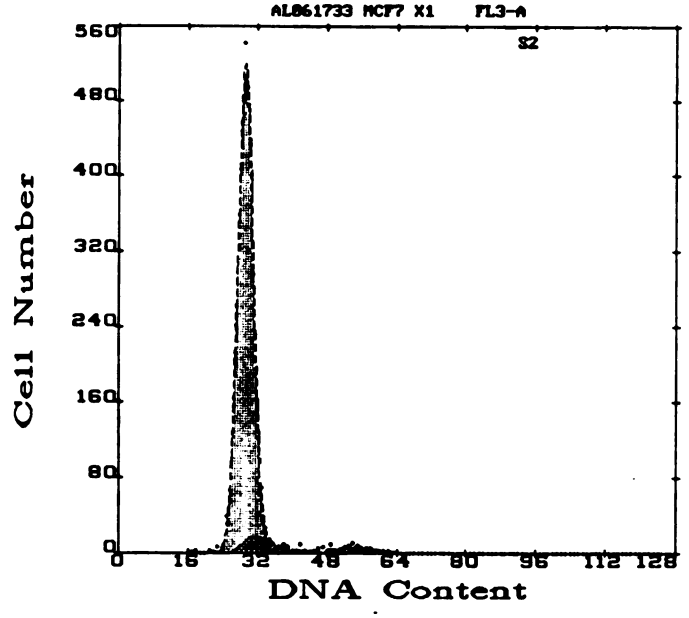


Fig 11. Cell cycle--MCF-7 A: control, B: 4 Gy irradiation
DNA content was measured by a flow cytometer. The G1 peak in irradiated cells is higher than that in control group.

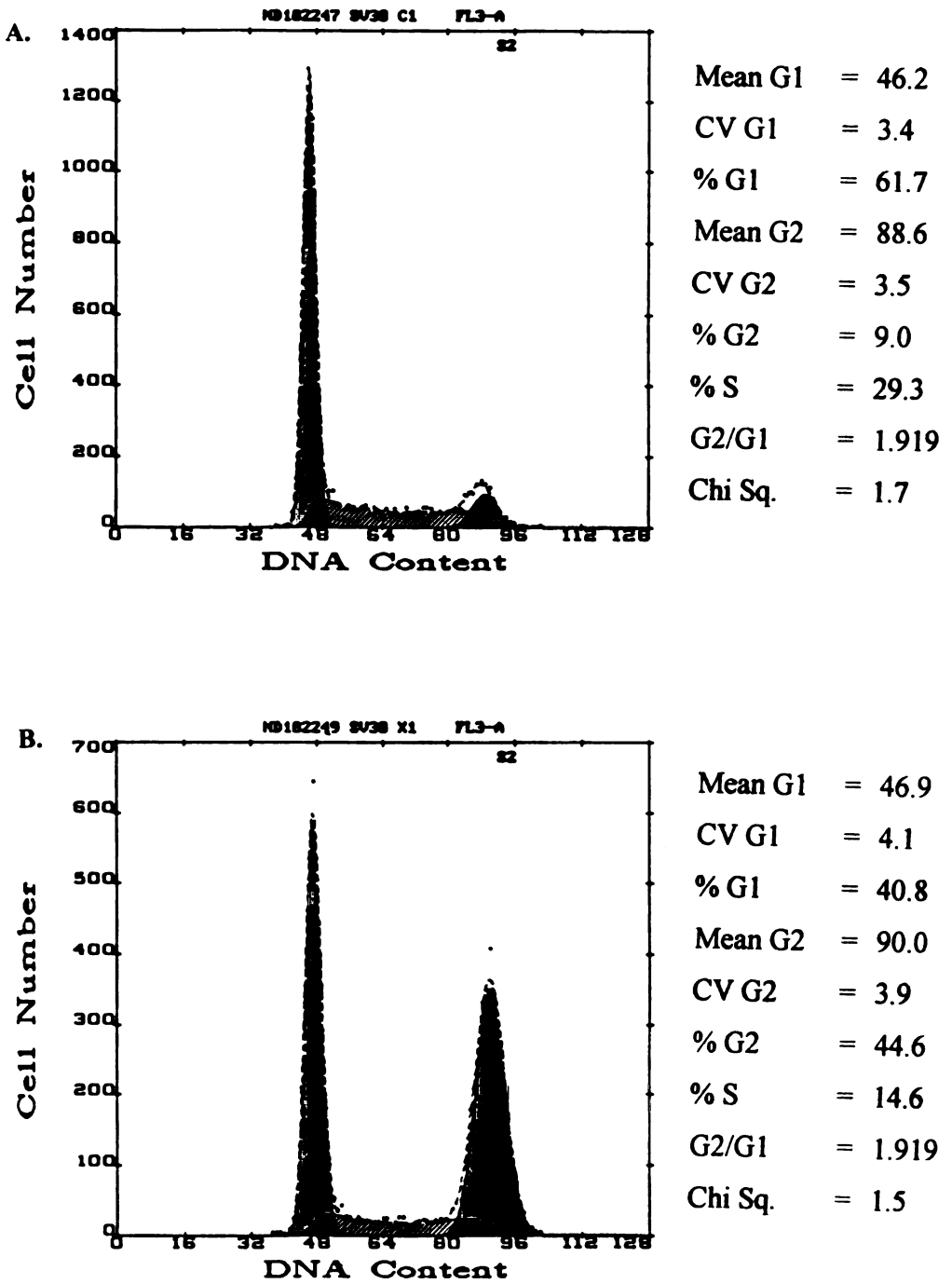


Fig 12. Cell cycle--M15SV30 A: control, B: 4 Gy irradiation

DNA content was measured by a flow cytometer. The G1 peak in irradiated cells dropped while G2 peak increased.

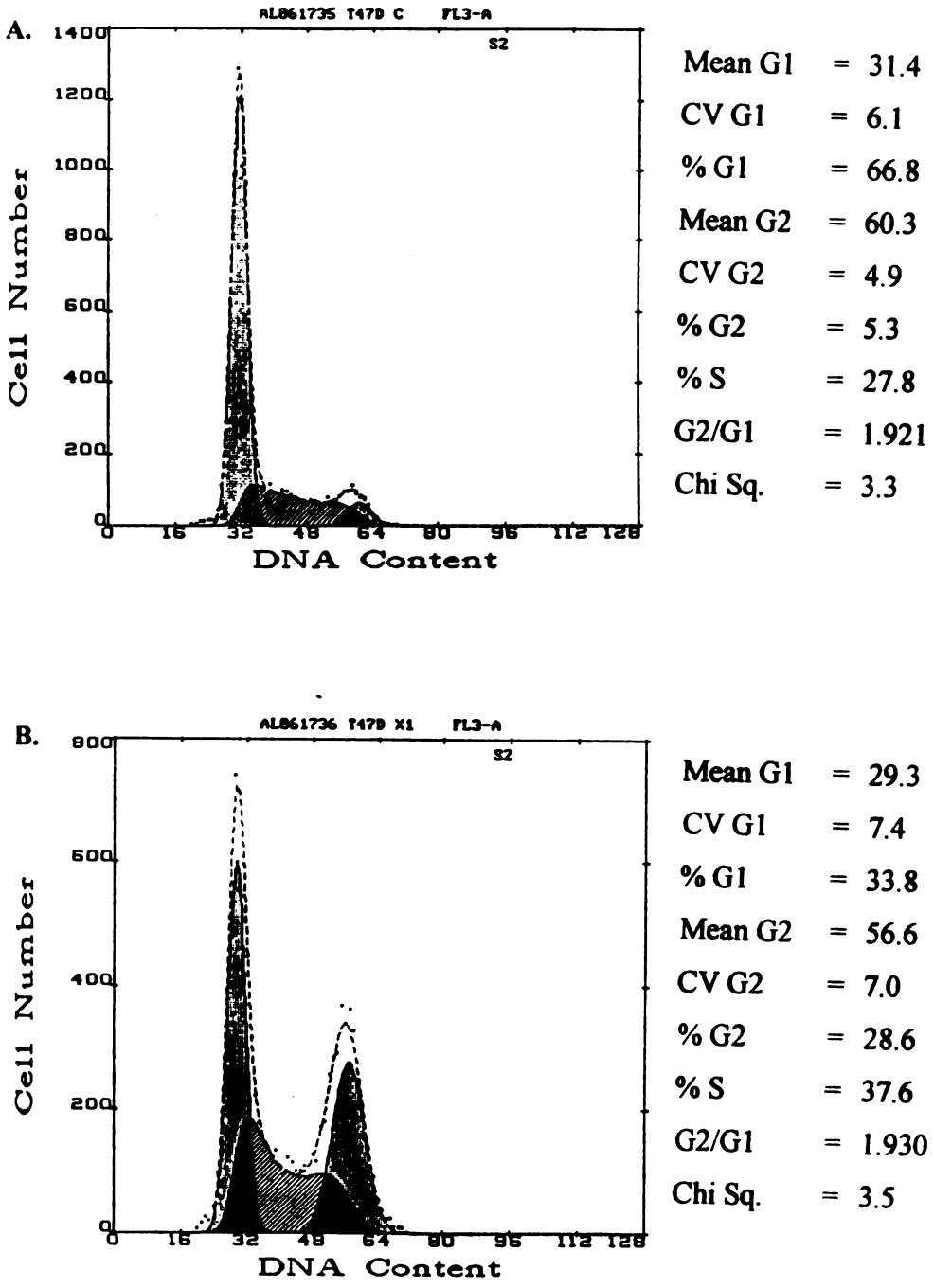
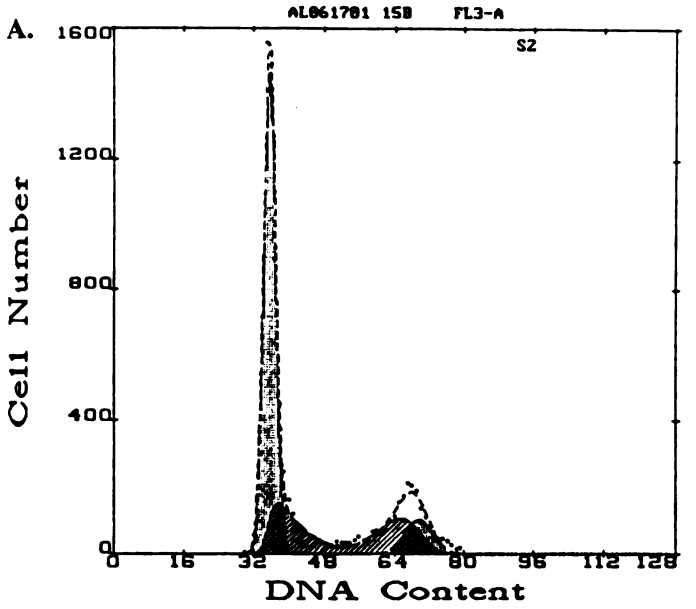
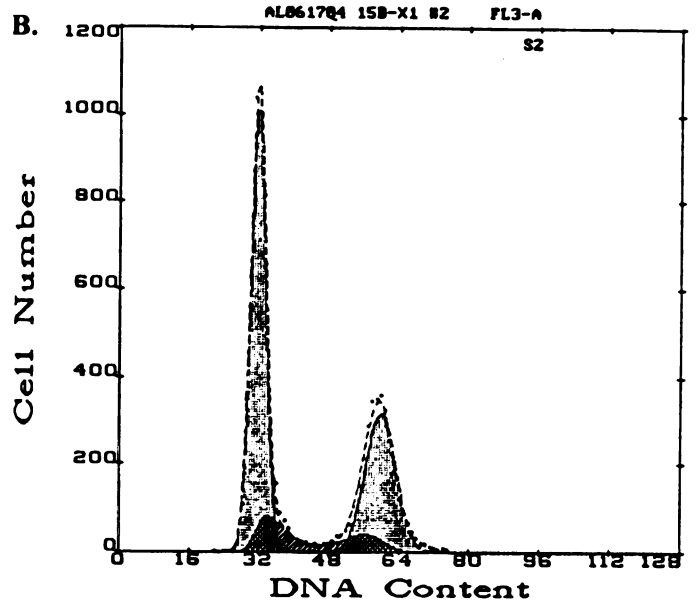


Fig 13. Cell cycle–T47D A: control, B: 4 Gy irradiation

DNA content was measured by a flow cytometer. The G1 peak in irradiated cells dropped dramaticly while G2 peak increased.

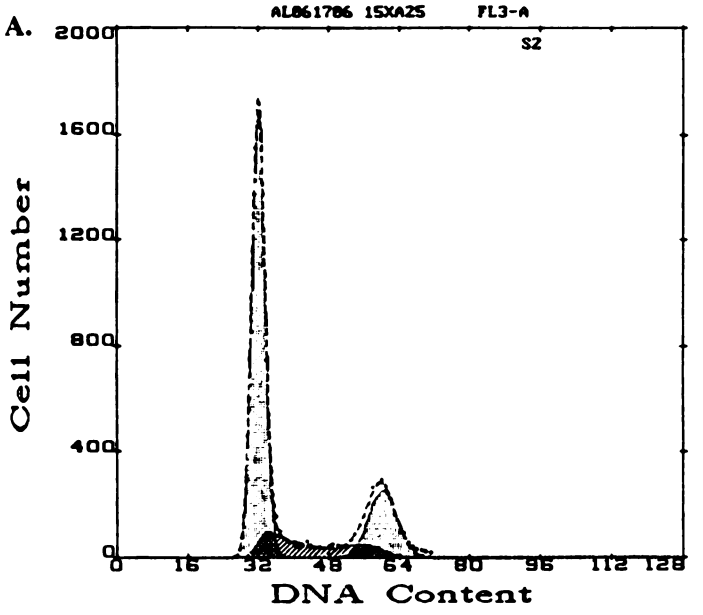


Mean G1 = 35.5
CV G1 = 3.9
% G1 = 58.6
Mean G2 = 69.2
CV G2 = 4.6
% G2 = 9.5
% S = 32.0
G2/G1 = 1.947
Chi Sq. = 3.7

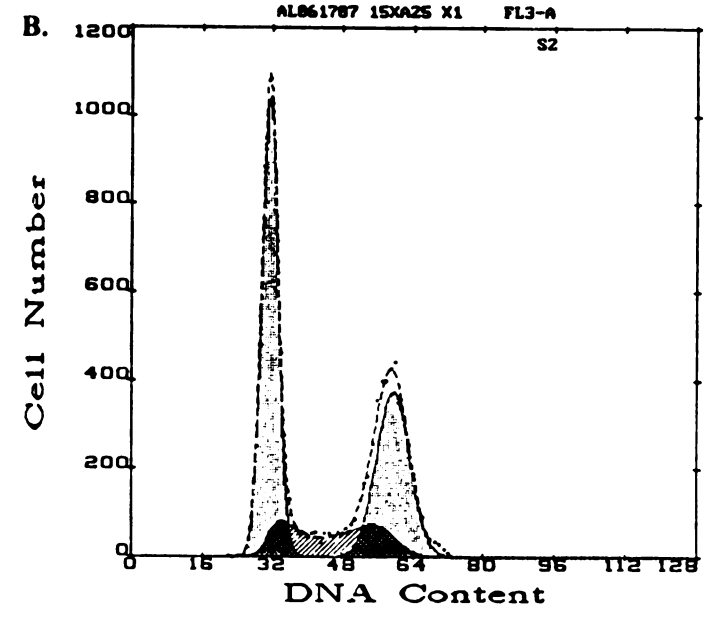


Mean G1 = 31.7
CV G1 = 5.5
% G1 = 53.0
Mean G2 = 59.7
CV G2 = 5.7
% G2 = 32.3
% S = 14.7
G2/G1 = 1.886
Chi Sq. = 2.7

Fig 14. Cell cycle--HME15B A: control, B: 4 Gy irradiation
DNA content was measured by a flow cytometer. The G1 peak in irradiated cells dropped while G2 peak increased.



Mean G1 = 31.9
CV G1 = 4.8
% G1 = 63.3
Mean G2 = 60.1
CV G2 = 5.4
% G2 = 20.1
% S = 16.6
G2/G1 = 1.881
Chi Sq. = 1.8



Mean G1 = 31.2
CV G1 = 6.0
% G1 = 47.1
Mean G2 = 59.4
CV G2 = 6.3
% G2 = 34.0
% S = 18.8
G2/G1 = 1.901
Chi Sq. = 1.1

Fig 15. Cell cycle--M15XA25 A: control, B: 4 Gy irradiation
DNA content was measured by a flow cytometer. The G1 peak in irradiated cells dropped while G2 peak increased.

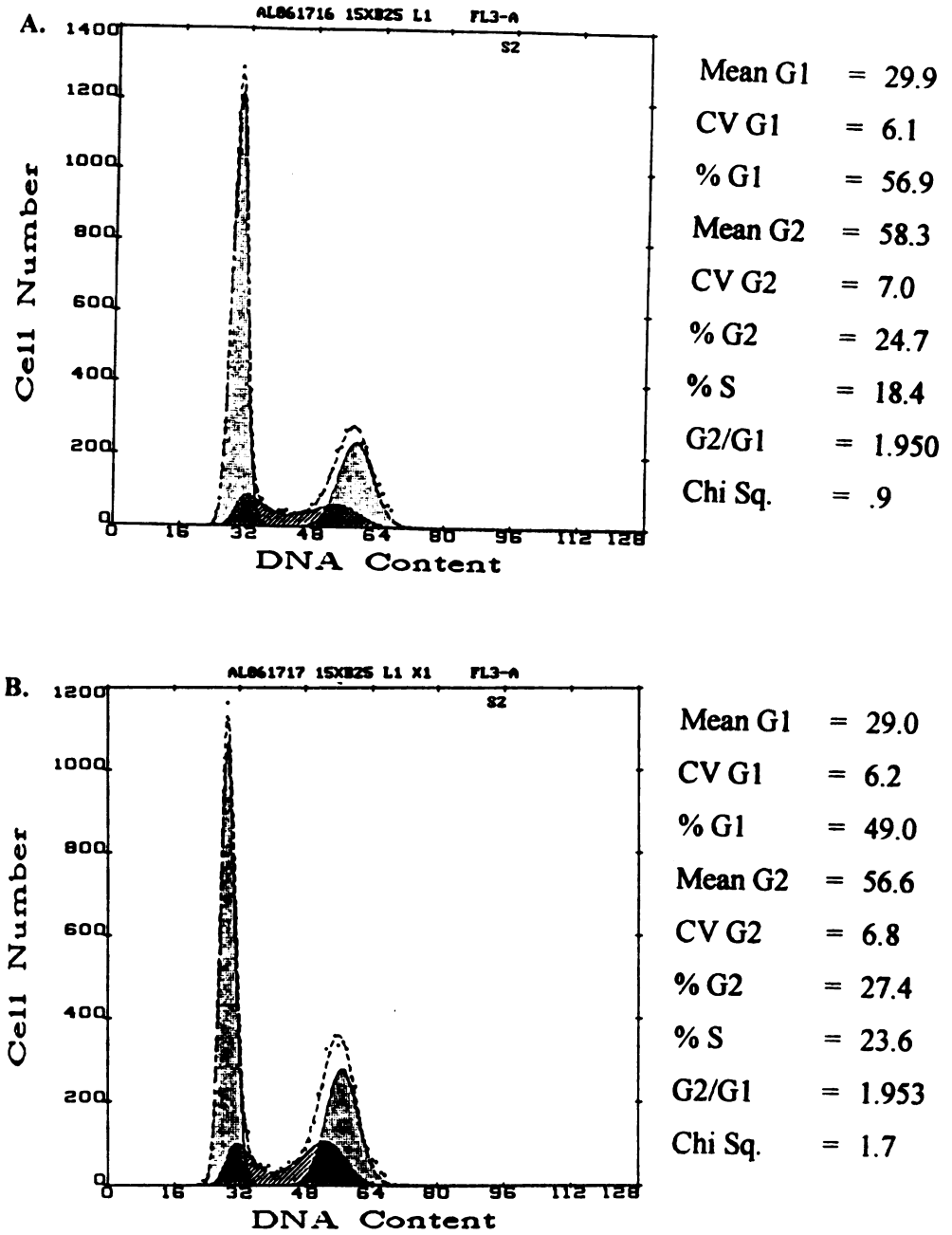
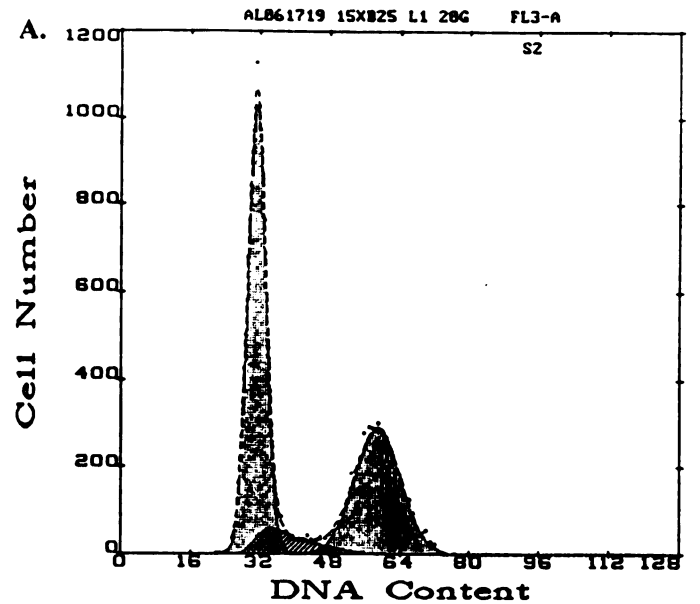


Fig 16. Cell cycle--M15XB25L1 A: control, B: 4 Gy irradiation
DNA content was measured by a flow cytometer. The G1 peak in irradiated cells dropped while G2 peak increased.



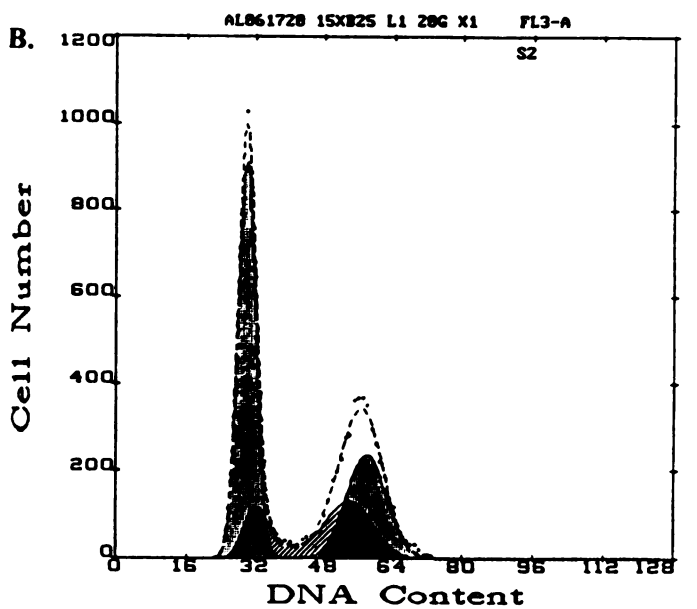
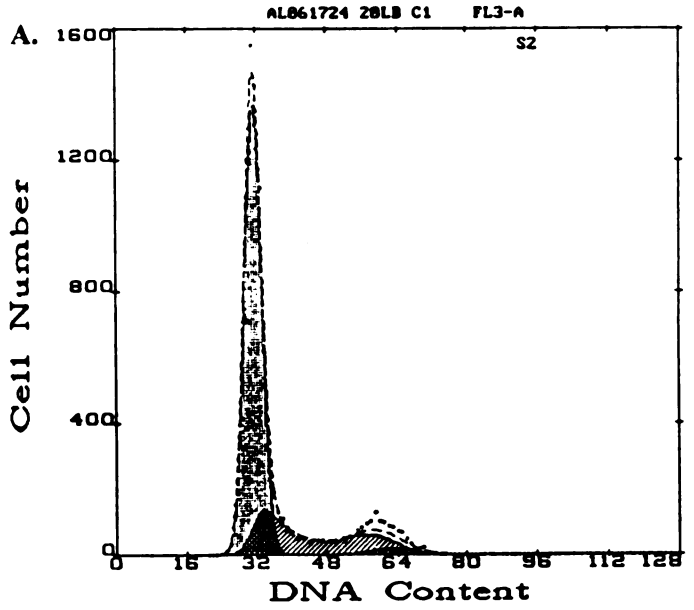
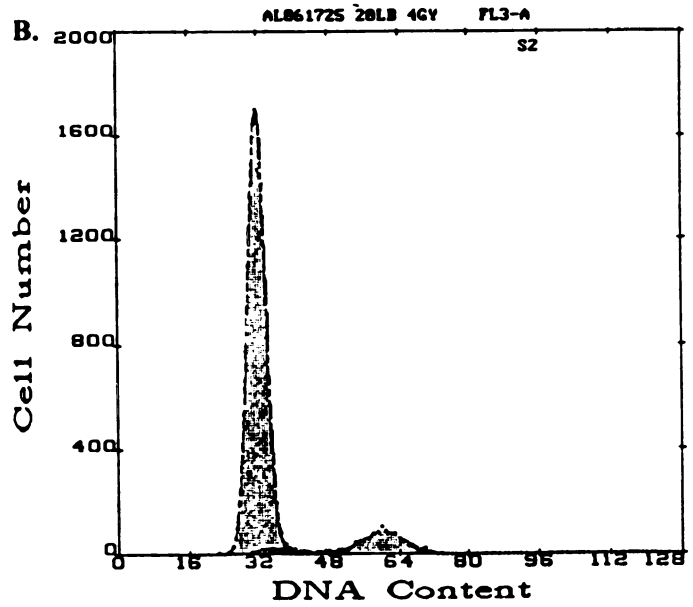


Fig 17. Cell cycle--M15XB25L1 20 Gy A: control, B: 4 Gy irradiation
DNA content was measured by a flow cytometer. The G1 peak in irradiated cells dropped while G2 peak increased.

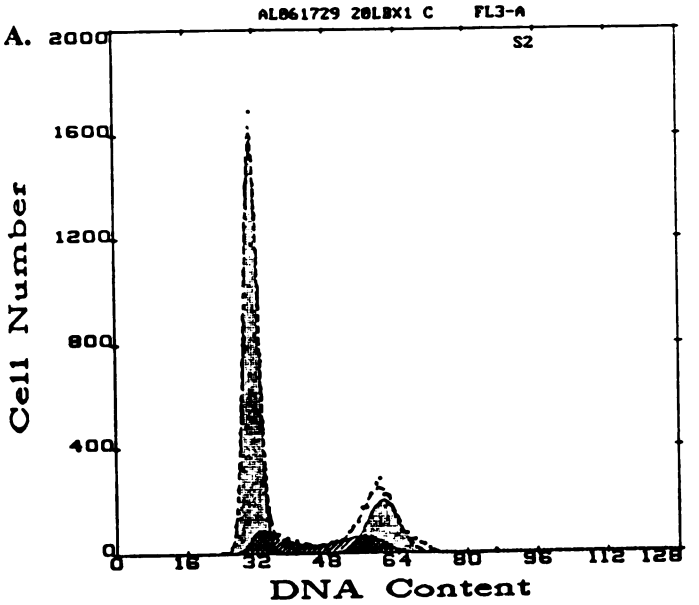


Mean G1	=	31.2
CV G1	=	6.3
% G1	=	71.3
Mean G2	=	62.3
CV G2	=	8.3
% G2	=	2.5
% S	=	26.3
G2/G1	=	1.996
Chi Sq.	=	3.4



Mean G1	=	31.6
CV G1	=	6.4
% G1	=	87.0
Mean G2	=	60.0
CV G2	=	8.1
% G2	=	10.4
% S	=	2.6
G2/G1	=	1.899
Chi Sq.	=	1.3

Fig 18. Cell cycle--HME 20 LB A: control, B: 4 Gy irradiation
DNA content was measured by a flow cytometer. The G1 peak in irradiated cells is higher than that in control group..



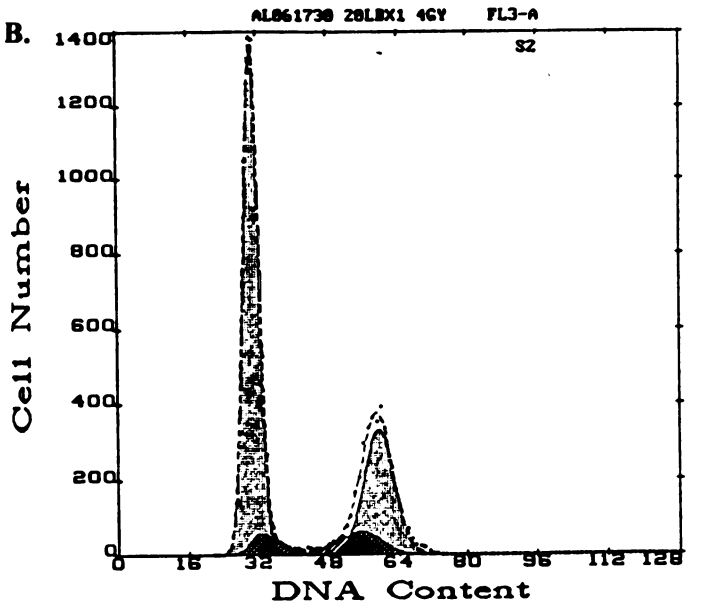


Fig 19. Cell cycle--20LBX1 A: control, B: 4 Gy irradiation
DNA content was measured by a flow cytometer. The G1 peak in irradiated cells dropped while G2 peak increased.

B. p21

Induction of p21 after irradiation requires functional p53. M15SV30, which presumably contains the inactivated p53, showed only low level of p21 expression (Fig 20). There is no or trace expression of p21 protein in T47D and M15SV30, consistent with the presence of inactivated p53 in these cells. Both parental normal Type II cells (HME15 and HME20) expressed small amount of p21. In contrast, most extended lifespan clones showed an increase in the expression level of p21. In total, fifteen and five E. L. clones (or its sub-populations receiving different dose of X-rays) from HME15 (Fig. 20, 21) and HME20 (Fig. 22) were analyzed. In the HME 15 E. L. series, the p21 proteins in ten clones were clearly elevated (Fig. 20, 21). For HME20 E. L. clones, the p21 proteins were greatly elevated in four of the five clones tested (Fig. 22). A clone at different stage of the X-ray irradiation may show great difference in p21 expression (i.e. high expression in XB23L4 and low expression in XB23L4 20 Gy)(Fig. 20). The p21 proteins, similar to p53, were primarily found in the nuclear fraction as shown in one clone studied (Fig. 22). The expression level of p21 is also correlated with the level of p53 in extended life cells. Clones that highly expressed the p53 were also found to contain relatively high level of p21 expression (Fig. 23). The chart is from the Fig. 9a. and Fig. 21. The two gels were run at the same time using same samples. After completion of Western blot and chemifluorescent detection, the films were scanned into a computer and the density of the bands was analyzed by Sigma gel program. The concomitant expression of high level of both protein suggests that p53 in extended life clones is functional.

C. Cyclin D1

The cyclin D1 proteins are expressed at relatively high levels in both parental Type II cells (HME15 AND HME20) compared to that in MCF-7 and T47D cancer cell lines or in the SV40 immortalized Type II cell line (M15SV30) (Fig. 24, 25). Except for A few clones (i.e. XA6, XA25L1, XA25L4, and XA25L7), most of the E. L. clones tested (total fifteen) are not greatly different from the parental cells in cyclin D1 expression.

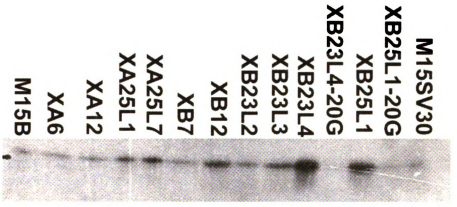


Figure 20. P21 expression in E. L. clones derived from HME15

Normal Type II HBEC (M15B), immortal Type II HBEC (M15SV30), and the E. L. clones of HME15 were studied by Western blot.

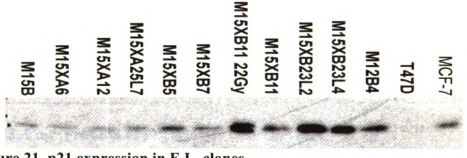


Figure 21. p21 expression in E.L. clones

Normal Type II HBEC (M15B), breast cancer cells (T47D, MCF-7), E.L. from HME15 by x-ray and E.L. from HME12 by chemical(M12B4) were studied by Western blot.

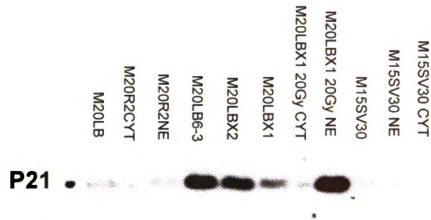


Figure 22. p21 expression in E. L. clones derived from HME20
Different cells fractions were studied: Normal Type II HBEC total protein (M20LB), cytoplasmic fraction (M20R2CYT), and nuclear fraction (M20R2NE). the positive control total protein (M15SV30), cytoplasmic fraction (M15SV30 CYT), and nuclear fraction (M15SV30NE).

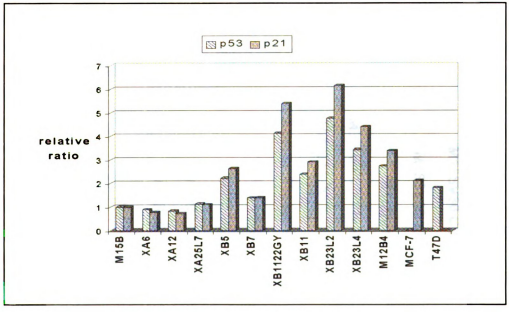


Fig 23. Co-expression of p53 and p21 in normal and E.L. cells.
Data are from Fig 9A. and Fig 21. Two western blots were scanned into a computer and the density of the bands was analyzed by Sigma gel program. The relative ratio is set as multiples of control (M15B=1).

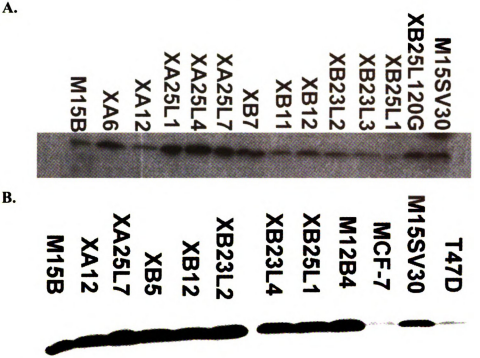


Figure 24. CyclinD1 expression in E. L. clones

Normal Type II HBEC (M15B) ,two breast cancer cells (T47D,MCF-7), immortal Type II HBEC (M15SV30) and E.L. clones in between.

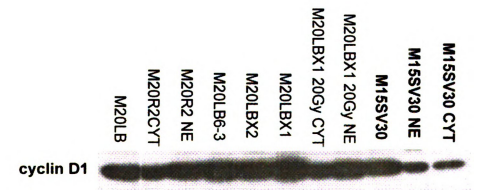


Figure 25. Expression and localization of cyclin D1 in E. L. clone derived from HME20

CYT : cytoplasmic fraction; NE: nuclear fraction, others are total protein

D. RB

The molecular weight of pRb is about 105-110Kda; the amount of these two different molecular forms depends on the status of phosphorylation. The phosphorylated form is inactive and can be found in proliferating cells. There appears no significant change in total amount of Rb between E. L. clones and their parental normal cells. However, there is a difference in phosphorylation of Rb (Fig. 26, 27). There is only one upper band (hyperphosphorylated form) in normal Type II cells (15B) and in the immortal cell line (M15SV30). All except one (XB7) of the E. L. clones showed two bands of Rb, representing hyper and unphosphorylated form. XB7 only has hyperphosphorylated band (Fig. 27).

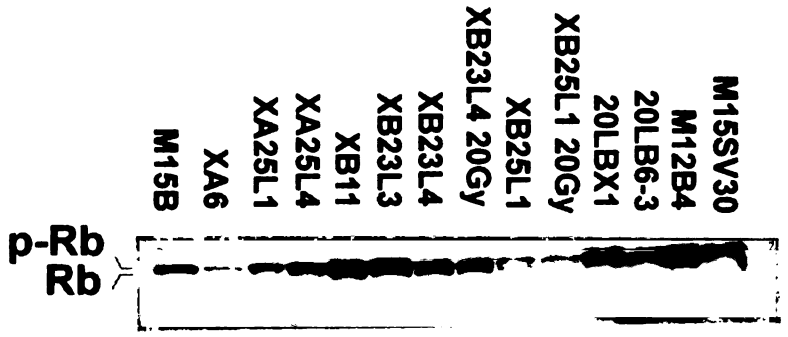


Figure 26. Rb expression and phosphorylation

Normal Type II HBEC (15B), immortal Type II HBEC (M15SV30), and E.L. clones were studied by Western blot. Positions of phosphorylated and non-phosphorylated Rb are indicated in left.

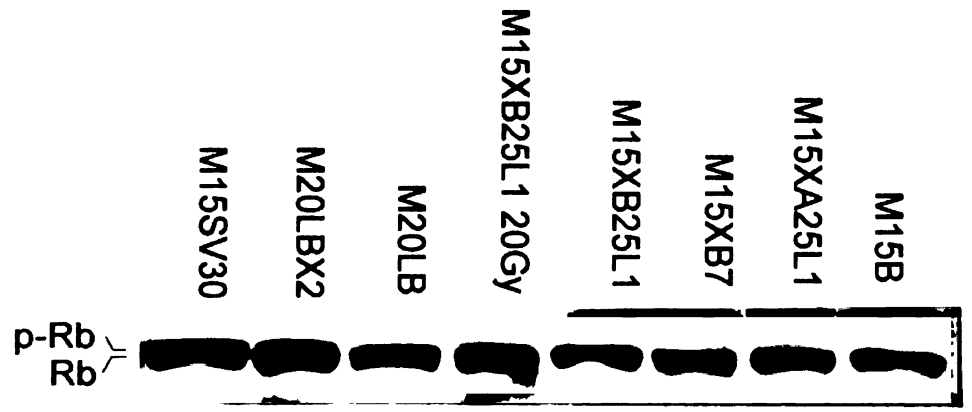


Figure 27. Rb phosphorylation in normal and E. L. clones

Normal Type II HBEC (15B), immortal Type II HBEC (M15SV30), and E.L. clones were Studied by Western blot. positions of phosphorylated and non-phosphorylated Rb are indicated in left.

E. P16 ^{ink4a}

Recently, p16^{ink4a} has been reported to play a role in the early event of immortalization (Reznikoff et al., 1996; Noble et al., 1996). To understand whether p16 is involved in lifespan extension, the expression of p16 was examined. In the first experiment studying HME20 E. L. clones (Fig. 28), there is detectable level of p16 in normal Type II cells (20LB and 20RII). In these cells, p16 is localized in the cytoplasm. The immortal M15SV30 cell line increased the level of p16 possibly because of inactivated pRb (Fig. 29). The E. L. clones derived from HME20 did not express p16. In the second experiment, HME15 normal Type II cells were, unexpectedly, found not to express p16 (Fig. 29). Among the extended lifespan clones studied (seven clones), only XB7 expressed the p16. Another normal Type II cell culture (HME14) did express p16. HME14 is one of four cell subjects that could not be successfully transformed. We wondered if there might be a correlation between p16 expression and the ability to acquire extended lifespan after X-ray irradiation. Therefore, the three normal Type II

cells derived from different women that were used for X-ray irradiation were assayed for p16 expression. The results showed that HME5 and HME12 clearly express p16 (Fig. 30)

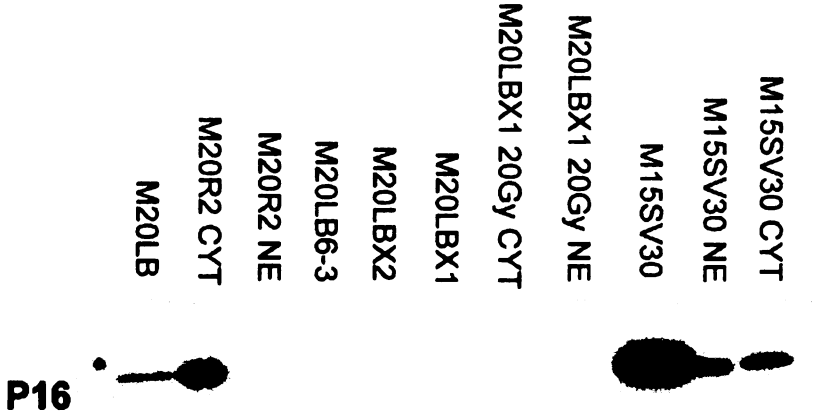


Figure 28. P16 expression and localization in E. L. clones derived from HME20
Protein fraction indicated CYT: cytoplasmic ; NE: nuclear

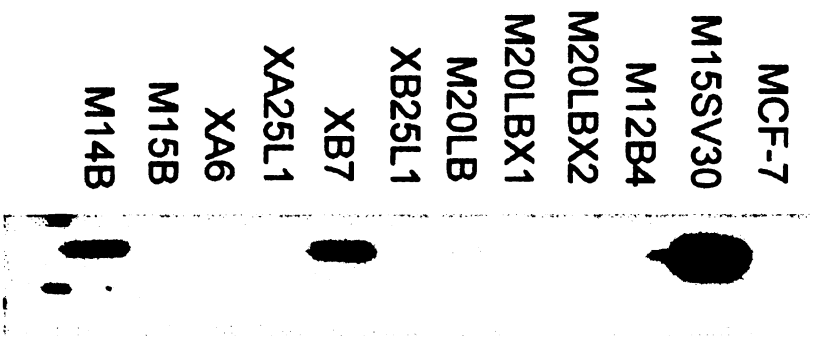


Figure 29. P16 expression in normal and E. L. clones
Normal Type II HBEC (M14B, M15B, M20LB), immortal Type II HBEC(M15SV30).

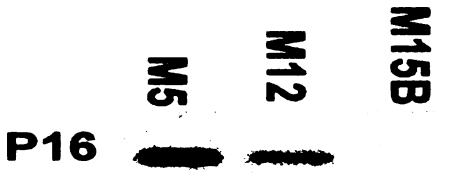


Figure 30. Expression of p16 in different normal Type II HBEC

Discussion

Based on substantial evidence that ionizing radiation is a breast carcinogen and the observation that Type I HBEC is more susceptible to neoplastic transformation than Type II HBEC (Kao et al., 1995), experiments were carried out to test if X-rays are effective initiator of neoplastic transformation of a specific type of HBEC. Furthermore, by characterizing the transformed cell clones with extended or infinite lifespan, an insight into the genetic and molecular mechanisms for radiation -induced breast carcinogenesis might be gained. The initial focus of the characterization is on certain genes known to regulate the cell cycle and to maintain genome stability.

The major results of this study may be summarized as follows:

1. Clones with extended lifespan have been induced and isolated from Type II HBEC after repeated low dose X-ray treatment. Twenty-two and two E. L. clones have been isolated from HME15 and HME20, respectively. However, no E. L. clone was isolated from four other different primary HBEC cultures.

2. These Type II E. L. clones failed to become immortal after prolonged culture or with additional X-ray irradiation.

3. No E. L. clone was isolated from Type I HBEC with similar X-ray treatment.

4. The p53 and p21 proteins were frequently and concomitantly elevated in these E. L. clones derived from Type II HBEC. However, these clones appear to contain functional wild type p53, since they showed radiation-induced G1 arrest.

5. While the other Type II HBEC used in this study expressed the p16/ink4a protein, the HME15 which gave rise to many X-ray induced E.L. clones were unexpectedly

deficient in the expression of p16/ink4a. However, one of the E. L. clones derived from this cell culture (XB7) re-expressed the p16 protein indicating that the p16 gene is suppressed but not mutated. The experiment also confirmed that p16 may be up-regulated in cells with inactivated Rb. The other E. L. clones examined showed non or weak expression of p16.

6. While the parental Type II HBEC expressed the hyper-phosphorylated Rb, the E. L. clones expressed both phosphorylated and unphosphorylated Rb.

These results have several implications and raise many new questions

a. Why do Type II E. L. clones fail to become immortal spontaneously or after additional X-ray irradiation?

Most of the E. L. clones were isolated from HME15. Using the same cells, it was found that both Type I and Type II cells were equally susceptible to transformation by SV40 large T antigen to become E. L. clones. However, the Type II E. L. clones were not easily immortalized (1/10) campared to Type I E. L. clones (10/11) after prolonged growth (Chang, C. C. et al. unpublished results). Thus Type II HBEC are instrinsically not easily to become immortalized. Wazer et al. (1994) did report the isolation of a immortal HBEC line after X-ray irradiation. The total dose used in their experiments (30 Gy) is higher than that used in this study. It is possible that, with continual repeated low dose X-ray treatment of large population of cells, our E. L. clones will become immortal. The frequency is, however, expected to be low as shown by Wazer et al. (1994).

b.What might be the reason for the inability for X-Rays to induce extended lifespan in Type I HBEC?

The failure to obtain E. L. clones from Type I HBEC after X-ray irradiation may be due to several reasons. First, the total number of Type I cells used in the experiments is considerably less than Type II cells (about 1/4 - 1/5). Unlike SV40 large T antigen which can inactivate p53 and Rb completely by complexing with the gene products, the X-rays need to delete both alleles to transform if the target gene is a tumor suppressor gene. The second possibility is that the protocol for X-ray treatment may not be right. We did observe that Type I cells were more sensitive to radiation-induced inhibition of cell proliferation. Perhaps a more frequent treatment with much lower dose each time would be more effective. The third possibility is the difference between *in vitro* and *in vivo*. *In vivo*, Type I HBEC receive signals by attaching to the extra-cellular matrix. While *in vitro*, the cells were plated on plastic surface that sent different signals to the cells (Trosko. JE, personal communication). In addition to that, Type I HBEC interact with other types of cell *in vivo* may provide protection against radiation killing. Alternatively, X-rays may function as an effective carcinogen at later stage but not at the initial stage. There is evidence that X-rays are effective in converting immortal HBEC to weakly tumorigenic cells (Kang et al., 1997)

c. Why were Type II HBEC derived from different women not equally transformable by X-rays to acquire extended lifespan?

E.L. clones were isolated from only two of six different Type II HBEC treated with X-ray. HME15 was the most transformable. The reason for the discrepancy in transformability among different cultures is not clear. Since HME15 is the only one

among the five HBEC (HME5, 12, 14, 15 and 20) not expressing p16, it is tempting to speculate that p16 deficiency may contribute to a cell culture's susceptibility to be transformed by X-rays.

d. Is there a role of elevated p53 in the expression of extended lifespan and in the failure of E. L. clones to become immortal?

The E. L. Type II HBEC clones are frequently found to contain elevated level of p53 protein. The increased amount of p53 is the wild type form as indicated by the concomitant increase of p21 and by the radiation-induced G1 arrest in these cells. Unless there are different mechanisms to confer extended lifespan, the result of this studies with X-rays are difficult to reconcile with the results of SV40 studies where the elimination of p53 function leads to extended lifespan. The failure of Type II HBEC E. L. clone to become immortal may not be due to the presence of elevated functional p53 since SV40 transformed Type II HBEC also were infrequently to become immortal. These cells contain no functional p53 due to the presence of the SV40 large T antigen.

e. Why, unlike normal or immortal HBEC which express the phosphorylated form (non-functional) of Rb, do the E. L. clones of Type II express both phosphorylated and unphosphorylated form (functional) of Rb?

Consistent with what might be expected of elevated p53 and p21, the E. L. clones were found to contain the unphosphorylated form of Rb in addition to the phosphorylated form. This may reflect that most immortal and normal cells at the stage of analysis are actively proliferating whereas E. L. clones contain considerable non-cycling cells.

f. Why does the level of p21 increase in the E.L. clones?

Since p21 are associated with senescence, and deficiency in p21 in human fibroblast lead to escape from senescence (Brown et al., 1997), it was expected that lower expression of p21 might be found in E.L. clones in this study. The possible explanations for the opposite result is that different types of cell may have different mechanisms to enter senescence. In this case, p16 may play the major role in senescence in our study. Another possibility is that both p21 and p16 are required for cells to enter senescence, inactivation of either one could result in bypass of senescence. The presence of elevated levels of p53 and p21 in E. L. clones may indicate that these cells contain both proliferating and senescent cells.

The results of this study have several implications. First, suppose the failure to transform Type I HBEC by X-rays is due to the use of small population of cells and the use of improper protocol, new effort to obtain E. L. and immortal Type I HBEC should use a different protocol taking these factors and other variable into consideration. for example, organoids formed by Type I cells on Matrigel may be used for neoplastic transformation by X-rays. Second, the failure of Type II E. L. clones to become immortal is consistent with the results form SV40 studies, indicating that Type II HBEC may not be consider as target cells for neoplastic transformation. Third, the deficient of p16 found in the most transformable culture (HME15) suggests that p16 may not be expressed in all normal HBEC and that this deficiency may be correlated with susceptibility to environmental agents induced neoplastic transformation. This should be tested in future studies

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